



2019

Protective Actions of Oxytocin on Aortic Wistar and SHR VSMCs

Varun Kumar Soti
Nova Southeastern University

Follow this and additional works at: https://nsuworks.nova.edu/hpd_corx_stuetd



Part of the [Pharmacy and Pharmaceutical Sciences Commons](#)

All rights reserved. This publication is intended for use solely by faculty, students, and staff of Nova Southeastern University. No part of this publication may be reproduced, distributed, or transmitted in any form or by any means, now known or later developed, including but not limited to photocopying, recording, or other electronic or mechanical methods, without the prior written permission of the author or the publisher.

NSUWorks Citation

Varun Kumar Soti. 2019. *Protective Actions of Oxytocin on Aortic Wistar and SHR VSMCs*. Doctoral dissertation. Nova Southeastern University. Retrieved from NSUWorks, College of Pharmacy. (4) https://nsuworks.nova.edu/hpd_corx_stuetd/4.

This Dissertation is brought to you by the College of Pharmacy at NSUWorks. It has been accepted for inclusion in Student Theses, Dissertations and Capstones by an authorized administrator of NSUWorks. For more information, please contact nsuworks@nova.edu.

**PROTECTIVE ACTIONS OF OXYTOCIN ON AORTIC WISTAR AND SHR
VSMCS**

By

VARUN KUMAR SOTI

A dissertation submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

College of Pharmacy

Nova Southeastern University

Fort Lauderdale, Florida 33328

August 2019

Dissertation Advisors: Ana M. Castejon, Ph.D. and Luigi X. Cubeddu, M.D., Ph.D.

**Nova Southeastern University
Health Professions Division
College of Pharmacy
Pharmaceutical Sciences
Fort Lauderdale, FL**

CERTIFICATE OF APPROVAL

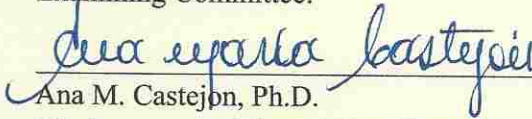
Ph.D. Dissertation

This is to certify that the Ph.D. Dissertation of

VARUN KUMAR SOTI

With a major in Pharmaceutical Sciences with a specialization in Molecular Medicine and Pharmacogenomics has been approved by the Examining Committee on May 31, 2019 as satisfactory for the dissertation requirement for the Doctor of Philosophy degree.

Examining Committee:



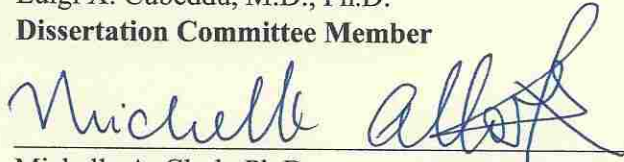
Ana M. Castejon, Ph.D.
Chairperson of Dissertation Committee

5/31/2019
Date



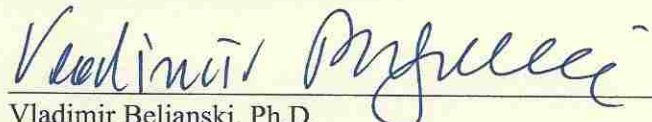
Luigi X. Cubeddu, M.D., Ph.D.
Dissertation Committee Member

5/31/2019
Date



Michelle A. Clark, Ph.D.
Dissertation Committee Member

5/31/2019
Date



Vladimir Beljanski, Ph.D.
Dissertation Committee Member

8/26/2019
Date

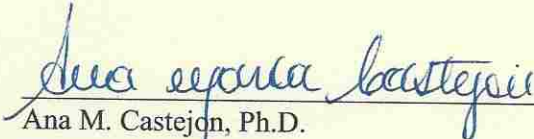
Nova Southeastern University

**Health Professions Division
College of Pharmacy
Pharmaceutical Sciences
Fort Lauderdale, FL**

CERTIFICATION

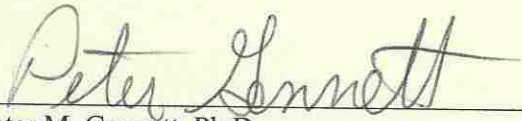
We hereby certify that this dissertation, submitted by Varun Kumar Soti, conforms to acceptable standards and is fully adequate in scope and quality to fulfill the dissertation requirement for the Doctor of Philosophy degree.

Approved:



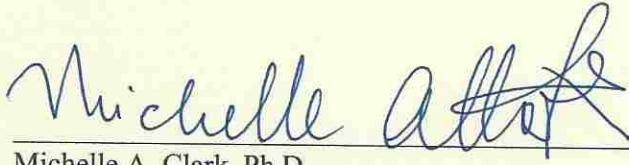
Ana M. Castejon, Ph.D.
Department Chairperson

8/26/2019
Date



Peter M. Gannett, Ph.D.
Associate Dean, Research and Graduate Education

9/9/2019
Date



Michelle A. Clark, Ph.D.
Dean

9/9/2019
Date

Abstract

An Abstract of a Dissertation Submitted to Nova Southeastern University in
Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

PROTECTIVE ACTIONS OF OXYTOCIN ON AORTIC WISTAR AND SHR VSMCS

By

VARUN KUMAR SOTI

AUGUST 2019

Background. Oxytocin, known as a female reproductive hormone, has emerged as a cardiovascular hormone showing cardioprotective effects, for instance, negative chronotropic and inotropic effects and ANP release in cardiomyocytes and endothelium. However, oxytocin's effect in VSMCs remains to be explored. **Objectives.** This study had three goals: characterizing oxytocin's effect on proliferation, inflammation, oxidative stress, and vasocontraction pathway in Wistar VSMCs; determining oxytocin's effect on Ang II; describing oxytocin's effect and its interaction with Ang II in SHR VSMCs. **Methods:** Aortic VSMCs were isolated from Wistar rats and SHRs. Hemocytometer, MTT and ³H-Thymidine incorporation assays, and flow cytometry were employed for cell proliferation studies. Two key proliferation pathways — ERK1/2 and PI3K/Akt pathways, and vasocontraction (ROCK) pathway were investigated for mechanistic studies utilizing Western blotting. IL-6 and ROS were measured to assess inflammation and oxidative stress, respectively. **Results.** Cell proliferation studies and Western blotting outcomes in Wistar and SHR VSMCs, demonstrated that oxytocin (100 nM) significantly decreased cell proliferation and reduced phosphorylation of ERK1/2, PI3Kp110 α , Akt, ROCK-1, and ROCK-2, respectively. OXTA antagonized these effects of oxytocin. Flow cytometric measurements indicated that oxytocin-induced apoptosis and necrosis. IL-6 assay and ROS assay results displayed that oxytocin increased IL-6 and ROS, respectively. Oxytocin's pre-treatment with Ang II in Wistar and SHR VSMCs caused: decreased cell proliferation; reduced phospho-ERK1/2, phospho-PI3Kp110 α , phospho-Akt, ROCK-1, and ROCK-2; decreased IL-6; increased ROS. Comparison between Wistar and SHR VSMCs illustrated that oxytocin greatly reduced PI3Kp110 α phosphorylation in Wistar VSMCs than SHR VSMCs ($P < 0.01$). Oxytocin caused higher IL-6 secretion in SHR VSMCs ($P < 0.01$) and higher ROS production in Wistar VSMCs ($P < 0.001$). Oxytocin's pre-treatment with Ang II showed: higher reduction in ERK1/2 phosphorylation in SHR VSMCs ($P < 0.001$); higher reduction in PI3Kp110 α phosphorylation ($P < 0.05$) and higher increase in ROS in Wistar VSMCs ($P < 0.001$). **Conclusion.** Oxytocin showed an anti-proliferative effect on aortic VSMCs, likely through inhibition of ERK1/2 and PI3K/Akt phosphorylation. Oxytocin-mediated decreased phosphorylation of ROCK-1 and ROCK-2 suggest oxytocin's anti-vasoconstrictive action in VSMCs. Oxytocin's antagonistic actions against Ang II indicate that oxytocin may exert beneficial vascular effects in conditions associated with high levels of Ang II.

Dedication

This is dedicated:

To the late Mrs. Chandrawati Devi, my beloved Grandmother. I miss you every second of
my life;

To my greatest inspiration, Mrs. Sushma Sharma, my loving Mother;

To the strongest man I know, Dr. Gopal Krishna Soti, my dear Father;

To my enormous support, Ms. Bhawan Soti, my loving Sister.

Acknowledgements

First of all, I am incredibly thankful to God for making everything possible in my life. Next, I would like to express my sincerest appreciation to my research advisors, Dr. Ana M. Castejon, and Dr. Luigi Cubeddu. It is your guidance, vision, research expertise, enthusiasm, patience, and constant motivation that helped me immensely in materializing this dissertation research. I sincerely thank you both for believing in me and standing by me when I was challenged, in particular, throughout this research study and, in general, my graduate studies. I feel privileged to work alongside and under both of your tutelages in the Cardiovascular and Metabolic Research Laboratory.

I would like to offer my special thanks to Dr. Michelle A. Clark. I am grateful to you for providing me with your esteemed research guidance and material support with regards to equipment, chemicals, and lab facilities whenever needed. I express my sincere gratitude to you for your valuable contributions not only to this research but also to my graduate studies at Nova Southeastern University.

I would like to place on record my sincere thanks to Dr. Vladimir Beljanski. I thank you for your valuable guidance and timely advice. It was a great learning experience working with you at the coveted NSU Center for Collaborative Research. I am thankful to Dr. Fiorella Rossi for providing me the assistance with Flow experiments. I would like to offer my special thanks to the HPD Research Grant Committee for funding this research.

I am especially appreciative of my senior colleague and my dearest friend Dr. Dhanush Haspula. I express my heartfelt thanks to you for providing me with the training and assistance with various experimental and laboratory techniques. Personally, I am

forever indebted to you, for you stood by me during the most challenging times I faced in my life, let alone my graduate studies.

I would like to mention my special thanks to my senior colleagues Dr. Ahmed Z. Alanazi, Dr. Shmuel Negussie, Dr. Ana O'Connor, my lab mates Dr. Jordan Spaw and Dr. Mohammad Alanazi, and Dr. Francisco Puerta, the former Post-Doc. I profoundly appreciate the technical assistance provided by Mr. Michael Dressler and Ms. Waad Abdul Kareem S Samman.

I am incredibly thankful to the late Dr. Hugh McLean, former Associate Dean, Research and Graduate Education, for giving me the once in a lifetime opportunity to pursue the doctoral degree in the United States of America.

I sincerely thank Dr. Peter M. Gannet, Associate Dean, Research and Graduate Education. I am grateful to you for your inspiration, leadership, and valuable help.

It is my great honor and privilege to express my highest appreciation to Ms. Kim Simmons. You have been my constant source of encouragement and wisdom and have been the reliable support to me. I am forever indebted to you for being with me through my thick and thins. You showed me respect, love, compassion, and treated me as your own son. You are my beacon of light. Had it not been for you, I would not have been able to finish my graduate studies.

I sincerely appreciate the valuable contribution Ms. Althea Harvey made. You considered and treated me as your family. You have invariably inspired me to remain optimistic.

It gives me great pleasure to acknowledge on record the significant contribution my dearest friend and sister Dr. Bushra Hijazi made. I also sincerely thank my most beloved

friends Ms. Carmen DeJesus and Ms. Priya Dondapati, for their incredible support throughout.

I would like to express my profound gratitude to Ms. Sonja Karen Hall. You welcomed me in your home and showed compassion, generosity, and care. You provided me with the stability that enabled me to focus on my studies. I am fortunate to have your back. You are deeply appreciated.

Lastly, and most importantly, I express my most significant and the highest appreciation to my loving family. I express my sincerest gratitude and profound thanks to my mother, Mrs. Sushma Sharma. I owe you all that I have achieved in my life. You taught me the discipline of working hard with integrity, compassion, and to never give up in any circumstance or situation that life throws at. Mummy, you are my greatest inspiration.

I sincerely acknowledge my father, Dr. Gopal Krishna Soti. Thank you so much, Papa, for all that you have done for me, for it would not have been possible without you. Thank you for trusting in me and graciously providing me with the space to make my own decisions in life.

I am incredibly thankful to my loving sister, Ms. Bhawna Soti. To me, you are the greatest blessing of God. I am profoundly grateful to you for being there with me in my darkest of times and lightest of moments. I thank God for having you as my sister.

Table of Contents

| | |
|---|-----------|
| Abstract | iii |
| Dedication | iv |
| Acknowledgements | v |
| Table of Contents | viii |
| List of Figures | xi |
| List of Tables | xiv |
| List of Abbreviations | xv |
| | |
| Chapter 1. Introduction | 1 |
| 1.1. Background | 1 |
| 1.2. Traditional Roles of Oxytocin | 4 |
| 1.3. Emerging Role of Oxytocin in the CVS | 5 |
| 1.3.1. Effect of Oxytocin on Heart | 5 |
| 1.3.2. Effect of Oxytocin on Cardiomyocytes | 6 |
| 1.3.3. Effect of Oxytocin on Blood Vessels (ECs and VSMCs) | 8 |
| 1.3.4. Effect of Oxytocin on Pathophysiological conditions of CVS | 10 |
| 1.4. Role of Ang II in the CVS and its Pathophysiology | 12 |
| 1.5. The Gap in the Literature, Research Hypotheses, and Study Aims | 15 |
| 1.5.1. Hypothesis 1 | 15 |
| 1.5.2. Hypothesis 2 | 16 |
| 1.5.3. Specific Aim 1 | 16 |
| 1.5.4. Specific Aim 2 | 16 |
| 1.5.5. Specific Aim 3 | 17 |
| 1.6. Role of VSMCs in the CVS and its Pathophysiology | 18 |
| 1.7. Study Significance | 21 |
| 1.8. Barriers, Issues, Limitations, and Delimitations | 22 |
| | |
| Chapter 2. Literature Review | 24 |
| 2.1. MAPK/ERK1/2 Pathway in the CVS | 24 |
| 2.2. PI3K/Akt Signaling in the CVS | 29 |
| 2.3. Rho Kinases (ROCK-1 and ROCK-2) Signaling in the CVS | 35 |
| 2.4. Interleukin-6 in the CVS | 41 |
| 2.5. Reactive oxygen species (ROS) in the CVS | 44 |
| 2.6. Summary | 48 |
| | |
| Chapter 3. Methodology | 49 |
| 3.1. Wistar rats and Spontaneously Hypertensive Rats (SHRs) | 49 |
| 3.2. Isolation and Culture of Primary Rat Aortic VSMCs | 49 |
| 3.3. VSMCs Proliferation Assessment by the Hemocytometer Method | 51 |
| 3.3.1. The Principle of the Hemocytometer Method | 51 |
| 3.3.2. The Procedure of Cell Counting by the Hemocytometer Method | 52 |
| 3.4. VSMCs viability determination by the MTT Assay | 53 |

| | |
|---|-----------|
| 3.4.1. The Principle of the MTT Assay | 53 |
| 3.4.2. The Procedure of the MTT Assay | 53 |
| 3.5. VSMCs Growth Measurement by the ³ H-Thymidine Incorporation Assay | 54 |
| 3.5.1. The Principle of the ³ H-Thymidine Incorporation Assay | 54 |
| 3.5.2. The Procedure of the ³ H-Thymidine Incorporation Assay | 54 |
| 3.6. VSMCs Apoptosis and Necrosis Measurement by Flow Cytometry | 56 |
| 3.6.1. The Principle of Flow Cytometry | 56 |
| 3.6.2. The Procedure of Flow Cytometry | 59 |
| 3.7. IL-6 Measurement by the ELISA | 61 |
| 3.7.1. The Principle of IL-6 ELISA | 61 |
| 3.7.2. The Procedure of IL-6 ELISA | 63 |
| 3.8. ROS Assay | 64 |
| 3.8.1. The Principle of ROS Assay | 64 |
| 3.8.2. ROS Assay Procedure | 64 |
| 3.9. Western Blotting | 66 |
| 3.9.1. The Principle of Western Blotting | 66 |
| 3.9.2. Preparation of Cell Lysate and Procedure of Western Blotting | 66 |
| 3.10. Statistical Analysis | 74 |
| 3.11. Specific Aim 1 and 2, Strategies, and Experimental Design | 75 |
| 3.12. Specific Aim 3, Strategies, and Experimental Design | 82 |
| Chapter 4. Results | 83 |
| 4.1. Normotensive Wistar Rats (Specific Aims 1 and 2) | 83 |
| 4.1.1. Effects of oxytocin and Ang II on Proliferation of Aortic Wistar VSMCs | 83 |
| 4.1.1.1. Cell counting by the Hemocytometer Method in Wistar VSMCs | 83 |
| 4.1.1.2. Assessment of Cell Viability with MTT-assay in Wistar VSMCs | 87 |
| 4.1.1.3. Cell Growth Assessment with ³ H-Thymidine Incorporation assay in Wistar VSMCs | 90 |
| 4.1.1.4. Assessment of Cell Viability by Flow Cytometry in Wistar VSMCs | 92 |
| 4.1.2. Effect of Oxytocin and its Pre-treatment with Ang II on the Phosphorylation of ERK1/2 in Aortic Wistar VSMCs | 96 |
| 4.1.3. Effect of Oxytocin and its Pre-treatment with Ang II on the Phosphorylation of PI3K p110a and Akt in Aortic Wistar VSMCs | 98 |
| 4.1.4. Effect of Oxytocin and its Pre-treatment with Ang II on the Activation of ROCK -1 and ROCK-2 in aortic Wistar VSMCs | 101 |
| 4.1.5. Effect of Oxytocin and its Pre-treatment with Ang II on IL-6 secretion in Aortic Wistar VSMCs | 105 |
| 4.1.6. Effect of Oxytocin and its Pre-treatment with Ang II on ROS in Aortic Wistar VSMCs | 108 |
| 4.2. Spontaneously Hypertensive Rats (Specific Aim 3) | 111 |
| 4.2.1. Effect of Oxytocin and its Pre-treatment with Ang II on Aortic SHR VSMCs proliferation | 111 |
| 4.2.1.1. Cell counting by the Hemocytometer method in SHR VSMCs | 111 |
| 4.2.1.2. Assessment of Cell Viability with MTT-assay in SHR VSMCs | 114 |

| | |
|---|------------|
| 4.2.1.3. Cell Growth Assessment with ³ H-Thymidine Incorporation Assay in SHR VSMCs | 117 119 |
| 4.2.1.4. Assessment of Cell Viability by Flow Cytometry in SHR VSMCs | |
| 4.2.2. Effect of Oxytocin and its Pre-treatment with Ang II on the Phosphorylation of ERK1/2 in Aortic SHR VSMCs | 122 |
| 4.2.3. Effect of Oxytocin and its Pre-treatment with Ang II on the Phosphorylation of PI3K p110 α and Akt in Aortic SHRs VSMCs | 124 |
| 4.2.4. Effect of Oxytocin and its Pre-treatment with Ang II on the Activation of ROCK -1 and ROCK-2 in aortic SHR VSMCs | |
| 4.2.5. Effect of Oxytocin and its Pre-treatment with on IL-6 secretion in Aortic SHR VSMCs | 127 |
| 4.2.6. Effect of Oxytocin and its Pre-treatment with Ang II on ROS production in Aortic SHR VSMCs | 130 |
| 4.3. Comparison of the Effects of Oxytocin and its Pre-treatment with Ang II on aortic Wistar VSMCs versus SHR VSMCs | 133 |
| 4.3.1. Comparison of the Effect of Oxytocin and its Pre-treatment with on cell proliferation in Aortic Wistar VSMCs and SHR VSMCs | 136 |
| 4.3.2. Comparison of the Effect of Oxytocin and its Pre-treatment with Ang II on Cell Viability in Aortic Wistar VSMCs and SHR VSMCs | 136 |
| 4.3.3. Comparison of the Effect of Oxytocin on Apoptosis and Necrosis in Aortic Wistar VSMCs between and SHR VSMCs | 139 |
| 4.3.4. Comparison of the Effect of Oxytocin and its Pre-treatment with Ang II on the Phosphorylation of ERK1/2, PI3K p110 α , and AKT in Aortic Wistar VSMCs and SHR VSMCs | 142 |
| 4.3.5. Comparison of the Effect of Oxytocin and its Pre-treatment with Ang II on Vasocontraction Pathway: ROCK-1 and ROCK-2 in Aortic VSMCs Isolated from Wistar rats and SHRs | 146 |
| 4.3.6. Comparison of the Effect of Oxytocin and its Pre-treatment with Ang II on IL-6 in Aortic Wistar VSMCs and SHR VSMCs | 150 |
| 4.3.7. Comparison of the Effect of Oxytocin and its Pre-treatment with Ang II on ROS production in Aortic Wistar VSMCs and SHR VSMCs | 153 |
| | 157 |
| Chapter 5. Discussion | 159 |
| 5.1. Oxytocin Reduced the Proliferation in Aortic VSMCs | 162 |
| 5.2. Oxytocin Deactivated the Proliferation Signal Transduction Pathways (ERK1/2 Pathway and PI3K/Akt Pathway) in Aortic VSMCs | 164 |
| 5.3. Oxytocin Deactivated the ROCK Pathway in Aortic VSMCs | 168 |
| 5.4. Oxytocin Increased IL-6 in Aortic VSMCs | 171 |
| 5.5. Oxytocin Increased ROS in Aortic VSMCs | 174 |
| 5.6. Summary of the Dissertation Research | 177 |
| 5.7. Conclusion | 181 |
| 5.8. Future recommendation | 182 |
| Bibliography | 184 |

List of Figures

| | | |
|-------------|---|-----|
| Figure 1 | Histology of a Blood Vessel | 18 |
| Figure 2.1 | Activation of MAPK Signaling Pathway in the CVS | 25 |
| Figure 2.2 | The PI3K/Akt Signaling Pathway in the CVS | 31 |
| Figure 2.3 | ROCK Signaling Pathway in the Contraction of VSMCs | 40 |
| Figure 2.4 | Redox Signaling in the CVS | 46 |
| Figure 3.1 | Hemocytometer and the Grid lines of a Chamber in a Hemocytometer Microscopic Slide | 51 |
| Figure 3.2 | Flow Cytometer | 57 |
| Figure 3.3 | Presentation of Markers for Detection by Healthy, Apoptotic, Late-Apoptotic and Necrotic Cells | 58 |
| Figure 3.4 | A Typical ELISA Sandwich | 62 |
| Figure 3.5 | A Schematic Representation of the Various Steps in Western Blotting | 68 |
| Figure 4.1 | The Effect of Oxytocin and Different Treatment Groups on Aortic VSMCs Proliferation in Wistar rats as Assessed by the Hemocytometer Method | 85 |
| Figure 4.2 | The Effect of Oxytocin and Different Treatment Groups on aortic VSMCs Proliferation Rate of Aortic VSMCs in Wistar rats as Assessed by the Hemocytometer Method | 86 |
| Figure 4.3 | The Effect of Oxytocin and Different Treatment Groups on Wistar aortic VSMCs Viability as Assessed by the MTT Assay | 88 |
| Figure 4.4 | The Effect of Oxytocin and Different Treatment Groups on Wistar aortic VSMCs Viability Rate as Assessed by the MTT Assay | 89 |
| Figure 4.5 | The Effect of Oxytocin and Different Treatment Groups on Wistar aortic VSMCs Growth as Assessed by the ³ H-Thymidine Incorporation Assay | 91 |
| Figure 4.6 | The Effect of Oxytocin on Apoptosis and Necrosis in Aortic Wistar VSMCs as Assessed by Flow Cytometry | 94 |
| Figure 4.7 | The Effect of Oxytocin and Different Treatment Groups on the Phosphorylation of ERK1/2 in Aortic Wistar VSMCs | 97 |
| Figure 4.8 | The Effect of Oxytocin and Different Treatment Groups on the Phosphorylation of PI3K p110 α in Aortic Wistar VSMCs | 99 |
| Figure 4.9 | The Effect of Oxytocin and Different Treatment Groups on the Phosphorylation of Akt in Aortic Wistar VSMCs | 100 |
| Figure 4.10 | The Effect of Oxytocin and Different Treatment Groups on the Activation of ROCK- 1 in Aortic Wistar VSMCs | 102 |
| Figure 4.11 | The Effect of Oxytocin and Different Treatment Groups on the Activation of ROCK- 2 in Aortic Wistar VSMCs | 103 |
| Figure 4.12 | The Effect of Oxytocin and Different Treatment Groups on IL-6 Secretion in Aortic Wistar VSMCs | 107 |
| Figure 4.13 | The Effect of Oxytocin and Different Treatment Groups on ROS Production in Aortic Wistar VSMCs | 110 |

| | | |
|-------------|--|-----|
| Figure 4.14 | The Effect of Oxytocin and Different Treatment Groups on Aortic VSMCs Proliferation in SHRs as Assessed by the Hemocytometer Method | 112 |
| Figure 4.15 | The Effect of Oxytocin and Different Treatment Groups on the Proliferation Rate of Aortic VSMCs in SHRs as assessed by the Hemocytometer method | 113 |
| Figure 4.16 | The Effect of Oxytocin and Different Treatment Groups on Aortic SHR VSMCs Viability as Assessed by the MTT assay | 115 |
| Figure 4.17 | The Effect of Oxytocin and Different Treatment Groups on Aortic SHR VSMCs Viability Rate as Assessed by the MTT assay | 116 |
| Figure 4.18 | The Effect of Oxytocin and Different Treatment Groups on Aortic SHR VSMCs Growth as Assessed by the ³ H-Thymidine Incorporation Assay | 118 |
| Figure 4.19 | The Effect of Oxytocin on Apoptosis and Necrosis in aortic SHR VSMCs as Assessed by Flow Cytometry | 120 |
| Figure 4.20 | The Effect of Oxytocin and Different Treatment Groups on the Phosphorylation of ERK1/2 in Aortic SHR VSMCs | 123 |
| Figure 4.21 | The Effect of Oxytocin and Different Treatment Groups on the Phosphorylation of PI3K p110 α in Aortic SHR VSMCs | 125 |
| Figure 4.22 | The Effect of Oxytocin and Different Treatment Groups on the Phosphorylation of Akt in Aortic SHR VSMCs | 126 |
| Figure 4.23 | The Effect of Oxytocin and Different Treatment Groups on the Activation of ROCK- 1 in Aortic SHR VSMCs | 128 |
| Figure 4.24 | The Effect of Oxytocin and Different Treatment Groups on the Activation of ROCK- 2 in Aortic SHR VSMCs | 129 |
| Figure 4.25 | The Effect of Oxytocin and Different Treatment Groups on IL-6 Secretion in Aortic SHR VSMCs | 132 |
| Figure 4.26 | The Effect of Oxytocin and Different Treatment Groups ROS Production in aortic SHR VSMCs | 135 |
| Figure 4.27 | The Comparison of the Effects of Oxytocin, Ang II, and the Pre-treatment of Oxytocin with Ang II on Proliferation of Aortic VSMCs Between Wistar VSMCs and SHR VSMCs as Assessed by the Hemocytometer Method | 138 |
| Figure 4.28 | The Comparison of the Effects of Oxytocin, Ang II, and the Pre-treatment of Oxytocin with Ang II on Viability of Aortic VSMCs Between Wistar VSMCs and SHR VSMCs as Assessed by the MTT Assay | 141 |
| Figure 4.29 | The Comparison of the Effects of Oxytocin on Live cells, Apoptosis, and Necrosis Between Wistar VSMCs and SHR VSMCs as Assessed by the Flow Cytometry | 144 |
| Figure 4.30 | The Comparison of the Effects of Oxytocin, Ang II, and the Pre-treatment of Oxytocin with Ang II on the Phosphorylation of ERK1/2 (figure a, b, and c), PI3K p110 α (figure d, e, and f), and Akt (figure g, h, and i) on Aortic VSMCs Between Wistar VSMCs and SHR VSMCs | 149 |

| | | |
|-------------|---|-----|
| Figure 4.31 | The Comparison of the Effects of Oxytocin, Ang II, and the Pre-treatment of Oxytocin with Ang II on the Phosphorylation of ROCK-1 (figure a, b, and c) and ROCK-2 (figure d, e, and f) on Aortic VSMCs Between Wistar VSMCs and SHR VSMCs | 152 |
| Figure 4.32 | The Comparison of the Effect of Oxytocin (10 nM, 100 nM, and 1000nM), Ang II (100 nM), Pre-treatment of Oxytocin with Ang II on IL-6 Secretion between Wistar VSMCs and SHR Aortic VSMCs | 155 |
| Figure 4.33 | The Comparison of the Effect of Oxytocin (100 nM), Ang II (100 nM), Pre-treatment of Oxytocin (100 nM) with Ang II (100 nM) on IL-6 Secretion between Wistar VSMCs and SHR Aortic VSMCs | 156 |
| Figure 4.44 | The Comparison of the Effect of Oxytocin, Ang II, Pre-treatment of Oxytocin with Ang II on ROS Production between Wistar VSMCs and SHR Aortic VSMCs | 158 |
| Figure 5 | Inhibition of the ERK1/2 Pathway, the PI3K/Akt Pathway, and the Rho-kinase Pathway by Oxytocin in Aortic VSMCs | 170 |

List of Tables

| | | |
|-------------|--|----|
| Table 3.1. | Primary and Secondary Antibodies used in Western Blotting | 69 |
| Table 3.2. | Concentrations of Primary and Secondary Antibodies used in Western Blotting | 71 |
| Table 3.3. | Composition of Buffers used in Western Blotting | 72 |
| Table 3.4. | Composition of 10% Resolving (Separating) Gel used in Western Blotting | 73 |
| Table 3.5. | Composition of 5% Stacking Gel used in Western Blotting | 73 |
| Table 3.6. | Treatment Groups in Hemocytometer Method, MTT, and ³ H-Thymidine Incorporation Assays | 76 |
| Table 3.7. | Treatment Groups for ERK1/2 and PI3K/Akt Pathway Experiments | 77 |
| Table 3.8. | Treatment Groups for the ROS Assay | 78 |
| Table 3.9. | Treatment Groups for the IL-6 Assay | 79 |
| Table 3.10. | Treatment Groups for Rho Kinase Pathway Experiments | 80 |

List of Abbreviations

| | |
|--------------------------|---|
| °C | Degree in Celsius |
| α1-ADR | Alpha-1 Adrenergic Receptors |
| ³ H-Thymidine | Tritiated Thymidine (Radioactive Thymidine) |
| 4E-BP1 | 4E-Binding Protein 1 |
| 7-AAD | 7-Amino Actinomycin D |
| AC | Adenylyl Cyclase |
| ACE | Angiotensin Converting Enzyme |
| Ang I | Angiotensin I |
| Ang II | Angiotensin II |
| ANOVA | Analysis of Variance |
| ANP | Atrial Natriuretic Peptide |
| AP-1 | Activator Protein-1 |
| APS | Ammonium Per Sulfate |
| AT-1R | Angiotensin Type 1 Receptors |
| ATP | Adenosine Triphosphate |
| BCA | Bicinchoninic Acid |
| BH ₄ | Tetrahydrobiopterin |
| cAMP | Cyclic Adenosine Monophosphate |
| Cat / CAT | Catalase |
| CMs | Cardiomyocytes |
| CNS | Central Nervous System |
| CO ₂ | Carbon Dioxide |
| CPI-17 | C-Kinases Potentiated Protein Phosphatase 1 Inhibitor -17 |
| cpm | Counts Per Minute |
| CRP | C Reactive Protein |
| CVD | Cardiovascular Diseases |
| CVS | Cardiovascular System |
| CXCL | Chemokine (C-X-C Motif) Ligand |
| DAG | 1, 2-Diacylglycerol |
| DCF | 2',7'-Dichlorofluorescein |
| DCFDA | 2',7'- Dichlorofluorescein Diacetate |
| DMEM | Dulbecco's Modification of Eagle's Medium |
| DMSO | Dimethyl Sulfoxide |
| DNA | Deoxyribonucleic Acid |
| ECL | Enhanced Chemiluminescence |
| ECM | Extracellular Matrix |
| ECs | Endothelial Cells |
| eEF2 | Eukaryotic Elongation Factor 2 |
| eIF4 | Eukaryotic Initiation Factor 4 |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| eNOS | Endothelial Nitric Oxide Synthase |
| ERK | Extracellular Signal-Regulated Kinase |
| ET | Endothelin |
| FBS | Fetal Bovine Serum |

| | |
|---------------------------------|--|
| FKBP | FK506-Binding Protein |
| g | Gram |
| GAPDH | Glyceraldehyde-3-Phosphate Dehydrogenase |
| GDI | Guanine Nucleotide Dissociation Inhibitor |
| GEF | Guanine Nucleotide Exchange Factor |
| GLUT | Glucose Transporter |
| gp130 | Glycoprotein 130 |
| GPCRs | G-Protein Coupled Receptors |
| GPx | Glutathione Peroxidase |
| GRx | Glutathione Reductase |
| GSH | Glutathione (Reduced) |
| GSK | Glycogen Synthase Kinase |
| GST | Glutathione S-Transferase |
| GTP | Guanosine Triphosphate |
| GTP | Guanosine-5'-Triphosphate |
| G α_i | G Alpha Inhibitory Protein |
| h-CD | H-Caldesmon |
| H ₂ O ₂ | Hydrogen Peroxide |
| HCl | Hydrochloric Acid |
| HEK293 | Human Embryonic Kidney |
| HO-1 | Heme Oxygenase-1 |
| HRP | Horseradish Peroxidase |
| HSPG | Heparan Sulfate Proteoglycan |
| ICAM-1 | Intercellular Adhesion Molecule-1 |
| IGF | Insulin-Like Growth Factor |
| IL-6 | Interleukin-6 |
| IL-6R | Interleukin -6 Receptor |
| IP3 | Inositol-3-Phosphate |
| JNK | C-Jun N-Terminal Kinases |
| KCl | Potassium Chloride |
| K _D | Equilibrium Dissociation Constant |
| KD | Kilo Dalton |
| KH ₂ PO ₄ | Potassium Dihydrogen Phosphate |
| KLF2 | Kruppel-Like Factor 2 |
| L-VGCC | L-Type Voltage-Gated Calcium Channel |
| M | Molar |
| MAPK | Mitogen-Activated Protein Kinase |
| MAPKK | Mitogen-Activated Protein Kinase Kinase |
| MAPKKK | Mitogen-Activated Protein Kinase Kinase Kinase |
| MCP-1 | Monocyte Chemoattractant Protein-1 |
| MI | Myocardial Infarction |
| MIP-2 α | Macrophage Inflammatory Protein-2 Alpha |
| mL | Milliliter |
| MLC | Myosin Light Chain |
| MLCK | Myosin Light Chain Kinase |
| MLCP | Myosin Light Chain Phosphatases |
| mm | Millimeter |

| | |
|----------------------------------|--|
| mM | Micro Molar |
| MMP | Matrix Metalloproteinase |
| MnSOD | Manganese Superoxide Dismutase |
| MSCs | Mesenchymal Stem Cells |
| mTOR | Mammalian Target of Rapamycin |
| MTT | 3-(4,5-Dimethylthiazo-2-Yl)-2,5-Diphenyl Tetrazolium Bromide |
| MYPT-1 | Myosin Phosphatase Target Subunit-1 |
| Na ₂ HPO ₄ | Disodium Hydrogen Phosphate |
| NaCl | Sodium Chloride |
| NADPH | Nicotinamide Adenine Dinucleotide Phosphate |
| NaOH | Sodium Hydroxide |
| NE | Norepinephrine |
| NF-κB | Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells |
| NK | Natural Killer |
| nm | Nano Meter |
| nM | Nano Molar |
| NO | Nitric Oxide |
| NOS | Nitric Oxide Synthase |
| Nrf2 | Nuclear Factor (Erythroid-Derived 2) -Like 2 |
| NSU-IACUC | Nova Southeastern University Institutional Animal Care and Use Committee |
| O ₂ ⁻ | Superoxide |
| OD | Optical Density |
| OXTA | Oxytocin Receptor Antagonist – L-371,257 |
| OXTRG | Oxytocin Receptor Gene |
| P value | Probability Value |
| p70S6K | P70s6 Kinase |
| PAH | Pulmonary Arterial Hypertension |
| PAI-1 | Plasminogen Activator Inhibitor-1 |
| PBS | Phosphate-Buffered Saline |
| PDK | Phosphoinositide-Dependent Kinase |
| pg | Picogram |
| Phospho-Akt | Phosphorylated Akt |
| Phospho-ERK1/2 or pERK | Phosphorylated ERK1/2 |
| Phospho-PI3K | Phosphorylated PI3K |
| PI | Phosphatidylinositol |
| PI-3-P | Phosphatidylinositol-3-Phosphate |
| PI-3,4-P ₂ | Phosphatidylinositol-3,4-Biphosphate |
| PI3K | Phosphatidylinositol-3-Kinases |
| PIP ₃ | Phosphatidylinositol Triphosphate |
| PKB | Protein Kinase B |
| PKC | Protein Kinase C |
| PLC | Phospholipase C |
| PP2A | Protein Phosphatase 2 A |

| | |
|---------------|---|
| PS | Phosphatidylserine |
| PtdIns | Phosphatidylinositol |
| PTEN | Phosphatase and Tensin Homolog Deleted on Chromosome Ten |
| Pyk-2 | Proline-Rich Tyrosine Kinase-2 |
| RA | Rheumatoid Arthritis |
| RAAS | Renin-Angiotensin-Aldosterone System |
| RBD | Rho-Binding Domain |
| Rho-GEFs | Rho Guanine Nucleotide Exchange Factors |
| RNA | Ribonucleic Acid |
| ROCK | Rho-Associated Coiled-Coil Kinase |
| ROS | Reactive Oxygen Species |
| rpm | Rotation Per Minute |
| RSK | Ribosomal S6 Kinase |
| RTKs | Receptor Tyrosine Kinases |
| SAPK | Stress-Activated Protein Kinases |
| SDS | Sodium Dodecyl Sulfate |
| SDS-PAGE | Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis |
| SFM | Serum-Free Media |
| SHR VSMCs | Vascular Smooth Muscle Cells Isolated from SHRs |
| SHRs | Spontaneously Hypertensive Rats |
| sIL-6R | Soluble Interleukin -6 Receptor |
| SOD | Superoxide Dismutase |
| STAT3 | Signal Transducer and Activator of Transcription 3 |
| TBST | Tween in Tris-Buffer Saline |
| TEMED | Tetramethylethylenediamine |
| TNF- α | Tumor Necrosis Factor-Alpha |
| TSC | Tuberous Sclerosis Complex |
| v/v | Volume by Volume |
| VCAM-1 | Vascular Cell Adhesion Molecule-1 |
| VEGF | Vascular Endothelial Growth Factor |
| VSMCs | Vascular Smooth Muscle Cells |
| Wistar VSMCs | Vascular Smooth Muscle Cells Isolated from Wistar Rats |
| ZIPK | Zipper-Interacting Protein Kinase |
| μ L | Micro Liter |
| μ M | Micro Molar |

Chapter 1: Introduction

1.1. Background

At the dawn of the 19th century, Sir Henry Dale achieved a breakthrough that would herald a new era in endocrinology. He discovered that a chemical extracted from the human posterior pituitary gland caused uterine contractions in pregnant cats, dogs and guinea pigs. He called this chemical ‘oxytocin,’ derived from the Greek words ‘*ογνξ*,’ ‘*τοχοξξ*,’ meaning ‘swift birth.’ Over the next 100 years, oxytocin became a subject for extensive research.¹

It is now established that oxytocin is a neurohypophysial nonapeptide secreted by magnocellular cells in the paraventricular and supraoptic nuclei of the hypothalamus. It is stored in the posterior lobe of the pituitary gland. A wide range of stimuli including sucking (milk ejection reflex), pre- (labor induction) and post-parturition, and stress trigger oxytocin release from the posterior pituitary into the blood circulation. There is an extensive projection of oxytocinergic neurons in the central nervous system (CNS) — brain and spinal cord including amygdala, ventromedial hypothalamus, nucleus acumben, septum, and brainstem.² Besides, oxytocin is also synthesized peripherally in tissues such as placenta, uterus, testis, kidney, heart, and blood vessels in both vascular endothelial cells (ECs) and vascular smooth muscle cells (VSMCs).³

The oxytocin receptor gene (OXTRG) has been mapped in the human chromosome at the locus 3p25-3p26.2 alongside a gene that encodes the vasopressin receptor. The distance between the two genes is 500-kilo bases (kb). The human OXTRG spreading over 17 kb consists of three introns and four exons. The third intron, being the largest spanning 12 kb, separates the transmembrane domain 6 from the coding region. Exons 1 and 2 frame the 5' prime non-coding domain, while the exons 3 and 4 code for the amino acids sequence for oxytocin receptors. Exon 4, in particular, codes for the seven transmembrane domain, carboxyl terminal and the 3' non-coding region.³

Both oxytocin and vasopressin are the neurohypophysial peptides, with each having a sequence of nine amino acids, and are stored in the posterior lobe of the pituitary gland. The point of difference between the structure of oxytocin and vasopressin is the amino acid at position 8. In oxytocin, leucine is present at number 8. Whereas, in vasopressin, either lysine or arginine is present at this position. The sequence of amino acid in oxytocin is as follows: *Cysteine (1) – Tyrosine (2) – Phenylalanine (3) – Glutamine (4) – Asparagine (5) – Cysteine (6) – Proline (7) – Leucine (8) – Glycine (9)*. Importantly, the presence of isoleucine at position 3 in the oxytocin family is required for the activation of oxytocin receptors.⁴

Oxytocin receptors belong to the class I G-protein coupled receptors (GPCRs) that predominantly bind to $G_{q/\alpha 11}$ class of guanosine triphosphate (GTP) binding proteins that activate phospholipase C (PLC). This brings about the formation of the two important secondary messengers: inositol-3-phosphate (IP3) and 1, 2-diacylglycerol (DAG). IP3 sparks the release of intracellular calcium

(Ca²⁺); DAG activates protein kinase C (PKC) that triggers the phosphorylation of targets leading to a further downstream signaling. The activation of these second messengers result in oxytocin-mediated physiological effects at different locations. When bound with *G_i* protein, oxytocin receptors inhibit the adenylyl cyclase (AC), leading to inhibition of the production of cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP). This inhibits cAMP pathway-dependent phosphorylation of targets and attenuates further downstream signaling. The effects of oxytocin varies widely and depends upon the target tissue(s), gland(s), organ(s) or system(s).^{3,5,6}

1.2. Traditional roles of oxytocin

Oxytocin is often referred to as a ‘female reproductive hormone.’ It is known to contract pregnant uterus assisting parturition¹. It also affects lactating breasts by initiating milk ejection reflex⁷, promotes maternal-fetal bonding⁸ and plays a role in reproduction and in reaching an orgasm.⁹

Oxytocin became commercially available in 1980. Its agonist and antagonists have found applications in both clinical research and pre-clinical studies.^{10,11,12}

It has become common in medical practice to employ pharmacological interventions such as intravenous administration of Pitocin (a commercial brand name oxytocin), along with other mechanical methods (for example extra-amniotic Foley catheter) to induce labor.^{13,14}

Oxytocin has emerged as a versatile hormone affecting social bonding, trust, memory, learning, empathy, and depression.^{15,16,17,18}

From as recent as the year 2012, the intranasal administration of oxytocin has found an application in clinical behavioral studies.^{19,20,21}

1.3. Emerging role of oxytocin in the cardiovascular system (CVS)

In the last decade, the dynamics in oxytocin research have been completely shifted. A new role for oxytocin has now evolved in cardiovascular regulation.^{22,23} Oxytocin being considered initially as a female hormone is now gaining consensus as a ‘cardiovascular hormone.’²⁴ The present study is an endeavor to develop a deeper understanding of the role that oxytocin plays in cardiovascular physiology and pathophysiological conditions.

1.3.1. Effect of oxytocin on heart

Several studies in rats and humans have reported that oxytocin is synthesized locally in the heart. The studies demonstrating competition binding curves have revealed only one receptor subtype for oxytocin in the heart. All the chambers of the heart and the large blood vessels have oxytocin and its receptors.^{25,26}

The equilibrium dissociation constant (K_D) values are slightly different in rat heart ($K_D \sim 1.00$ nM), hypothalamus ($K_D \sim 0.02$ nM), and uterus ($K_D \sim 0.76$ nM). However, in the blood vessels there are no significant difference between the K_D values of rat vena cava ($K_D \sim 0.78$ nM) and aorta ($K_D \sim 0.59$ nM). The right atrium and the left ventricle have the highest and lowest concentrations of oxytocin, respectively. The right atrium’s oxytocin levels are equivalent to hypothalamic oxytocin levels.²⁵

Studies have shown that oxytocin can reduce the rate of cardiac blood flow and induce bradycardia. In isolated dog atriums, oxytocin produced negative inotropic and chronotropic effects via its receptors in the heart.^{27,28}

1.3.2. Effect of oxytocin on cardiomyocytes

Oxytocin plays a role in the regulation of atrial natriuretic peptide (ANP).^{29,30} The ANP is a peptide hormone consisting of 28-amino acids. It is synthesized, stored and released locally in cardiomyocytes. The factors that cause the secretion of ANP include atrial wall distension, increased blood volume (in pathological conditions such as congestive heart failure), angiotensin II (Ang II), endothelin, and sympathetic activation (norepinephrine). The ANP elicits a cardioprotective effect by decreasing the central venous pressure, thereby reducing blood volume and decreasing ventricular pre-load, and thus decreasing cardiac output. Besides, ANP also inhibits renin release and aldosterone synthesis.³¹

One previous study has demonstrated the functional relevance of oxytocin and its receptors in rat heart and cultured cardiomyocytes. The study has evidenced that oxytocin (10^{-6} M, a physiologically relevant concentration) stimulates ANP release from isolated perfused female rat hearts and cultured cardiomyocytes. In this study, not only had the oxytocin antagonist (10^{-6} M and 10^{-7} M) , in a concentration-dependent manner, inhibited the oxytocin-stimulated ANP release in the heart perfusion medium, but also inhibited the basal release.²⁹

The research findings of these studies indicate that oxytocin, via ANP release, reduces cardiac contraction - force and rate, and decreases circulating blood volume. This illustrates the protective effect of oxytocin in cardiovascular physiology, and its benefits can be further explored in cardiovascular pathophysiological conditions, for instance, congestive heart failure.

In recent years, oxytocin has also found relevance in stem cell research on cardiogenesis. A few studies have indicated that oxytocin has the potential to induce differentiation of embryonic stem cells (P-19ECs) to cardiomyocytes (CMs). Studies have brought to light that nitric oxide (NO) signaling is involved in oxytocin-induced differentiation of P-19ECs to CMs.³² There is also an upregulation of oxytocin receptors and Gata-4 (a protein required for the atria-ventricular formation and its functions) that are considered crucial to the oxytocin-mediated cardiomyocytes differentiation process.³³

1.3.3. Effect of oxytocin on blood vessels (endothelial cells and VSMCs)

Several pre-clinical studies have demonstrated that oxytocin has shown anti-oxidant and anti-inflammatory effects in vascular endothelial cells (derived from the aorta), THP-1 monocyte, and macrophages. The physiological levels of oxytocin have shown significant reduction in nicotinamide adenine dinucleotide phosphate (NADPH) superoxide production, and also decreased the levels of the interleukin-6 (IL-6) in monocytes, macrophages, and aortic endothelial cells.³⁴

It has been reported that in diabetes, an independent risk factor for many cardiovascular diseases (CVD),³⁵ neovascularization is significantly impaired after ischemia.³⁶ The endothelial cells lose the ability to recover significant blood flow due to endothelial dysfunction. Besides, the progenitor endothelial cells suffer reduced angiogenic functions.³⁷ Oxytocin has been shown to restore angiogenic functions in a streptozocin-induced diabetes rat model. The pre-treatment of cultured bone marrow-mesenchymal stem cells (MSCs) with oxytocin has shown significant restoration of kruppel-like factor 2 (KLF2) which is a crucial angiogenic factor.³⁸

Many in vitro studies have shown that oxytocin, in endothelial cells derived from the human dermal microvasculature, umbilical vein and breast cancer cells, induces an overexpression of vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMP-11, MMP-17 and MMP-26), integrin β 6 genes, and cathepsin D.^{39,40} Thereby, stimulating migration and proliferation (which are the crucial steps in angiogenesis) of endothelial cells.

These studies have suggested that oxytocin induces angiogenic effects via an oxytocin receptor stimulation of the phosphatidylinositol-3-kinases (PI3K)/Akt/endothelial nitric oxide synthase (eNOS) pathway. It has been determined that the ability of oxytocin to phosphorylate Akt and eNOS depends on the PLC activity.³⁹ A subsequent study has revealed that it is through the stimulation of PLC and calcium mobilization, oxytocin phosphorylates the tyrosine kinases: proline-rich tyrosine kinase-2 (Pyk-2) and Src. This activates the PI3K/Akt/eNOS pathway.⁴¹

It is interesting to note that most of the cardiovascular effects of oxytocin have been studied in vascular endothelial cells and cardiomyocytes. However, in VSMCs, only the presence of oxytocin receptors has been established thus far. There is very little information regarding oxytocin effects in VSMCs.^{42,43}

1.3.4. Effect of oxytocin in various pathophysiological cardiovascular conditions

Inflammation and oxidative stress are the two critical pathophysiological events in atherosclerosis.⁴⁴ In a rat model of atherosclerosis, the administration of oxytocin showed an improvement in the lipid profile and decreased the plasma levels of IL-6, monocyte chemoattractant protein-1 (MCP-1) and C reactive protein (CRP). It also improved the plasma levels of reduced glutathione (GSH) and nitric oxide (NO). Besides, oxytocin treatment significantly improved the histological structure of the aorta detected by a significantly decreased expression of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) in a rat model of atherosclerosis.⁴⁵

The ability of oxytocin to counteract oxidative stress and reduce inflammation underscores the importance of oxytocin in many other cardiovascular pathophysiological conditions including atherosclerosis.⁴⁵

In myocardial infarction (MI), oxytocin has displayed a cardioprotective role. In a rabbit model of MI, oxytocin has been reported to protect against myocardial injury through the phosphorylation of kinases such as extracellular signal-regulated kinase (ERK), signal transducer and activator of transcription 3 (STAT3), Akt, MMP and eNOS. Akt, ERK, and STAT3 are all pro-survival signals that may play an important role in cardiac ischemic preconditioning, thereby reducing ischemic damage to the heart.⁴⁶

In a rat model, oxytocin has mediated improvement in myocardial injury immediately after heart transplant by downregulating the chemotactic agents:

chemokine (C-X-C motif) ligand 1 (CXCL1), and macrophage inflammatory protein-2 alpha (MIP-2 α)/Chemokine (C-X-C motif) ligand 2 (CXCL2). These agents are known to recruit neutrophils in the myocardium.⁴⁷ In ovariectomized rats, the oxytocin pre-treatment protected from injury after MI by reducing plasminogen activator inhibitor-1 (PAI-1) concentrations.⁴⁸

1.4. Role of angiotensin II (Ang II) in the CVS and its pathophysiology

The emergence of the different functional aspects of oxytocin in the CVS continues to fascinate the scientific community. It is noteworthy that a central component of the CVS is the renin-angiotensin-aldosterone system (RAAS). The principal effector hormone of the RAAS is Ang II. It is an octapeptide, and an enzyme known as the angiotensin converting enzyme (ACE) catalyzes the conversion of angiotensin I (Ang I) into Ang II. Ang II affects all major organs of the body – heart, kidney, vasculature, and brain. In particular, Ang II has a diverse range of effects in the CVS.⁴⁹

Ang II causes vasoconstriction and maintains the blood pressure in the normal physiological conditions.⁵⁰ Besides, it is also involved in many CVD such as atherosclerosis, congestive heart failure, diabetes, hypertension, inflammation and MI.⁵¹ It mainly binds to the angiotensin type 1 receptors (AT-1R) in the CVS and execute its actions via stimulation of the mitogen-activated protein kinases (MAPKs) pathways, PI3K/Akt/mammalian target of rapamycin (mTOR) pathways, and Rho kinase pathways.^{51,52} These signaling pathways and kinases are involved in Ang II-mediated contraction of VSMCs, hypertrophy, hyperplasia, endothelial dysfunction, vascular diseases, and their progression.^{53,54}

In pathological conditions such as hypertension and atherosclerosis, Ang II significantly impacts the repair and the remodeling of VSMCs in large blood vessels. It phosphorylates ERK1/2 and p38 MAPKs, thereby inducing expression of immediate early response genes: c-fos, c-myc and c-jun. Thus, Ang II mediates cell proliferation, migration, and hypertrophy in VSMCs.^{55,56} Besides, Ang II

causes the migration of VSMCs on receiving pathogenic stimuli by inducing STAT3 phosphorylation, and its subsequent nuclear translocation induces expression of the genes specific to cell proliferation, hypertrophy, and migration of VSMCs.^{57,58}

Ang II is also a known pro-oxidant.⁵⁹ It causes production of reactive oxygen species (ROS) via the activation of NADPH oxidase, leading to increased oxidative stress. The Ang II-induced oxidative stress causes endothelial dysfunction and vascular inflammation by the activation of NF- κ B, a redox-sensitive nuclear transcription factor, and upregulation of adhesion molecules: intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), selectin, integrin, cytokines [IL-6, IL-12, IL-1, tumor necrosis factor- α (TNF- α)], and chemokines (MCP-1, IL-8, osteopontin).⁶⁰ It has been reported that glutathione s-transferase (GST) attenuates Ang II-induced STAT3 phosphorylation and exerts a protective effect in VSMCs. GST conjugates the reduced form of glutathione (a cellular antioxidant) with reactive oxygen species (ROS) and detoxifies it.⁵⁸

Ang II has been shown to influence fibrinolysis activity.^{51,61} A key regulator of endogenous fibrinolytic activity and thrombolysis resistance is PAI-1. It physiologically inhibits plasminogen activation. In response to vascular injury, PAI inhibits cellular migration and matrix degradation.^{62,63} Ang II infusion in normotensive and hypertensive patients showed increased circulating levels of PAI-1.⁶⁴ In a rat model, Ang II via interactions with AT1R, increased the expression of PAI in VSMCs from isolated aorta, thereby influencing endogenous fibrinolytic

activity.^{65,66} PAI-1 is an important cardiovascular risk factor in atherosclerosis. Besides, an increased PAI-1 also manifests amongst the metabolic abnormalities associated with hypertension.^{67,68}

The antagonizing of Ang II effects by employing ACE inhibitors is a well-established therapy commonly used in CVD. The ACE inhibitors are in clinical practice in the management of hypertension, coronary artery disease, heart failure, diabetes, and chronic kidney diseases.⁶⁹ They are one of the few treatment options that we have currently for these diseases. However, there are many side effects attributed to ACE inhibitors' use in CVD.⁷⁰ Despite the long clinical use of these agents, treatment of hypertension has not been all that successful. The need of the present times is to identify alternative targets and unveil better therapeutic approaches to better treat CVD.

1.5. The gap in the literature, research hypotheses, and study aims

As mentioned earlier in the background, oxytocin has demonstrated cardioprotective effects in various in-vitro and in-vivo studies performed on vascular endothelial cells and cardiomyocytes.²³ However, there is a gap in the literature. Even though previous researches have established that oxytocin receptors are found in VSMCs, interestingly, oxytocin effects in VSMCs have not been fully characterized. On the contrary, the effects of Ang II, another crucial cardiovascular peptide, have been well established in VSMCs and the CVS to a significant extent. As stated before, Ang II is known to induce vascular oxidative stress, phosphorylate ERKs, PI3Ks, and Rho kinases, leading to marked VSMCs proliferation, hypertrophy, hyper-contraction, inflammation, and increased oxidative stress.⁵¹ Therefore, to fill in the gap regarding the lack of research on the effect of oxytocin on VSMCs and its interaction with Ang II, the present study was conducted.

In the context of the protective effects of oxytocin in the cardiovascular system and from the results of the preliminary experiments, the following research hypotheses were proposed:

1.5.1. Hypothesis 1

By its ability to reduce proliferation, inflammation, oxidative stress, and vasoconstriction pathway, oxytocin antagonizes Ang II-induced proliferation, inflammation, and oxidative stress and dysregulates Ang II-induced vasoconstriction pathway in primary Wistar aortic VSMCs.

1.5.2. Hypothesis 2

Oxytocin signaling pathways in aortic VSMCs are altered in hypertensive conditions.

The hypotheses mentioned above were tested by pursuing the following specific aims:

1.5.3. Specific Aim 1

To characterize the effect of oxytocin on proliferation, inflammation, oxidative stress and vasocontraction in primary aortic VSMCs obtained from normotensive Wistar rats. The VSMCs proliferation was determined by employing cell proliferation, cell viability, and cell growth assays. To further understand the mechanism of how oxytocin reduces aortic VSMC proliferation, the two critical signaling pathways involved in VSMCs proliferation were investigated: ERK1/2 pathway and PI3K/Akt pathway. The oxidative stress was measured as the function of the ROS levels, and inflammation was assessed by measuring IL-6 levels. The effect of oxytocin on vasocontraction was assessed indirectly by investigating oxytocin's effect on the Rho-kinase pathway [Rho-associated coiled-coil kinase-1 (ROCK-1) and -2 (ROCK-2)] involved in the vasocontraction of VSMCs.

1.5.4. Specific Aim 2

To determine of the interaction between oxytocin and Ang II in primary aortic VSMCs obtained from normotensive Wistar rats. The interaction of oxytocin with Ang II was investigated by studying the effect of pretreatment of oxytocin on Ang II-induced cell proliferation and proliferation

pathways (the ERK1/2 pathway and PI3K/Akt pathway), inflammation, oxidative stress, and the Rho-kinase pathway in aortic VSMCs obtained from normotensive Wistar rats.

1.5.5. Specific Aim 3

To characterize the effect of oxytocin on proliferation, inflammation, oxidative stress and vasocontraction in primary aortic VSMCs obtained from spontaneously hypertensive rats (SHRs). The effects of oxytocin on VSMC proliferation (ERK1/2, PI3K, Akt), oxidative stress (ROS), inflammation (IL-6) and vasocontraction (ROCK-1 and ROCK-2) were characterized on VSMCs isolated from SHRs. The comparison was made, and the analysis was carried out between the effects of oxytocin on its own and its pre-treatment with Ang II in aortic VSMCs Wistar rats and SHRs.

1.6. Role of VSMCs in the CVS and its pathophysiology

VSMCs constitute tunica media, the middle layer of blood vessels, with endothelial cells and connective tissues making tunica intima and tunica externa, respectively (**Figure 1**). VSMCs are the stromal cells which perform a variety of functions and play a crucial role in the CVS.⁷¹

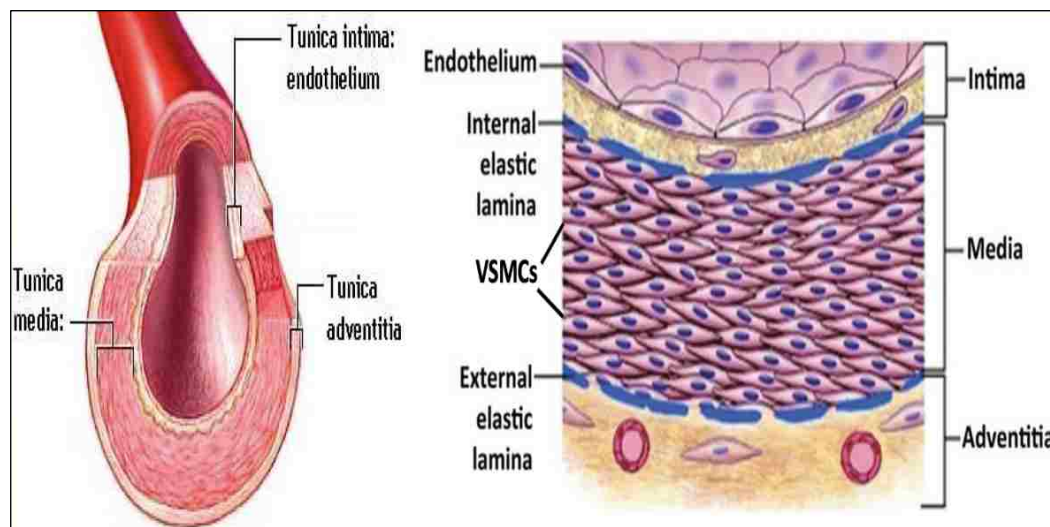


Figure 1. Histology of a blood vessel. VSMCs form the middle layer (tunica media) of a blood vessel. Adapted from *Muscle cell and Tissue*, Intech Open. 2018;230.

VSMCs maintain the contractile tone of the arterial wall via intracellular calcium release causing the myosin-actin interactions, regulating blood pressure and flow to meet the metabolic demands of the body. They secrete molecules that comprise the extracellular matrix (ECM) which enable arteries to withstand the high pressure of the circulating blood.⁷²

In normal physiological conditions, VSMCs remain quiescent and adhere to their phenotype essential in regulating contractile tone of arteries. Molecules such as laminin, perlecan, heparan sulfate proteoglycan (HSPG), and collagen type

IV, composing the basement of VSMCs are attributed to keeping VSMCs quiescent.⁷³

However, in the event(s) of pathological or pathophysiological condition(s) such as arterial injury, an excessive overload of lipids, pressure-related stress, hyperglycemic conditions, VSMCs undergo a variety of adaptations and remodeling. They become active and transition into mitogen-responsive phenotypes in the presence of fibronectin.⁷⁴ They start interacting with the intracellular signaling pathways, involving the ERKs, PI3Ks, and Rho kinases.^{75,76}

The activation of these pathways leads to hypercontraction, proliferation and migration of VSMCs often leading to the thickening of the tunica intima, hyperplasia, precipitating aneurysm, hemorrhage, and restenosis, and worsening the other cardiovascular diseases including atherosclerosis, hypertension and increasing the complications post-vascular surgical procedures.^{72,77}

The activated phenotype of VSMCs also produce ROS which play a role in the vascular pathology through its redox potential.⁷⁸ The principal enzymatic source of the production of ROS in VSMCs is NOX. In hypertension, vasoactive agents such as Ang II activate the NOX, leading to the increased production of ROS, ultimately resulting in increased oxidative stress and increased expression of inflammatory mediators such as cytokines (example IL-6).⁷⁹

The functions of VSMCs in physiology and their role in the pathophysiology of the CVS are crucial and render them a potential target of therapeutic approaches in cardiovascular research. Anti-proliferative therapies

such as rapamycin and paclitaxel targeting VSMCs' hyperplasia in animal models have failed to show similar effects in patients.⁸⁰

Therefore, there is a need to develop therapies that are not only targeting the most notable pathophysiological features of CVD such as proliferation, migration, hypercontraction, oxidative stress, and inflammation of VSMCs but are also maintaining contractile phenotype of VSMCs.

1.7. Study significance

It was envisioned that this study would be a step towards the long-term goal of identifying oxytocin as an alternative therapeutic target in the treatment of CVD. The present study was an attempt to determine the potential role of oxytocin with regards to its cardioprotective effects against Ang II-induced deleterious effects. Previous studies have established that at sustained high levels, Ang II acts as the ‘aggressor’ in the cardiovascular system.⁵³ On the contrary, pre-clinical evidence have, so far, indicated a ‘protective’ role of oxytocin in the cardiovascular system.²³

This in vitro study was the first of its kind to investigate and understand the protective effects of oxytocin against the harmful actions of Ang II, with relation to VSMCs, on the cardiovascular system. The study findings contributed towards a better understanding of oxytocin actions on blood vessels (VSMCs), and identification of secondary messenger pathways involved in this oxytocin effect.

The research findings demonstrated that oxytocin blocked Ang II-mediated hyperproliferation of VSMCs and proliferation and vasoconstriction pathways in VSMCs from SHRs, thus providing evidence of oxytocin’s vital role in cardiovascular pathophysiology and it shows the potential therapeutic benefits it might have in the management of hypertension. The study generated significant pre-clinical evidence regarding the beneficial effects of oxytocin in a hypertensive rat model, laying a platform foundation for future mechanistic in vivo studies and pilot clinical studies.

1.8. Barriers, issues, limitations, and delimitations

The present study involved the use of primary rat aortic VSMCs. The advantage of using laboratory-cultured primary VSMCs is that they retain many cellular functions and markers quite similar to the ones observed *in vivo*. Whereas, commercially available VSMCs cell lines may differ phenotypically and genetically from their origin. However, there were specific challenges that occurred during the course of this study.

Firstly, the procurement of significant yield of high purity VSMCs was challenging. The techniques utilized in this research to harvest primary cells was the ‘explant method.’⁸¹ Although this technique is cost-effective and routinely applied to obtain a high yield of VSMCs, its major drawback is the purity of primary cells obtained. That VSMCs are prone to natural contamination with intimal endothelial or adventitial fibroblasts, the VSMCs isolation protocol was optimized, and the cells were used from passages between 3-15 for maintaining the phenotypical uniformity and the purity of cells.⁸¹ The endothelial cells and fibroblast eventually die out by the time VSMCs underwent the third passage.

Secondly, the effect of many cell passages was a challenge. For avoiding this, care was taken to make sure that all the experiments in both Wistar rats and SHR were conducted using the same passage number. For example, if a cell proliferation experiment was carried out with VSMCs in the passage 4 in Wistar rats, the VSMCs from the similar passage number was utilized to perform a cell proliferation experiment in SHRs.

Lastly, the magnitude of the response of cells to the treatments obtained from one rat was different from the other rat. Therefore, to reduce the variation in data and increase the reproducibility of the data, the experiments were repeated numerous times using multiple rats to increase the number of replicates. However, the repetition of all the experiments was tough, time-consuming and challenging.

Chapter 2: Literature Review

2.1. MAPK/ERK1/2 pathway in the CVS

MAPKs belong to the serine-threonine specific class of kinases. They constitute a signal transduction pathway known as the MAPK pathway. The MAPK pathway is a cascade that converts signals from external mechanical stimuli and growth factors into signal transduction, leading to the regulation of genes involved primarily in protein synthesis.⁸² It occurs in sequential phosphorylation of the three core protein kinases from cytosol: mitogen-activated protein kinase kinase kinase (MAPKKK), mitogen-activated protein kinase kinase (MAPKK), and mitogen-activated protein kinase (MAPK).⁸³ A schematic representation of the activation of MAPK signaling pathway is shown in the **Figure 2.1**.⁸⁴

It is the MAPKs which carry out intracellular signal transduction through three distinct pathways. The first one is through the phosphorylation and activation of extracellular signal-regulated kinase (ERK), which has two isoforms: ERK1 and ERK2. The phosphorylated ERK1 and 2 (active form) lead to the phosphorylation and activation of various regulatory proteins in the cytosol as well as in the nucleus, which is critical for protein synthesis and cell survival.⁸³

The second MAPK pathway involves the phosphorylation and activation of stress-activated protein kinases (SAPK). This cascade of MAPK

is triggered in response to external stimuli such as ultraviolet rays, stress (heat shock), altered osmolarity, hypoxia, and exposure to reactive oxygen species.⁸⁵

The triggering of the second MAPK pathway causes the phosphorylation and activation of c-Jun N-terminal kinases (JNK) which is critical in proliferation, apoptosis, inflammation, and in response to deoxyribonucleic acid (DNA) damage.^{86–88}

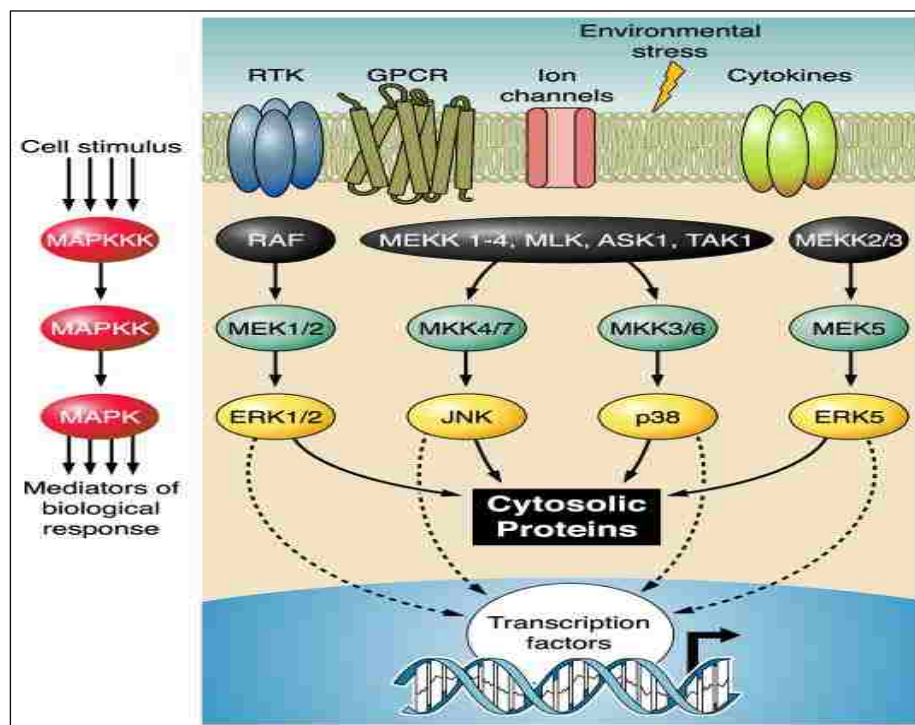


Figure 2.1. Activation of mitogen-activated protein kinase (MAPK) signaling pathway in the cardiovascular system. Adapted from *Physiol Rev.* 2010;90(4);1508. GPCR indicates G-protein coupled receptor; RTK, receptor tyrosine kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; RAF, rapidly accelerated fibrosarcoma ; MLK, mixed-lineage kinases; ASK-1, apoptosis signal-regulating kinase 1; TAK-1, transforming growth factor- β -activated kinase 1; MAPKKK/MEKK, mitogen-activated protein kinase kinase kinase; MAPKK/MEK/MKK, mitogen-activated protein kinase kinase; MAPK, mitogen-activated protein kinase.

The third pathway consists of p38 MAPK. Like the JNK pathway, this pathway can also be triggered in response to stress induced by a change in cellular osmolarity, exposure to free radicals, heat shock, and inflammation.⁸⁹ The p38 MAPK signaling is involved in cellular processes such as proliferation, differentiation, migration and immune response.⁹⁰⁻⁹²

The ERK1/2 pathway is one of the first MAPK pathways to be characterized in mammals. In VSMCs, the stimulation of AT1R by Ang II, causes the phosphorylation of ERK1/2. The phosphorylated ERK1/2 (active form) then translocate to the nucleus, and subsequently result in the phosphorylation of transcription factors including c-Fos, c-Jun, and activator protein-1 transcription factors, ultimately carrying out cell synthesis, proliferation, differentiation and migration of VSMCs. In addition, ERK1/2 targets the ribosomal S6 kinase (RSK). Once phosphorylated, the activated ERK1/2, in turn, phosphorylates RSK via the transfer of ribonucleic acid (RNA)-binding factor. This is a key step in the initiation of translation, leading to protein synthesis, regulation, and progression of the cell cycle of VSMCs.⁹³⁻⁹⁵

Phosphorylated ERK1/2 is also known to be involved in the regulation of vascular tone of VSMCs. It has been established that h-Caldesmon (h-CD) is a downstream target of the phosphorylated ERK1/2.⁹⁶ An h-CD is a high molecular weight contractile regulatory protein involved in the contractile responses of vascular walls.⁹⁷

Numerous studies have implicated ERK1/2 signaling in the pathophysiology of CVDs such as hypertension.^{98,99} In an in vivo study,

increased activity of ERK1/2 was reported after transient elevation of blood pressure with Ang II in femoral arteries and rat aortas.¹⁰⁰

The pharmacological inhibition of ERK1/2 signaling was shown to improve endothelial function, and significantly reduced Ang II-induced contraction of mesenteric resistance arteries in spontaneously hypertensive rats (SHRs).¹⁰⁰ In transgenic mice models, ERK1/2 signaling was shown to stimulate hypertrophy in cardiomyocytes via phosphorylation of factors such as elk-1, necessary for the activation of the c-Fos gene.¹⁰¹

Oxytocin was demonstrated to exert differential effects on the ERK1/2 signaling pathway. For example, oxytocin-induced phosphorylation of ERK1/2 increased the proliferation of cardiomyocytes and vascular endothelial cells.^{40,102} In other instances, such as in human embryonic kidney (HEK293) cells, oxytocin exhibited proliferative as well as anti-proliferative actions. The dual action of oxytocin on the proliferation of HEK293 cells was determined by the location of the oxytocin receptors. Oxytocin reduced the phosphorylation of ERK1/2, and as a result, inhibited cell proliferation when the oxytocin receptors were present outside the caveolin-1-enriched microdomains. And, oxytocin augmented phosphorylation of ERK1/2, resulting in stimulated cell proliferation when the oxytocin receptors were inside caveolin-1-enriched microdomains.¹⁰³

It is imperative to understand the role of caveolins. Caveolins are scaffolding proteins involved in the regulation of GPCR signaling. They facilitate GPCR regulation via scaffolding and partitioning of different receptors, G-proteins trafficking, and signaling.¹⁰⁴

As stated earlier, oxytocin's effect on cell proliferation and ERK1/2 activity has been studied in cardiomyocytes and vascular endothelial cells. However, little to no information is available on the effects of oxytocin on ERK1/2 signaling pathway and on its effects on the proliferation of VSMCs.

The present study characterized the effect of oxytocin and its impact on Ang II (known for stimulating cell proliferation via the ERK1/2 signaling⁵⁵) on cell proliferation in aortic VSMCs from normotensive rats (Wistar rats). Further, oxytocin's impact on aortic VSMCs proliferation and on ERK1/2 signaling, and oxytocin's effect on Ang II-mediated cell proliferation and Ang II-stimulated ERK1/2 signaling were also investigated in aortic VSMCs from SHRs.

2.2. PI3K/Akt signaling in the CVS

The phosphoinositide-3-kinase (PI3K)/Akt pathway is an essential intracellular signaling transduction pathway involved in the regulation of the CVS.¹⁰⁵ It is crucial in the proliferation of VSMCs,¹⁰⁶ control of the vascular myogenic tone, and inflammation.^{107,108} Over the last decade, the PI3K/Akt signaling has been implicated in various pathological conditions of the CVS¹⁰⁹ such as cardiac hypertrophy (a critical risk factor of heart failure),¹¹⁰ and hypertension.¹⁰⁷

Phosphoinositide-3-kinases (PI3Ks) are intracellular enzymes which catalyze the phosphorylation of phosphatidylinositol (PI). PI are lipids (phosphatidyl glycerides).¹¹¹ PI3Ks phosphorylate PI at the 3-hydroxyl group of the inositol ring. Based on the products of the catalysis, PI3Ks have been grouped into three classes: Class I PI3Ks, which form phosphatidylinositol-3,4-bisphosphate (PI-3,4-P₂); Class II PI3Ks, which form phosphatidylinositol-3-phosphate (PI-3-P), PI-3,4-P₂, and phosphatidylinositol triphosphate (PIP₃); and Class III PI3Ks, which generate PI-3-P. All classes of PI3Ks are present in cardiomyocytes, vascular endothelial cells, and VSMCs.¹¹²

A general structure of a PI3K includes three domains: C-2 domain, helical domain, and a catalytic domain. The C-2 domain binds to the membranes, the helical domain serves regulatory functions, and the catalytic domain carries out the kinase activity of PI3Ks. Based on the regulatory domain, the Class I PI3Ks are further subdivided into two categories: Class I A PI3Ks which bind to receptor tyrosine kinases (RTKs), and Class I B PI3Ks which bind to GPCRs. The Class I

A PI3Ks exist as heterodimers of different isoforms of α , β , and δ of the p110 catalytic domain. Whereas, the Class I B PI3Ks exist as heterodimers of p110 γ and p101 catalytic domains.¹¹³ All Class I PI3Ks are significant in the CVS and its pathophysiology.¹¹⁴ Their actions are antagonized by phosphatase and tensin homolog deleted on chromosome ten (PTEN).¹¹⁵

Akt is a serine-threonine kinase and also known as protein kinase B (PKB). Akt is a downstream signaling target of PI3Ks. There are three isoforms of Akt: Akt 1 (PKB α), Akt 2 (PKB β), Akt 3 (PKB γ). All forms of Akt are present throughout blood vessels, heart, lungs, and brain.¹¹⁶ The stimulation of GPCRs by peptides such as Ang II or growth factors results in the activation of PI3Ks. The activated PI3Ks lead to the formation of PI-3,4-P₂ and PIP₃, which in turn phosphorylate Akt (active form).¹¹⁷

The phosphorylated form of Akt triggers downstream signaling via the phosphorylation of downstream signaling molecules such as mTOR. mTOR is a serine-threonine kinase. The phosphorylation of mTOR by phosphorylated Akt is vital to protein synthesis by the PI3K/Akt signaling pathway. mTOR has two isoforms: mTORC1 and mTORC2. The phosphorylated mTORC1 stimulates cell proliferation via the phosphorylation of p70S6 kinase, eukaryotic initiation factor 4E (eIF4E), and 4E-binding protein 1 (4E-BP1). The activated p70S6 kinase phosphorylates and activates S6 ribosomal protein. The activated eIF4E acts as translator initiator. It phosphorylates 4E-BP1. A phosphorylated 4E-BP1 is required for protein translation. The phosphorylated mTORC2 stimulates the

phosphorylation of Akt and is involved in the autoregulation of mTOR.¹¹⁸ The PI3K/Akt signaling pathway is illustrated in the following figure (**Figure 2.2**):

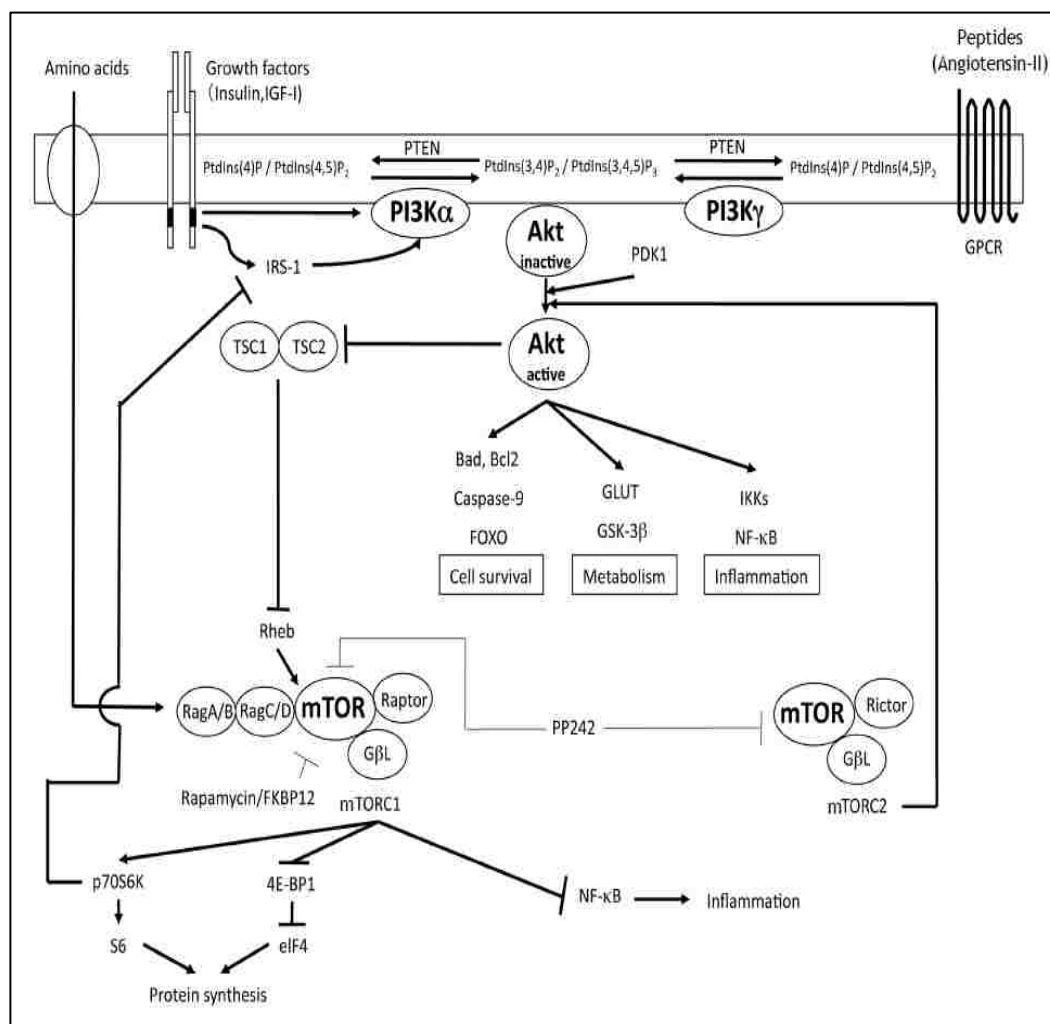


Figure 2.2. The PI3K/Akt signaling pathway in the cardiovascular system. A schematic representation of the PI3K/Akt signaling pathway in cardiomyocytes, vascular endothelial cells, and vascular smooth muscle cells (VSMCs). Adapted from *Curr Pharm Des.* 2011;17(18):1819. FKBP indicates FK506-binding protein; GLUT, glucose transporter; GPCR, G-protein coupled receptor; GSK, glycogen synthase kinase; IGF, insulin-like growth factor; PDK, phosphoinositide-dependent kinase; PI3K, phosphoinositide 3-kinase; PtdIns, phosphatidylinositol; PTEN, phosphatase and tensin homolog deleted on chromosome ten; p70S6K, p70S6 kinase; TSC, tuberous sclerosis complex; eIF4, eukaryotic initiation factor 4.

The implication of PI3K/Akt signaling cascade in many cardiovascular pathophysiological conditions has been reported by previous studies.¹¹⁹ In hypertension, it has been shown that the PI3K/Akt pathway alters vascular function mainly by increasing the myogenic tone of resistance arteries and by enhancing the constricting activity of VSMCs. A myogenic tone can be described as the functional behavior of resistance arteries in response to perfusion pressure.¹⁰⁷ On the activation of GPCRs by cardiovascular peptides such as Ang II, the PI3K/Akt signaling results in an increased intracellular level of calcium via the L-type voltage-gated calcium channel (L-VGCC).^{120,121}

It is noteworthy that L-VGCC dysfunction is an important characteristic feature of CVDs such as hypertension and cardiac arrhythmia, and its targeting by the compounds known as calcium channel blockers has been a long-standing clinical practice in the management of hypertension and arrhythmia.^{122,123}

Several studies have documented the relevance of PI3Ks in hypertension.^{107,124,125} A study conducted using a transgenic mice model revealed that the chronic administration of Ang II did not produce hypertension in mice lacking PI3K γ . The lack of PI3K γ not only reduced the L-VGCC-mediated intracellular calcium reflux but also decreased VSMCs contractility when stimulated with Ang II.¹²¹

Another relevant aspect of the PI3K/Akt signaling in the physiology and pathophysiology of the CVS is its role in cardiac hypertrophy.¹¹⁰ It is pertinent to highlight that cardiac hypertrophy can be both beneficial and harmful. Beneficial

cardiac hypertrophy is called ‘physiological cardiac hypertrophy,’ whereas the harmful hypertrophy is known as ‘pathological cardiac hypertrophy.’¹²⁶

Physiological cardiac hypertrophy is usually seen and is associated with natural post-natal development, pregnancy, and exercise. Thus, this is an adaptive growth of cardiomyocytes. The characteristic features of physiological cardiac hypertrophy are the preservation of contractile functions of the sarcomere (the contractile unit of cardiomyocytes) and a lack of interstitial fibrosis.^{127,128} On the other hand, pathological cardiac hypertrophy is ‘maladaptive hypertrophy.’ It is characterized by increased cell volume of cardiomyocytes, the new formation of sarcomeres, interstitial fibrosis and inflammation.¹²⁹

Several studies have demonstrated PI3K/Akt-mediated cell signaling in both physiological and pathological cardiac hypertrophy.^{130,131} In a study employing a transgenic mice model, mice with dominant negative PI3K showed a significant reduction in exercise-induced physiological cardiac hypertrophy compared to wild type mice (control).¹³² In another study employing a similar transgenic mice model, mice deficient in PI3K showed protection against isoproterenol-induced pathological cardiac hypertrophy and thus protection from heart failure.¹³³

It is thought-provoking that oxytocin is being touted as a ‘cardioprotective’ hormone,²³ yet it has not been investigated for its effects on PI3K/Akt signaling in cardiomyocytes, vascular endothelial cells, and VSMCs. To partially address this gap in the scientific literature, the present study used an in vitro model to investigate

the effects of oxytocin alone and its pre-treatment with Ang II on the PI3K/Akt signaling pathway in VSMCs in both normotensive and hypertensive conditions.

2.3. Rho kinases (ROCK-1 and ROCK-2) signaling in the CVS

Rho-associated coiled-coil kinases (ROCK) -1 and -2 belong to the serine/threonine category of kinases. They are the targets of small GTPases (that bind and hydrolyze the guanosine triphosphates) : Rho A, Rho B, and Rho C.¹³⁴ These GTPases are found abundantly in VSMCs and are rapidly activated by vasoconstricting peptides such as Ang II.¹³⁵ Both ROCK-1 and ROCK-2 are involved in the organization and remodeling of actin cytoskeleton, extracellular matrix, and in the contraction of VSMCs.^{136,137} They are also critical in cell adhesion, motility, and proliferation.^{138,139} Pre-clinical research and clinical evidence have implicated ROCK signaling in the pathogenesis of various diseases of the CVS including angina pectoris, hypertension, pulmonary arterial hypertension, heart failure, and stroke.¹⁴⁰⁻¹⁴⁴

The structural features of ROCKs include phosphorylation and cleavage sites. The structure of both ROCK-1 and ROCK-2 has an N-terminal containing a kinase domain, coiled-coil region, Rho-binding domain (RBD), and a C-terminal comprising of a PH-domain containing a cysteine-rich C1 domain. An RBD possesses three zones known as a 'switch': Switch I, Switch II, and Switch III, with each having a strong binding affinity for Rho A, Rho B, and Rho C, respectively. The C1-domain of C-terminal is involved in the autoinhibition of ROCKs. The two isoforms of ROCK have the highest homology in their kinase domain (about 92 percent) and the least homology in their coiled-coil domain (about 55 percent).

The activation of ROCKs depends on the catalytic activity of both the N-terminal and C-terminal regions. The ROCKs form homodimers, and dimerization depends upon kinase domains, an extension of N-terminals, and coiled-coil regions. Following an interaction of RBD with guanosine-5'-triphosphate (GTP)-loaded Rho proteins (Rho-A, -B, -C), the phosphorylation of the hydrophobic motif near the C-terminal region occurs, causing the kinase domain to undergo conformational changes from a closed (inactive) to an open (active) state.^{147,148} The active state of ROCKs is essential in carrying out the downstream cell signaling in the following three different ways:

First, the activated form of ROCKs directly augments the phosphorylation of myosin phosphatase target subunit-1 (MYPT-1). Second, it stimulates the phosphorylation of zipper-interacting protein kinase (ZIPK) which in turn phosphorylates MYPT-1.^{149,150} Third, activated ROCKs phosphorylate C-kinases potentiated protein phosphatase 1 inhibitor -17 (CPI-17). A CPI-17 is a direct inhibitor of MYPT-1.^{151,152}

The phosphorylation of MYPT-1 prevents the binding of myosin light chain phosphatases (MLCP) with myosin light chain (MLC), thus boosting myosin light chain kinase (MLCK)-induced phosphorylation of actin leading to sustained contraction of VSMCs. The stimulation of the ROCK signaling results in decreased activation of MLCP, and increased contraction of VSMCs which ultimately leads to elevated blood pressure.^{151,152} The ROCK-mediated contraction of VSMCs is depicted in the **Figure 2.3**.

In animal models of hypertension, an increased ROCK signaling has been observed which is further enhanced by the intake of a high-salt diet.¹⁵³ An increased ROCK signaling in hypertension has been attributed to the dysregulation of Rho guanine nucleotide exchange factors (Rho-GEFs).^{154,155} In clinical studies, an elevated phosphorylation level of MYPT-1 has been reported in patients diagnosed with essential hypertension. Ang II-induced hypertension and cardiac hypertrophy have also been linked with hyper-ROCKs signaling.^{156,157}

In samples of heart-tissues obtained from patients with heart failure, truncated ROCKs have been found. A cleavage of C-terminal of ROCKs by the activation of pro-apoptotic enzymes such as caspases abolishes the autoinhibitory capability of ROCKs. This leads to constitutive activation of ROCKs, resulting in a sustained hypercontraction of blood vessels, fibrotic cardiomyopathy, and diastolic dysfunction.¹⁵⁸

These detrimental effects of truncated ROCKs were further supported by findings from a study carried out on cardiomyocytes from mice. The cardiomyocytes showed an increased expression of C-terminally nicked ROCK-1, and the histopathological findings presented the characteristics of fibrotic cardiomyopathy.¹⁵⁹

Over the years many compounds have been developed to target the ROCK signaling. A wide range of those compounds are often non-selective ROCK inhibitors (i.e., they are non-specific in their binding to the two ROCK isoforms). They compete for ATP in the kinase domain of ROCKs, thus acting as competitive

inhibitors.¹⁶⁰ Fasudil is the only ROCK inhibitor that has been approved for clinical use for the treatment of various CVD including hypertension.¹⁶¹

Medical interventions employing fasudil in patients with hypertension, pulmonary arterial hypertension (PAH), angina pectoris, and heart failure have shown significant improvement in their medical conditions.^{142,162–164} The administration of fasudil in patients with congenital heart defects significantly reduced pulmonary artery pressure and pulmonary vascular resistance.¹⁶⁵

Evidence presenting an increased expression of ROCK-2 in the pulmonary arteries and pulmonary VSMCs in patients with idiopathic PAH has demonstrated the role of ROCK signaling in PAH.¹⁶⁶ This was further strengthened by another study in which patients with PAH treated with fasudil resulted in acute pulmonary vasodilation.¹⁶⁷ Chronic treatment with fasudil not only resulted in an improvement in pulmonary hypertension, but also reduced right ventricular hypertrophy, and prevented further remodeling in the pulmonary vasculature, along with significant decrease in VSMCs proliferation and hypercontraction, and lower endothelial dysfunction.¹⁶⁸

Previous studies have highlighted enhanced activity of ROCK in patients with acute and chronic heart failure.¹⁶⁹ ROCK activity measured in terms of phosphorylated levels of total MYPT-1 in circulating leukocytes indicates the involvement of ROCK signaling in heart failure.¹⁷⁰ Also, stimulated ROCK activity has been found in patients with myocardial infarction. A study in which fasudil was administered to patients with heart failure showed a significant decrease in vascular resistance in the forearm and a noticeable increase in forearm vasodilation.¹⁷¹

Another clinical study showed the beneficial effect of ROCK inhibitors in patients with angina. The treatment with fasudil resulted in reduced number of angina attacks per week in patients with stable angina and also increased their maximum exercise time.^{162,164}

The literature presented above underlines a crucial role of ROCK signaling pathway in physiological and pathophysiological conditions of CVS. Interestingly, oxytocin effect on ROCKs' signaling has not been previously studied. The present study endeavored to investigate the effect oxytocin, and its impact on Ang II-stimulated ROCK signaling in aortic VSMCs isolated from normotensive and hypertensive rats.

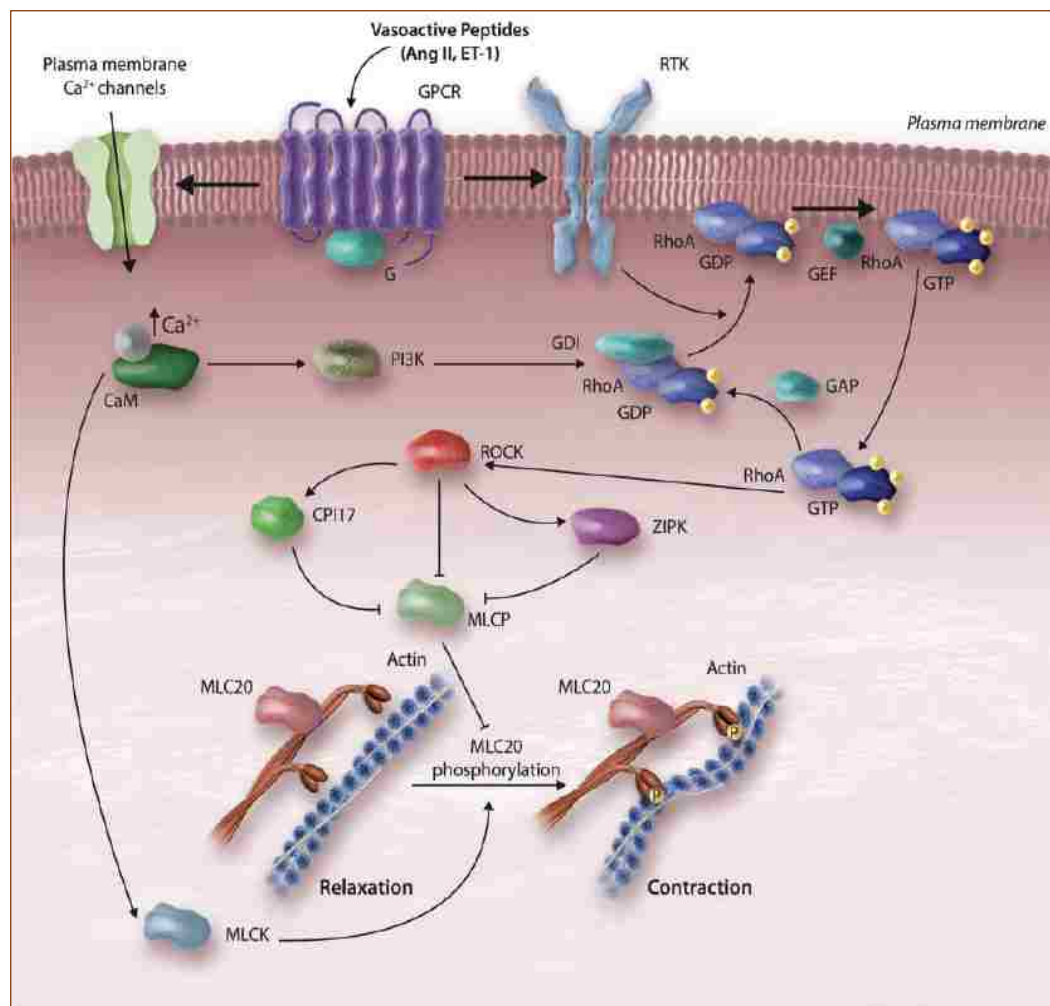


Figure 2.3. ROCK signaling pathway in the contraction of VSMCs. Adapted from *Cardiovasc Res.* 2018;114(4):534. Ang II indicates angiotensin II; ET-1, endothelin-1; GPCR, G-protein coupled receptor; RTK, receptor tyrosine kinase; GDP, guanosine diphosphate; GTP, guanosine triphosphate; GEF; guanine nucleotide exchange factor; GDI, guanine nucleotide dissociation inhibitor; PI3K, phosphoinositide-3-kinase; ROCK, rho-associated coiled-coil kinase; CPI-17, C-kinases potentiated protein phosphatase 1 inhibitor-17; MLCP, myosin light chain phosphatases; ZIPK, zipper-interacting protein kinase; MLCK, myosin light chain kinase.

2.4. Interleukin-6 in the CVS

IL-6 is a cytokine which has pleiotropic effects.¹⁷² It is crucial in the CVS and its diseases.¹⁷³ IL-6 is involved in the process of inflammation eliciting a myriad of immunological responses such as activation of thymocytes, stimulation and activation of T-cells and B-cell differentiation, differentiation and galvanization of macrophages, activation of natural killer (NK) cells, and stimulation of hepatocytes for the production of inflammatory proteins such as the CRP.¹⁷⁴⁻¹⁸¹

IL-6 binds to the IL-6 receptor complex to induce the signal transduction. The IL-6 receptor complex is comprised of two membrane-bound glycoproteins: IL-6R and glycoprotein 130 (gp130). The IL-6R is an 80 Kilo dalton (KD) component which binds to a ligand. The gp130 is the signal transducing part of the IL-6 receptor complex with an atomic mass of 130 KD.¹⁸² Also, there is a soluble form of IL-6R (termed sIL-6R). The binding of IL-6 to the membrane-bound IL-6R has been attributed to normal physiological immune responses of IL-6.¹⁸³

However, the binding of IL-6 to the soluble form (IL-6/sIL-6R) has been implicated in various pathological conditions,^{184,185} and has been clinically targeted by anti-IL-6 agents such as tocilizumab in severe cases of rheumatoid arthritis (RA).^{186,187,188} The IL-6 and its receptors both soluble and membrane-bound are present ubiquitously in all tissues, and abundantly in endothelial cells, VSMCs, and cardiomyocytes in particular.¹⁸⁹⁻¹⁹¹

The effects of IL-6 are both protective and detrimental.¹⁹² The duration of pathogenic stimuli or conditions determines these actions whether they would be

beneficial or deleterious.^{193,194} Short-term exposure to pathogenic stimuli results in protective effects of IL-6, whereas, a long-term exposure culminates in IL-6 driven harmful effects. A classic example of such consequences is the effects of IL-6 on the heart. IL-6 signaling in cardiomyocytes is cardioprotective in response to a short-term pathophysiological condition.¹⁹⁴⁻¹⁹⁶ However, when that pathophysiological condition remains for a long time, then the IL-6 signaling induces pathological cardiac hypertrophy, reduces cardiac contractility, ultimately leading to decreased cardiac function.^{193,197,198} Studies have highlighted IL-6 mediated decreased cardiac contractility is associated with the JAK/STAT pathway and not the ERK pathway.¹⁹⁹

IL-6 has been implicated in the pathogenesis of coronary artery disease such as atherosclerosis.²⁰⁰ Previous studies have shown the IL-6-mediated upregulation of MMPs contribute to vascular remodeling and atherosclerotic plaque disruption.²⁰¹ Many studies have demonstrated higher serum levels of IL-6 in patients diagnosed with and hospitalized for unstable angina.²⁰²

The increased expression of myocardial IL-6 has been associated with the progression of heart disease.^{203,204-206} Studies have established elevated levels of IL-6 in patients with heart failure. More specifically, the concentrations of IL-6 were directly related to the severity of left ventricular dysfunction and to the extent of the activation of the sympathetic system and RAAS.²⁰⁷

Ang II, an effector hormone of the RAAS, is known to induce inflammation in VSMCs via stimulation of IL-6 signaling.²⁰⁸ Oxytocin, on the other hand, has shown anti-inflammatory actions via decreasing IL-6 signaling in vascular

endothelial cells.³⁴ The effects of oxytocin on inflammation in VSMCs have not been studied. Interestingly, its interaction with Ang II with regards to inflammation has also not been investigated. This study undertook the investigation of oxytocin's effect on inflammation, and its interaction with Ang II in aortic VSMCs isolated from both normotensive rats and hypertensive rats.

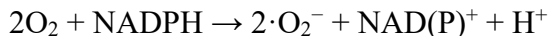
2.5. Reactive oxygen species (ROS) in the CVS

The ability of a living organism to convert oxygen into water through aerobic phosphorylation forms the core of the metabolism and production of energy. This very process of the metabolism of oxygen yields a byproduct known as 'ROS.'²⁰⁹ These toxic species are extremely deleterious in nature. They cause severe damage to cellular DNA, RNA, lipids, and proteins, resulting in cellular apoptosis, necrosis, and inflammation.²¹⁰⁻²¹² ROS play a significant role in the development of various pathological condition of the CVS including hypertension, cardiac hypertrophy, heart failure, diabetes, and atherosclerosis.^{78,213-216}

The primary source of ROS in cells in general, and in vascular endothelial cells, VSMCs, and cardiomyocytes, in particular, is an enzyme called 'nicotinamide adenine dinucleotide phosphate (NADPH) oxidase.'²¹⁷

The catalytic component of NADPH oxidase is NOX. There are seven isoforms of NOX that have been identified, and five of them are found in human: NOX1, NOX2, NOX3, NOX4, and NOX5.^{79,218}

In cardiovascular cells, the generation of ROS is stimulated by neurohumoral vasoconstrictor peptides such as Ang II and Endothelin -1 (ET-1), and by norepinephrine (NE). It is well known that activation of AT-1R by Ang II, triggers the production of superoxide (O_2^-) by NADPH oxidase. Studies have demonstrated the production of O_2^- , by NADPH oxidase, resulting in vasopressor action in response to Ang II infusion in normotensive conditions (Wistar rats).^{219,220} The reaction of NADPH oxidase-mediated production of highly reactive superoxide is as follows:



ET-1 is also known to induce ROS production via the activation of NADPH oxidase. ET-1 is a potent vasoconstrictor of the endothelium layer of blood vessels. There are two isoforms of ET receptors: ET_A and ET_B. The stimulation of ET_A receptors induces ROS production via NADPH oxidase activation and results in vasoconstriction of endothelium. The ET_B receptors have the opposite effect. They mediate vasorelaxation of endothelium.²²¹

ROS production in VSMCs and cardiomyocytes is also induced through the stimulation of alpha-1 adrenergic receptors (α 1-ADR) by NE.²²² The AT-1R, ET-1, and α 1-ADR activate NADPH oxidase via interaction with G-proteins and thus generate ROS.²²³

The other sources of ROS production are mitochondrial transport chain and eNOS uncoupling. In addition to ROS production by NADPH oxidase, ROS generated via NOS uncoupling and in mitochondria contribute to pathogenesis of the CVDs such as hypertension.^{224–227}

Under normal physiological conditions, the production of NO occurs via the coupling of L-arginine, in the presence of tetrahydrobiopterin (BH₄) acting as a co-factor, with eNOS. On stimulation by pathogenic stimuli, the levels of BH₄ are reduced, leading to the uncoupling of eNOS. eNOS uncoupling results in the release of O₂⁻ from the oxygenase domain of eNOS. The increased production of O₂⁻ causes endothelial dysfunction and eventually precipitates vascular damage.^{224,228,229}

The dysfunction of the mitochondrial respiratory chain due to vascular damage under pathophysiological conditions causes the transfer of electrons to molecular oxygen and bring about the formation of ROS such as O_2^- and hydrogen peroxide (H_2O_2).^{230–232} Increased mitochondrial ROS production damages mitochondrial antioxidants such as manganese superoxide dismutase (MnSOD), in turn, further initiating ROS production.²³³ The imbalance between ROS and antioxidants creates oxidative stress which is at the core of the pathogenesis of many CVD including hypertension.²³⁴ Redox signaling in the CVS is elucidated in the following figure:

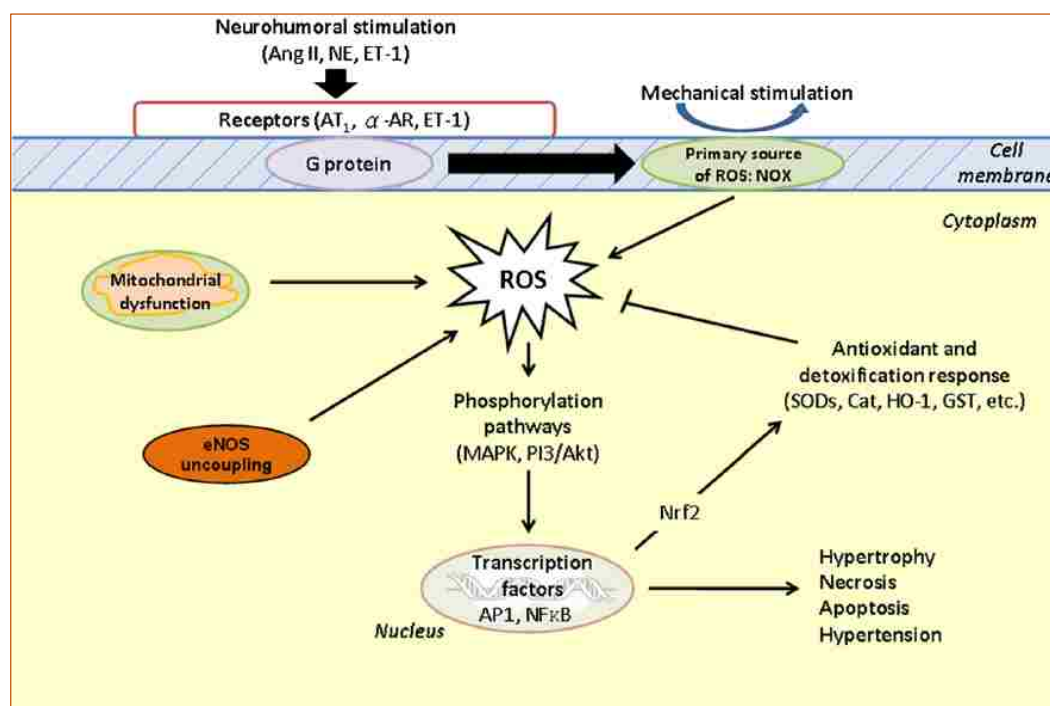


Figure 2.4. Redox signaling in the cardiovascular system. Adapted from *J Biomed Sci.*2013;20(69):4. AT-1 indicates angiotensin-1 receptors; α-AR, alpha-adrenergic receptors; ET-1, endothelin-1; Ang II, angiotensin II; NE, norepinephrine; ROS, reactive oxygen species; HO-1, heme oxygenase-1; Cat, catalase; AP-1, activator protein-1; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; Nrf2, nuclear factor (erythroid-derived 2)-like 2;

MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide-3-kinase; eNOS, endothelial nitric oxide synthase.

Oxidative stress is known to induce activation of MAPKs (ERK1/2, JNK, and p-38 MAPK).^{235,236} Many studies have demonstrated that the induction of oxidative stress under pathophysiological conditions increases ROS production, which in turn activates various MAPKs, leading to activation of several transcription factors, for example, activator protein-1 (AP-1), NF- κ B, and nuclear factor (erythroid-derived 2)-like 2 (Nrf2). These transcription factors are involved in hypertrophy of cardiomyocytes, endothelial cells and VSMCs, necrosis, apoptosis, and inflammation which are characteristic features of many CVD.^{237,238}

Previous research has shed light on the effect of oxytocin on oxidative stress in cardiomyocytes and vascular endothelial cells. However, the impact of oxytocin on oxidative stress in VSMCs has not been investigated thus far. The present study examined oxytocin's effect on oxidative stress in VSMCs via ROS measurement. Also, the interaction of oxytocin with Ang II, a known pro-oxidant, was studied in aortic VSMCs isolated from normotensive rats and hypertensive rats.

2.6. Summary

The present study was conducted to address the gap in the literature regarding oxytocin's effect on proliferation, proliferation pathways (ERK1/s and PI3K/Akt signaling pathways), and vasoconstriction pathways (ROCK pathway) in VSMCs. The study also evaluated the impact of oxytocin on oxidative stress (assessed by measuring ROS levels) and inflammation (analyzed by measuring IL-6 levels). To characterize these effects of oxytocin on VSMCs, this study employed aortic VSMCs isolated from Wistar rats (normotensive) and SHR (hypertensive rats).

Chapter 3: Methodology

3.1. Wistar rats and SHRs

The present study employed aortic VSMCs isolated from Wistar rats and SHRs. Hypertension has been extensively studied pre-clinically in SHRs models, often using normotensive Wistar rats as control.^{239,240} The SHR is a well-established genetic animal model of primary hypertension. The major advantage of the SHR model is the lack of inter-individual variation. Similar to the progression of hypertension in humans, SHRs have pre-hypertensive, developing and sustained hypertensive phases.^{241,239}

3.2. Isolation and culture of primary rat aortic VSMCs

The study protocol was approved by the Nova Southeastern University Institutional Animal Care and Use Committee (NSU-IACUC). Adult male Wistar rats (300-350g) and spontaneously hypertensive rats [(SHRs) 300-350g] were euthanized in a carbon dioxide (CO₂) chamber as per the established guidelines. VSMCs were isolated and cultured as per the previously established explant method.²⁴²

The thoracic cavity was cut-open, the aorta was isolated, and placed in a sterile 100 mm Petri dish containing sterile phosphate-buffered saline (PBS). Sterile forceps were utilized to clean the aorta by removing adjacent fat layers and coagulated blood.

Once cleaned, the aorta was slit from top to bottom and cut into two pieces using sterile scissors. Each piece was then cut into a 1 mm strip and each strip was placed in a sterile 100 mm Petri dish containing Dulbecco's Modification of Eagle's Medium (DMEM) [(Corning Cellgro, NY)], supplemented with 10% (v/v) fetal bovine serum (FBS) glutamine (2 mM/L), streptomycin (100 1g/mL) and penicillin (VWR Life Science, GA). Petri dishes containing strips of freshly isolated aortic VSMCs were incubated at 37°C under standard humidified conditions (95% air and 5% CO₂) to allow VSMCs to grow out from strips onto the surface of petri-dish. Once VSMCs were outgrown, strips were removed, and VSMCs were allowed to grow in petri-dish. On reaching confluency, VSMCs were passaged in T-75 flasks so that enough number of cells were obtained to be able to conduct experiments using 100-mm Petri dishes, 6-well plates, 24-well plates, and 96-well plates.

Before every experiment, VSMCs were subjected to trypsinization (Corning™ 0.25% Trypsin, Fischer Scientific, FL), counted using the Hemocytometer method, and were seeded in either 100 mm Petri dishes, 6-, 24-, or 96-well plates depending on the experimental design. They were incubated at 37°C under previously described standard humidified conditions. After every 48 hours, the culture medium was replenished until VSMCs were about 90% confluent. Once confluent, VSMCs were washed with sterile PBS. VSMCs were made quiescent by replenishing with the serum-free media (SFM) for 24 hours before commencing with the experimental treatments. Cell passages from 3-15 were used for all experiments. All experiments were performed using the primary aortic VSMCs isolated from at least three rats or more. ⁸¹

3.3. VSMCs proliferation assessment by the Hemocytometer method

3.3.1. The principle of the Hemocytometer method

The Hemocytometer method is the most widely used technique used to determine the number of cells per unit volume of a suspension.²⁴³ It is a valuable research tool utilized to count cells in cell proliferation studies. It consists of a microscopic slide which is divided into two chambers. Each chamber has grid lines which make nine large squares. Each large square has an area of 1mm^2 . The large squares are demarcated from each other by compact grid lines. The volume of the entire counting grid lines is 0.9mm^3 in each chamber. A glass cover-slip is placed over a chamber following which a suspension of cells is introduced into the chamber, and the cells are counted under the microscope. The cells are excluded from counting if they are touching the right-side and the bottom (**Figure 3.1**).

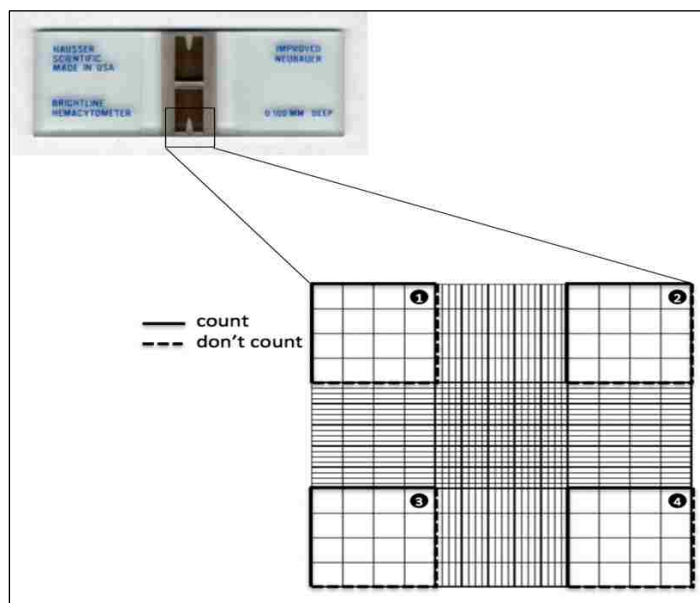


Figure 3.1. Hemocytometer and the grid lines of a chamber in a hemocytometer microscopic slide. Adapted from <https://www.hemocytometer.org/wp-content/uploads/2013/06/hemocytometer-squares-to-count.png?w=551>.

The hemocytometer technique employs the staining of cells with dyes such as trypan blue to identify live and dead cells. The cytoplasm of dead cells readily absorbs trypan blue and turns the appearance of dead cells to blue, making it easy to distinguish from live cells which have intact cytoplasm that does not take up trypan blue.²⁴³

3.3.2. The procedure of the cell counting by the hemocytometer method

Aortic VSMCs were seeded at 1×10^6 cells/well in 6-well plates and cultured as mentioned before. The cells were made quiescent by replacing serum-media with SFM, 24 hours prior to treatment. Post-treatment, the cells in each well were subjected to trypsinization and centrifuged at 1,500 rpm at 21°C for 10 minutes. The pellet was used to make the cell suspension with 1000 μ L SFM, of which 100 μ L was pipetted out and transferred in an Eppendorf tube, and stained with 900 μ L 0.4% trypan blue. From the trypan blue-stained cell suspension, 10 μ L was applied to the hemocytometer (Bright-Line Hemocytometer Set, Hausser Scientific, VWR Life Science, GA).

The cover-slip was placed on top to allow the complete and gentle filling of both chambers of the hemocytometer. The grid lines of the hemocytometer were focused under a microscope with a 10X objective. The cells were counted in all the 4 sets of large squares in the four corners of a chamber of the hemocytometer. The cells were excluded from counting when present on the right-hand boundary line or touching the bottom. The stained cells (dead cells), and unstained cells (live cells) were calculated and the dilution factor was corrected as per the manufacturer's protocol.^{243,244}

3.4. VSMCs viability determination by the MTT assay

3.4.1. The principle of the MTT assay

The MTT assay measures cell viability as a function of the mitochondrial activity. It is a colorimetric assay which relies on the reduction of the yellow colored 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial enzymes such NADPH oxidase and mitochondrial succinate, into an insoluble purple colored formazan. Organic solvents such as isopropanol and dimethyl sulfoxide (DMSO) are employed to solubilize the insoluble formazan, and the color change is measured as 'optical density' by a microplate reader. The optical density is a function of the mitochondrial activity. The higher optical density is indicative of higher mitochondrial activity, and the higher mitochondrial activity relates to the higher number of metabolically active cells.²⁴⁵

3.4.2. The procedure of the MTT assay

Aortic VSMCs were seeded at 1×10^4 cells/well in 96-well plates. The serum-media was replaced with SFM 24 hours prior to treatment. After carrying out the treatments with different groups, the SFM-containing the different treatments was aspirated, and 50 μ L of MTT (ACROS Organics, NJ) was added to each 96-well. The 96-well plates were incubated at 37°C under standard humidified conditions for 45 minutes. Following the incubation, 100 μ L of DMSO (Merck KGaA, Germany) was added to each well. The plates were read at 570 nm using a Biotek microplate reader (Biotek-Synergy H1 Hybrid Reader, VT).²⁴⁴

3.5. VSMCs growth measurement by the ³H-Thymidine incorporation assay

3.5.1. The principle of the ³H-Thymidine incorporation assay

The tritiated-Thymidine (³H-Thymidine) incorporation assay is considered as the gold-standard method to assess cell proliferation. This assay involves the direct measurement of cell proliferation by determining the synthesis of DNA. It employs the incorporation of the radioactive nucleoside tritiated (³H)-Thymidine into strands of chromosomal DNA of the actively dividing cells, during the mitotic phase of cell division. The radioactivity recovered from the cells is measured using a liquid scintillation counter as ‘counts per minute.’ The cell growth is assessed as the function counts per minute (cpm).

A higher radioactivity count reflects higher cell growth. The cells undergoing active division resulting in higher cell growth will have more incorporation of ³H-Thymidine into their DNA strands and consequently will have higher radioactivity count than those that are undergoing lesser cell division. Hence, a higher reading of cpm reflects a higher cell growth, and a lower reading of cpm represents a lower cell growth.²⁴⁶

3.5.2. The procedure of ³H-Thymidine incorporation assay

Aortic VSMCs were seeded at 5×10^4 cells/well in 24-well plates, cultured and made quiescent, as described previously, 24 hours before starting the treatments. Quiescent cells were subjected to different treatments. After the first 24 hours of treatment, 5 μ L of ³H-thymidine (1 μ Ci/mL) [Thymidine (Methyl-³H), MP Biomedicals, LLC, CA], was added to each well. The plates were incubated at 37°C under standard humidified conditions for another 24 hours. In the end, the SFM

containing the treatments and ^3H -thymidine was aspirated and washed with sterile cold-PBS (1,000 μL), and 500 μL of cold 10% trichloroacetic acid (TCA) was added to each well (this was done to precipitate out remnants of any radioactive material present outside cells). The 24-well plates were incubated overnight at 4°C .

TCA was removed and 500 μL of 0.1 N sodium hydroxide (NaOH) – 2% sodium dodecyl sulfate [(SDS), NaOH-SDS] solution, previously maintained at room temperature (25°C), was added to each well and incubated for one hour at 4°C . This step was carried out to cause alkaline lysis of cells resulting in protein denaturation (breaking of double-stranded DNA into a single-stranded DNA). SDS solubilizes the cell membrane, and NaOH disrupts the hydrogen bonding between the DNA bases and thus causing protein denaturation).

The samples were collected in vials (20 mL). To each sample, 6 mL of liquid scintillation cocktail (Ultima Gold™, PerkinElmer Inc., CA) was added, and the samples were quantified by a PerkinElmer liquid scintillation counter (Tri-Carb® 2810TR Liquid Scintillation Analyzer, PerkinElmer Inc., CA) as per the manufacturer's protocol.²⁴⁷

3.6. VSMCs apoptosis and necrosis assessment by flow cytometry

3.6.1. The principle of flow cytometry

Flow cytometry is a technique commonly used in determining the characteristics of a single cell in suspension. It involves the measurement of the optical and fluorescence properties of cells including their nuclei and chromosomes. The technique relies on the use of fluorescent dyes, for example Annexin V and propidium iodide, that are specific in their binding to cellular components such as membrane phospholipids, DNA, and RNA. When cells in a suspension labeled with fluorescent dyes are passed through a light (laser) source, they rise to a higher energy state. When cells return to their resting state, they emit light energy at higher wavelengths. Multiple fluorochromes with similar excitation and different emission wavelengths enable the simultaneous determination of several properties of cells.²⁴⁸

This technique is also valuable in cell sorting and is widely utilized in clinical hematology. When cells continue to move straight in the direction of the light source, the process is called ‘forward-scatter,’ and when they deviate from the direction of the light source, it is referred to as ‘side-scatter.’ The larger cells demonstrate forward-scatter whereas, the smaller cells show side-scatter.²⁴⁹ A flow diagram of a typical flow cytometer is shown in **Figure 3.2**.

Flow cytometry has profound applications in cell viability studies. It is used in the detection of apoptosis and necrosis.²⁵⁰ Apoptosis is defined as ‘programmed cell death’ whereas necrosis is ‘premature cell death’ due to external factors including injury and acute infection.²⁵¹ Apoptosis is characterized by specific

morphological changes such as loss of plasma membrane asymmetry, membrane blebbing, shrinking of cytoplasm and nucleus, and DNA cleavage in the nucleosome. One of the earliest features of a cell undergoing apoptosis is the loss of plasma membrane asymmetry. It is characterized by the translocation of phosphatidylserine (PS), a membrane phospholipid, from the inside to the outside of the plasma membrane (**Figure 3.3**).²⁵²

A fluorochrome-labeled dye such as Annexin V binds to the PS of plasma membranes of an apoptotic cell and is detected by flow cytometer. Similarly, a fluorescent 7-amino actinomycin D dye (7-AAD) binds to broken fragments of DNA, the hallmark of necrosis, and thus enable the detection of cells undergoing necrosis by flow cytometer.²⁵³

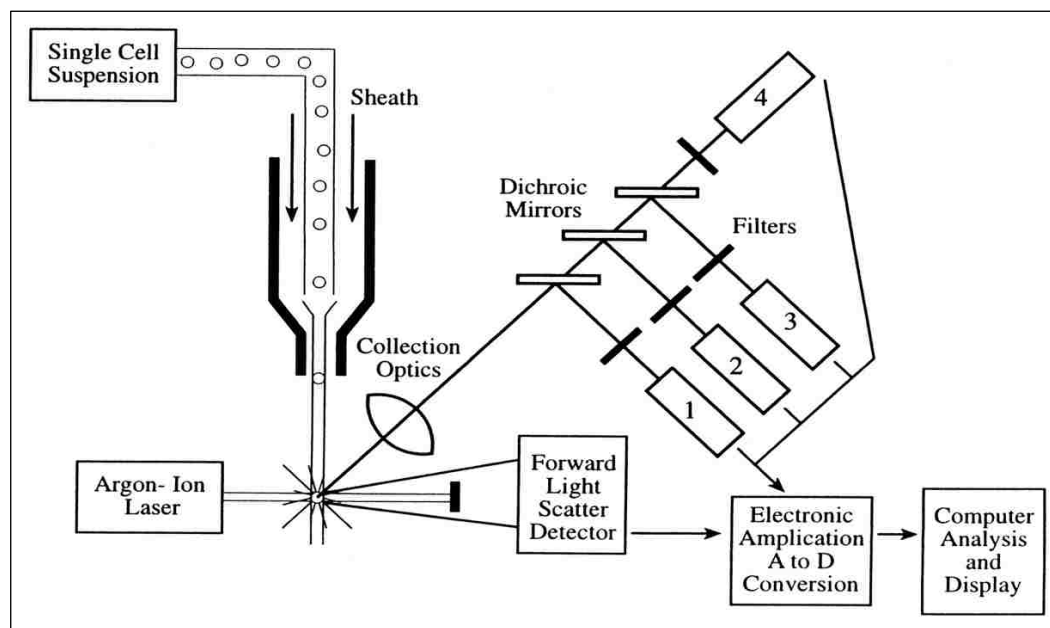


Figure 3.2. Flow cytometer. A flow diagram of a typical flow cytometer analyzing a single cell suspension. The sample containing a single suspension is projected with a sheath fluid. The argon-ion laser intersects the cells and causes the forward-scatter and the side-scatter. The signals are detected, amplified, registered, and digitally analyzed in a computer. Adapted from *Clin Chem.* 2000;46(8 Pt 2):1222.

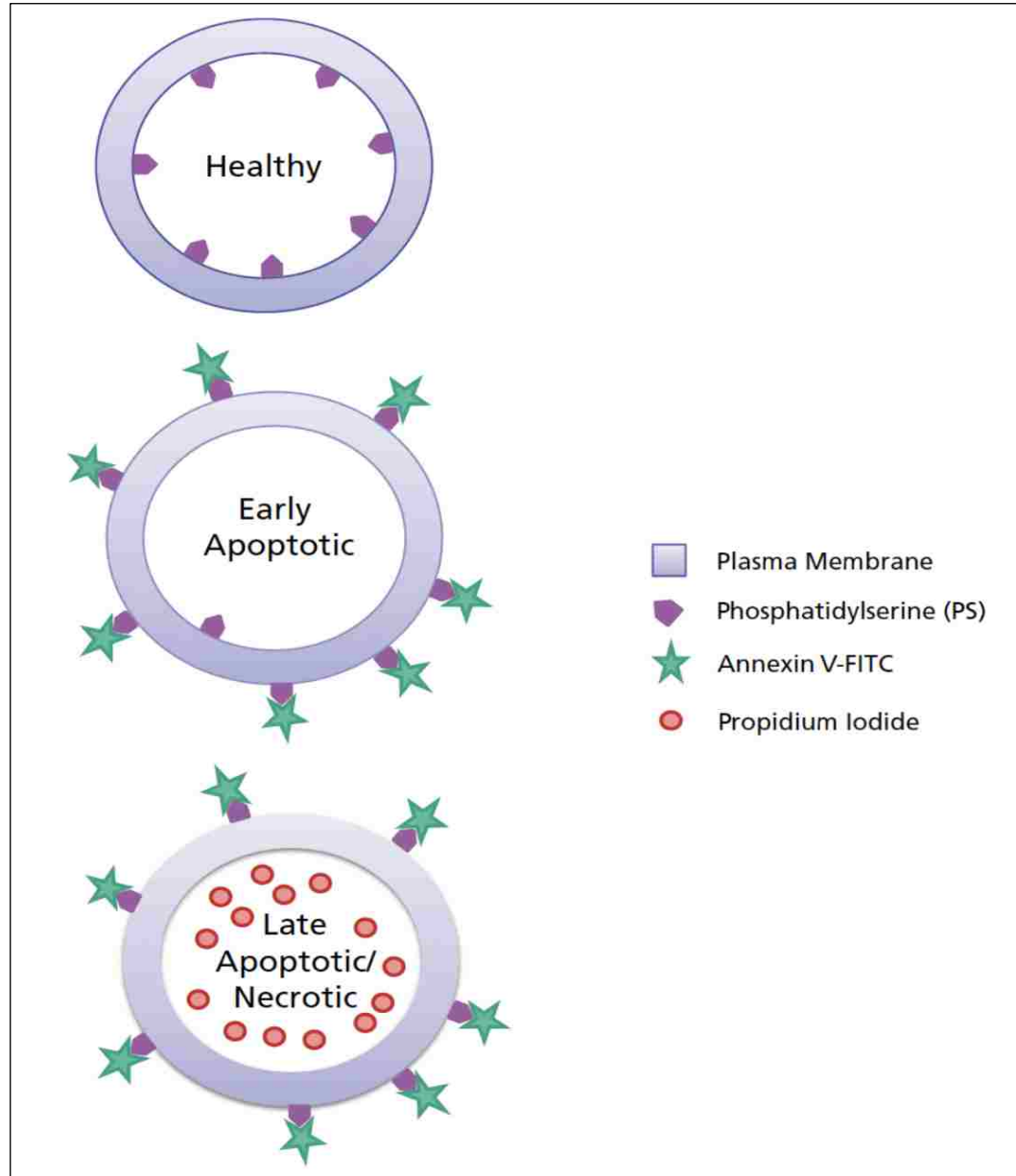


Figure 3.3. Presentation of markers for detection by healthy, apoptotic, late-apoptotic and necrotic cells. Adapted from BD Biosci. 2011; 8:2.

3.6.2. The procedure of apoptosis and necrosis assessment using flow cytometer

The present study utilized flow cytometry to study the effect of oxytocin on cell viability, apoptosis, and necrosis in aortic VSMCs from Wistar rats and SHR. Aortic VSMCs were made quiescent 24 hours before the treatments. On the day of the assay after replenishing with the serum-free media (SFM), the treatment was performed, and cells were placed under standard incubation conditions for 24 hours. Post-treatment, the supernatant was removed. The cells were washed with 1,000 μ L of sterilized PBS (previously maintained at the room temperature). The PBS was then removed, and 400 μ L of Accutase cell detachment solution (# A6964 Sigma-Aldrich[®], MO) was added to each well. The cells were then incubated under standard incubation conditions for about 5 -7 minutes.

Following the incubation, 1,000 μ L of fluorescent-activated cell sorting (FACS) buffer (# MB-086-0500 Rockland[™] antibody & assays) was added to each well, and the samples were collected in Eppendorf tubes (1.5 mL). The samples were then subjected to centrifugation at 1,500 rpm at 21°C for 10 minutes. The supernatant was discarded via decantation (the vacuum was not used to remove the supernatant in an effort to prevent cell loss). The pellet was re-suspended with the cold PBS (1,000 μ L), and samples were re-centrifuged at 10,000 rpm for 5 minutes.

The supernatant was discarded, and the pellet was re-suspended with 100 μ L Annexin V binding buffer and vortexed. To each prepared sample, 4 μ L of Annexin V and 4 μ L of 7-AAD (# 640922, FITC Annexin V Apoptosis Detection

Kit with 7-AAD, BioLegend[®], CA) was added, and gently mixed and allowed to stand for 30 minutes.

Subsequently, 400 μ L of binding buffer was added to each sample, and the samples were run at the medium speed (with the limit set at 10,000 events) using the BD LSRFortessa[™] X-20 cell analyzer as per the manufacturer's protocol.²⁵⁴

The data generated represents four sections: percentage of live cells; percentage of cells positive for Annexin V; percentage of cells positive for 7-AAD; and percentage of cells positive for both Annexin V and 7-AAD. The cells positive for Annexin V indicate the percentage of cells undergoing apoptosis. The cells positive for 7-AAD reflect the percentage of cells undergoing necrosis. And, the cells that were double positive for both Annexin-V and 7-AAD represent the cells undergoing both apoptosis and necrosis.

3.7. IL-6 measurement by the enzyme-linked immunosorbent assay (ELISA)

3.7.1. The principle of IL-6 ELISA

ELISA is an immunological assay used for the detection and quantification of peptides, antibodies, cytokines, and hormones in biological samples. The general principle of ELISA is based on the interaction of antigen present in the biological sample with the specific antibody immobilized to a solid surface, and then its interaction with a secondary antibody conjugated with an enzyme. The detection is carried out by measuring the activity of the enzyme-linked by incubating it with a substrate, yielding a quantifiable product.²⁵⁵

The present study employed ELISA to measure IL-6 levels secreted by VSMCs in the culture media. For this purpose, rat IL-6 ELISA kits were purchased (Rat IL-6 Immunoassay, # R6000B Quantikine® ELISA, R&D Inc., MN). The package comprised of microplates previously coated with monoclonal antibodies for rat IL-6. The samples were loaded into the microplates. A secondary antibody (specific to rat IL-6) was then added making a sandwich of immobilized antibody specific for rat IL-6, samples (containing IL-6 antigen), and secondary antibody.

The secondary antibody conjugated with horseradish peroxidase (HRP) produced a colored or fluorescent reaction when a chemical substrate was added. The fluorescent signals were detected by a Biotek microplate reader (Biotek-Synergy H1 Hybrid Reader, VT), thus determining the presence and the quantity of IL-6 antigen in the samples. A typical ELISA sandwich is depicted in the following figure:

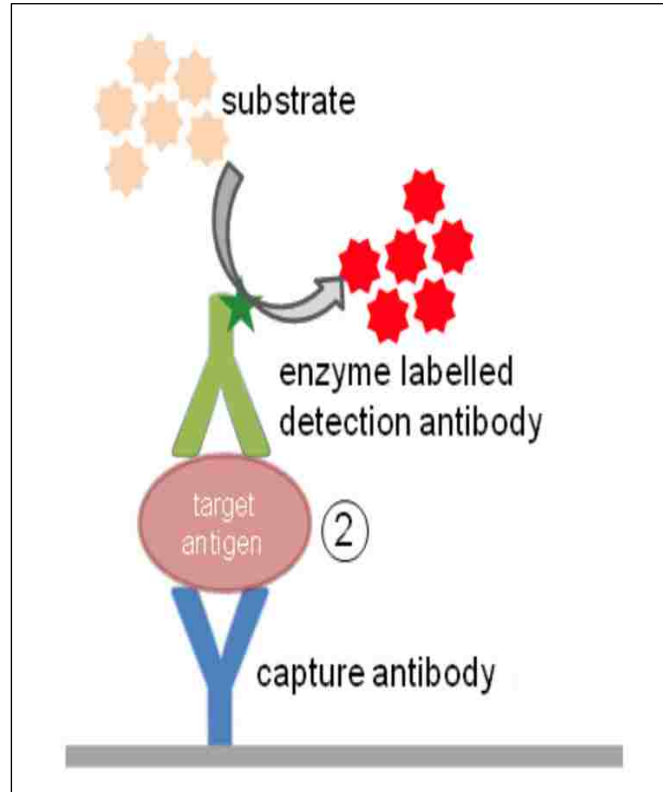


Figure 3.4. A typical enzyme-linked immunosorbent assay (ELISA) sandwich. Adapted from <https://www.immunology.org/public-information/bitesized-immunology/experimental-techniques/enzyme-linked-immunosorbent-assay>.

3.7.2. The procedure of IL-6 ELISA

The reagents contained in the rat IL-6 ELISA kit (Rat IL-6 Immunoassay, # R6000B Quantikine[®] ELISA, R&D Inc., MN) were prepared as per the manufacturer's protocol.

First, 50 μ L of assay diluent was added to each well in the assay plate, and then 50 μ L of standards, 50 μ L of each sample, and 50 μ L of assay control were added in their designated wells in the plate. Subsequently, the plate was incubated at room temperature (25°C) for 2 hours.

After incubation, the plate was washed five times with wash buffer and 10 μ L of rat IL-6 conjugate was added to each well.

The plate was incubated again for 2 hours at room temperature. After incubation, the rat IL-6 conjugate was aspirated, and each well was washed five times with wash buffer. 100 μ L of substrate solution was added to each well, and the plate was incubated under darkness for 30 minutes.

At the end of the 30 minutes, the stop solution was added to each well, and the plate was read by a Biotek microplate reader (Biotek-Synergy H1 Hybrid Reader, VT) at 450 nm.²⁵⁶

3.8. ROS assay

3.8.1. The principle of ROS assay

The ROS assay employs a fluorogenic dye named 2',7'-dichlorofluorescein diacetate (DCFDA).²⁵⁷ It measures the free radicals such as hydroxy, peroxy and other ROS within a cell. The assay principle is based on the process of deacetylation of DCFDA. The dye readily diffuses into cells and gets deacetylated into a 'non-fluorescent' compound by cellular esterase enzymes present in cells. The so formed non-fluorescent compound later gets oxidized by ROS present in cells into a 'highly fluorescent' compound called 2',7'-dichlorofluorescein (DCF) which is detected by fluorescence spectroscopy with maximum excitation at 485 nm and emission at 535 nm.²⁵⁸

3.8.2. ROS assay procedure

The ROS assay was carried out as per the manufacturer's protocol (# ab113851, DCFDA Cellular ROS Detection Assay Kit, Abcam[®], MA). Aortic VSMCs were seeded at 1×10^4 cells/well in 96-well plates, cultured and made quiescent by replacing serum media with SFM 24 hours prior to treatment. On the day of the experiment, cells were first washed with sterile PBS and then stained with 100 μ L of DCFDA (concentration of 25 μ M/well) for 45 minutes under standard incubation conditions.

After incubation, the cells were re-washed with sterile PBS, and 100 μ l of SFM was added to each well, and the treatment was carried out as per the experimental protocol. Post-treatment, the 96-well plates were read using a Biotek

microplate reader [(excitation at 485 nm and Emission at 535 nm), Biotek-Synergy H1 Hybrid Reader, VT].

3.9. Western blotting

3.9.1. The principle of Western blotting

Western blotting is also known as immunoblotting. It is a reliable and sensitive technique routinely employed in research for the detection and characterization of proteins. It is a qualitative and semi-quantitative method. Western blotting involves the electrophoretic separation of protein samples based on their size and molecular weight in a polyacrylamide gel. The proteins are electrically transferred from a gel to a nitrocellulose membrane. The electrical transfer of proteins from gel-to-membrane is called ‘blotting.’

Following the transfer, membranes are probed with a specific primary antibody and a secondary antibody conjugated with an enzyme (horseradish peroxidase) for the chemiluminescence detection of protein(s).²⁵⁹ The various steps in Western blotting is shown schematically in **Figure 3.5**.

3.9.2. Preparation of cell lysate and procedure of Western blotting

Cultured aortic VSMCs were lysed on ice with lysis buffer [sodium chloride (NaCl) 137 mM; Tris- hydrochloric acid (HCl) 20 mM; glycerol 10% v/v; Triton X-100 1% v/v; protease inhibitor cocktail—1 tablet per10 mL (Pierce™ Protease Inhibitor, Thermo Scientific, IL)], and centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatants were collected, and the bicinchoninic acid (BCA) assay (Pierce™ BCA Protein Assay Kit, Thermo Scientific, IL) was carried out for estimating total protein content.

Subsequently, samples were diluted with a modified laemmli sample buffer [2.5% SDS, 20% glycerol, 10% beta-mercaptoethanol, 0.004% bromphenol blue,

0.125 M Tris HCl, and pH 6.8 (Bio-Rad, CA)]. The preheated protein samples (equivalent to 20 µg proteins off each sample) were loaded in each well in 10% polyacrylamide gel and were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Nitrocellulose membranes were used to transfer the proteins from the gels. The membranes were blocked with 5% w/v non-fat milk (Blotting-Grade Blocker, Bio-Rad, CA) solution [in 0.1% Tween in Tris-buffer saline (TBST)] for one hour at room temperature (25°C).

The membranes were washed three times for five minutes with 0.1% TBST solution and were incubated with primary antibodies overnight at 4°C. After that, the membranes were probed with secondary antibodies conjugated with horseradish peroxidase for one hour at room temperature (25°C). The enhanced chemiluminescence (ECL) reagents (ECL Femto Max, Thermo Scientific, IL) were utilized for visualizing the membranes under chemiluminescence.

The bands were quantified with the Image J Software (National Institutes of Health, MD). The membranes were stripped with the stripping buffer (Restore™ Western Blot Stripping Buffer, VWR Life Science, GA), washed, and blocked in a similar manner as described above, and were incubated with primary antibodies for the housekeeping protein [glyceraldehyde-3-phosphate dehydrogenase (GAPDH)], probed with secondary antibodies, and visualized under chemiluminescence and quantified as stated previously.^{260,261}

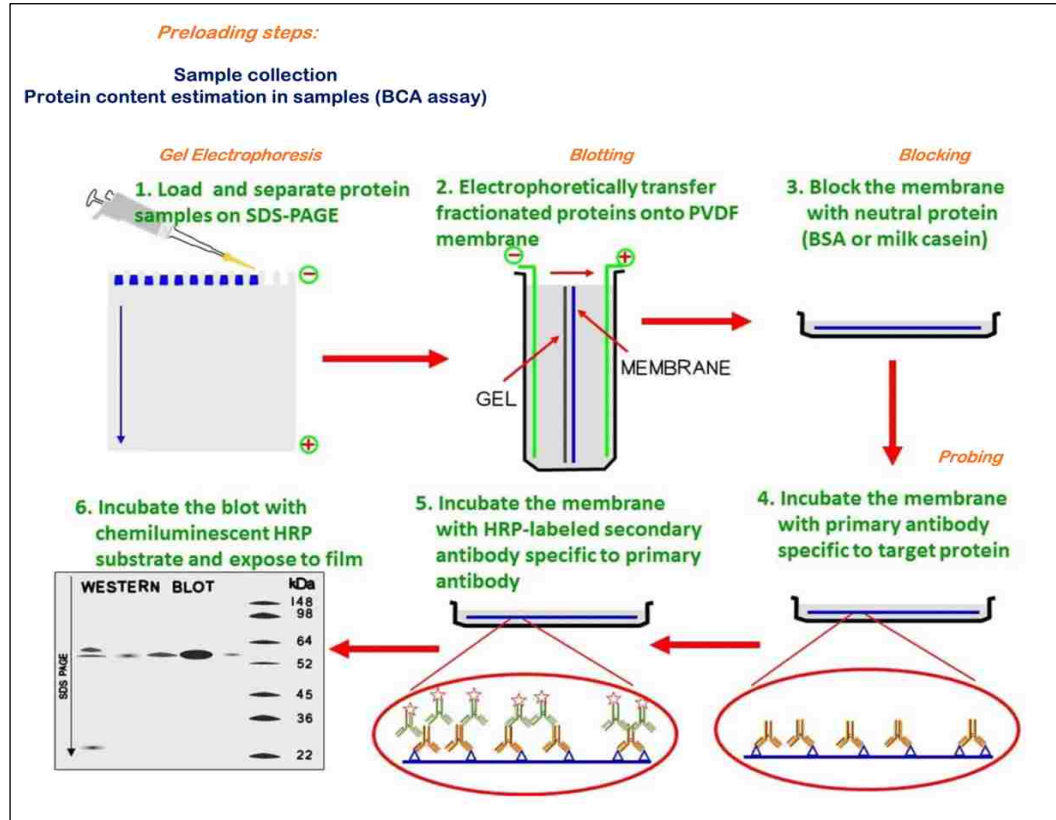


Figure 3.5. A schematic representation of the various steps in Western blotting.

Adapted from <https://microbeonline.com/western-blot-technique-principle-procedures-advantages-and-disadvantages/>

The primary and secondary antibodies employed in Western blotting are mentioned in the following table:

Table 3.1: Primary and secondary antibodies used in Western blotting

| # | <i>Antibody</i> | <i>Code #</i> | <i>Manufacturer</i> |
|---|--|-------------------|--|
| 1 | p44/42 MAPK (ERK1/2) (137F5) Rabbit mAb | 4695 | Cell Signaling Technology [®] , Inc., MA |
| 2 | Phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) | 9101 | Cell Signaling Technology [®] , Inc., MA |
| 3 | ROCK-1 (C8F7) Rabbit mAb | 4035 | Cell Signaling Technology [®] , Inc., MA |
| 4 | ROCK-2 Antibody | 8236 | Cell Signaling Technology [®] , Inc., MA |
| 5 | PI3-Kinase Flour [®] 680 p110 Antibody (D-4) Mouse | sc-8010 AF 680 | Santa Cruz Biotechnology Inc., TX |
| 6 | Phospho-AKT (Ser473) 587F11 Mouse mAb | 4051 | Cell Signaling Technology [®] , Inc., MA |
| 7 | GAPDH (D16H11) XP [®] Rabbit mAb HRP conjugate | 8884 | Cell Signaling Technology [®] , Inc., MA |
| 8 | Anti-Rabbit IgG HRP-linked Antibody | 7074 | Cell Signaling Technology [®] , Inc., MA |

| | | | |
|---|--|-------|---------------------------------|
| 9 | Anti-Mouse IgG (Fab specific)-Peroxidase Conjugate | A3682 | Sigma-Aldrich [®] , MO |
|---|--|-------|---------------------------------|

The concentrations of primary and secondary antibodies used in Western blotting are mentioned in the following table:

Table 3.2. Concentrations of primary and secondary antibodies used in Western blotting

| # | <i>Antibody</i> | <i>Dilution</i> | <i>Function</i> |
|---|-------------------------------------|---|--------------------------------|
| 1 | p44/42 MAPK (ERK1/2) | 1:1000 in TBST | Proliferation marker |
| 2 | Phospho-p44/42 MAPK (ERK1/2) | 1: 1000 in TBST | Proliferation marker |
| 3 | ROCK-1 | 1: 1000 in TBST | Vasocontraction marker |
| 4 | ROCK-2 | 1: 1000 in TBST | Vasocontraction marker |
| 5 | PI3-Kinase p110 α | 1:200 in TBST | Proliferation marker |
| 6 | Phospho-AKT | 1: 1000 in 5% w/v non-fat dry milk, 1x TBS, and 0.1% Tween-20 | Proliferation marker |
| 7 | GAPDH | 1: 1000 in TBST | Loading control (housekeeping) |
| 8 | Anti-Rabbit IgG HRP-linked Antibody | 1: 10000 in TBST | Secondary antibody |

| | | | |
|---|--|------------------|--------------------|
| 9 | Anti-Mouse IgG (Fab specific)-Peroxidase Conjugate | 1: 10000 in TBST | Secondary antibody |
|---|--|------------------|--------------------|

The compositions of buffers and polyacrylamide gels: separating and stacking gels used in Western blotting are mentioned in **Table 3.3**, **Table 3.4**, and **Table 3.5**, respectively.

Table 3.3: Composition of buffers used in Western blotting

| Buffers | 10X |
|---|--|
| Running buffer (for gel electrophoresis) | Tris: 15.20 g Glycine: 72.02 g Sodium dodecyl sulfate (SDS): 5.00 g <i>Make the volume up to 1,000 mL with distilled water.</i> |
| Transfer buffer (for blotting) | Tris: 15.20 g Glycine: 72.02 g <i>Make the volume up to 1,000 mL with distilled water.</i> |
| Tris-buffered saline (TBS) | Tris: 60.57g Sodium chloride (NaCl): 87.66 g <i>Make the volume up to 1,000 mL with distilled water.</i> |
| Phosphate-buffered saline (PBS) | Sodium chloride (NaCl): 82.00 g Potassium chloride (KCl): 2.00 g Disodium hydrogen phosphate (Na ₂ HPO ₄): 29.00 g Potassium dihydrogen phosphate (KH ₂ PO ₄): 2.00 g |

| | |
|--|---|
| | <i>Make the volume up to 1,000 mL with distilled water.</i> |
|--|---|

Table 3.4: Composition of 10% resolving (separating) gel used in Western blotting

| Ingredients | 2 gels | 4 gels |
|---------------------------------------|----------------|----------------|
| Acrylamide (30%) | 3.75 mL | 7.50 mL |
| SDS (10%) | 112.50 μ L | 225.00 μ L |
| Tris-HCl (1.5 M, pH 8.8) | 2.85 mL | 5.70 mL |
| Distilled water | 4.65 mL | 9.3 mL |
| Ammonium per sulfate [(APS), 10%] | 37.50 μ L | 75 μ L |
| Tetramethylethylenediamine (TEMED) | 7.50 μ L | 15 μ L |

Table 5: Composition of 5% stacking gel used in Western blotting

| Ingredients | 2 gels | 4 gels |
|--------------------------------------|----------------|-----------------|
| Acrylamide (30%) | 487.50 μ L | 975.00 μ L |
| SDS (10%) | 37.50 μ L | 75.00 μ L |
| Tris-HCl (1.5 M, pH 8.8) | 937.50 μ L | 1875.00 μ L |
| Distilled water | 2.25 mL | 4.50 mL |
| Ammonium per sulfate [(APS), 10%] | 19.00 μ L | 38.00 μ L |

| | | |
|----------------------------|--------------------|--------------------|
| Tetramethylethylenediamine | 4.00 μL | 8.00 μL |
|----------------------------|--------------------|--------------------|

3.10. Statistical Analysis

The analysis of variance (ANOVA) was used for comparison between Wistar rats and SHRs. The unpaired Student t-test was applied to determine whether differences between the two individual treatment groups were significant. The difference between the treatment groups was considered statistically significant if the probability value (P value) was less than 0.05.²⁶²

3.11. Specific aims 1 and 2, strategies, and experimental design

Specific aim 1: To characterize the effect of oxytocin on proliferation, inflammation, oxidative stress, and on vasocontraction pathway in primary aortic VSMCs obtained from normotensive Wistar rats.

Specific aim 2: To determine the interaction between oxytocin and Ang II with regards to proliferation, inflammation, oxidative stress, and on vasocontraction pathway in primary aortic VSMCs obtained from normotensive Wistar rats.

Strategies and experimental design for specific aims 1 and 2:

The following four strategies and experimental designs were devised for specific aims 1 and 2:

Strategy 1a:

To investigate the effect of oxytocin, and its interaction with Ang II on the proliferation of aortic VSMCs obtained from normotensive Wistar rats, the following techniques were utilized:

1. Cells were counted directly by employing the Hemocytometer method.
2. Cell viability was assessed using the MTT assay.
3. Cell growth was determined using the ³H-Thymidine incorporation assay.
4. Cell apoptosis and necrosis were detected by Flow Cytometry.

Experimental design for the strategy 1a:

VSMCs were seeded in 6-well plates, 24-well plates, and 96-well plates in the hemocytometer experiments, ³H-Thymidine incorporation assays, and MTT assays, respectively. For the hemocytometer method, MTT-assay, and ³H-

Thymidine incorporation assay, the treatment groups were identical and are stated in the following table:

Table 3.6: Treatment groups in Hemocytometer method, MTT, and ³H-Thymidine incorporation assays

| # | Treatment Groups |
|---|---|
| 1 | The control group (only SFM and no treatment) |
| 2 | Oxytocin 100 nM |
| 3 | Ang II 100 nM |
| 4 | Oxytocin (100 nM) + Ang II (100 nM) |
| 5 | L-371,257 [oxytocin receptor antagonist (OXTA)] 1 μ M |
| 6 | OXTA 1 μ M + Oxytocin (100 nM) + Ang II (100 nM) |
| 7 | OXTA 1 μ M + Oxytocin (100 nM) |

Cells were pre-treated with oxytocin (VWR Life Science, GA) ten minutes before treatment with Ang II (VWR Life Science, GA). OXTA (Santa Cruz Biotechnology Inc., TX) was added 30 minutes before the treatment with oxytocin. In both the hemocytometer method and MTT assay, the treatment was carried out every 24 hours through 72 hours. In the ³H-Thymidine incorporation assay, the treatment was carried out at 24 and 48 hours, respectively. The SFM was replenished (every 24 hours) before the start of treatments.

For the flow cytometer assay, 6-well plates were used to seed cells. There were three experimental groups: control (only SFM and no treatment), oxytocin (10 nM), and oxytocin (100 nM). The treatment was carried out only for 24 hours.

Strategy 1b:

To further understand the mechanism of oxytocin, and its interaction with Ang II on the proliferation of aortic VSMCs in Wistar rats, the two critical signaling pathways involved in VSMCs proliferation were investigated: ERK1/2 pathway and PI3K/Akt pathway. Western blotting technique was utilized as a part of strategy 1b.

Experimental design for Strategy 1b:

Cells were seeded in 6-well plates. The experimental design consisted of the following treatment groups mentioned in **Table 3.7**:

Table 3.7: Treatment groups for ERK1/2 and PI3K/Akt pathway experiments

| # | Treatment Groups |
|---|---|
| 1 | The control group (only SFM and no treatment) |
| 2 | Oxytocin 100 nM |
| 3 | Ang II 100 nM |
| 4 | Oxytocin (100 nM) + Ang II (100 nM) |
| 5 | L-371,257 [oxytocin receptor antagonist (OXTA)] 1 μ M |
| 6 | OXTA 1 μ M + Oxytocin (100 nM) + Ang II (100 nM) |

For investigating the effect of oxytocin, and its interaction with Ang II on the phosphorylation of ERK1/2, PI3K p110 α , and Akt, cells were treated with oxytocin for ten minutes, and also with Ang II also for ten minutes. In the combination group, cells were pre-treated with oxytocin for ten minutes before the

treatment with Ang II. OXTA was added 30 minutes before the treatment with oxytocin.

Strategy 2:

To investigate the effect of oxytocin, and its interaction with Ang II on oxidative stress in aortic VSMCs (Wistar rats), ROS levels were measured. A ROS assay was carried out to determine ROS levels.

Experimental design for strategy 2:

Cells were seeded in 96-well plates. The experimental design for the ROS assay comprised of the following treatment groups (**Table 3.8**):

Table 3.8: Treatment groups for the ROS assay

| # | Treatment Groups |
|---|---|
| 1 | The control group (SFM and no treatment) |
| 2 | Oxytocin 100 nM |
| 3 | Ang II 100 nM |
| 4 | Oxytocin (100 nM) + Ang II (100 nM) |
| 5 | L-371,257 [oxytocin receptor antagonist (OXTA)] 1 μ M |
| 6 | OXTA 1 μ M + Oxytocin (100 nM) + Ang II (100 nM) |
| 7 | OXTA 1 μ M + Oxytocin (100 nM) |
| 8 | OXTA 1 μ M + Ang II (100 nM) |

Cells were treated for ten minutes with oxytocin and Ang II. In the combination group, cells were pre-treated with oxytocin ten minutes before the

treatment with Ang II. OXTA was added 30 minutes before the treatment with oxytocin and Ang II.

Strategy 3:

To investigate the effect of oxytocin, and its interaction with Ang II on inflammation in aortic VSMCs (Wistar rats), the levels of IL-6, a critical inflammatory mediator, were measured. An IL-6 ELISA was utilized to measure IL-6 levels.

Experimental design for strategy 3:

Cells were seeded in 6-well plates. The experimental design for the IL-6 assay included the following treatment groups (**Table 3.9**):

Table 3.9: Treatment groups for the IL-6 assay

| # | Treatment Groups |
|---|---|
| 1 | The control group (only SFM and no treatment) |
| 2 | Oxytocin (10 nM) |
| 3 | Oxytocin (100 nM) |
| 4 | Oxytocin (1000 nM) |
| 5 | Ang II (100 nM) |
| 6 | Oxytocin (10 nM) + Ang II (100 nM) |
| 7 | Oxytocin (100 nM) + Ang II (100 nM) |
| 8 | Oxytocin (1000 nM) + Ang II (100 nM) |
| 9 | L-371,257 [oxytocin receptor antagonist (OXTA)] + Oxytocin (100 nM) + Ang II (100 nM) |

Cells were treated with different concentrations of oxytocin for ten minutes, and with Ang II for ten minutes. In the combination group, cells were pre-treated with oxytocin ten minutes before the treatment with Ang II. OXTA was added 30 minutes before the treatment with oxytocin and Ang II. Post-treatment, IL-6 secreted in the media (supernatant) was collected, and IL-6 ELISA was carried out as per the manufacturer's protocol.

Strategy 4:

The effect of oxytocin, and its interaction with Ang II on vasocontraction in aortic VSMCs in Wistar rats was assessed indirectly by investigating the Rho-kinase pathway (ROCK-1 and ROCK-2) a critical pathway implicated in vasocontraction of VSMCs. The Western blotting method was employed as part of strategy 4.

Experimental design for strategy 4:

Cells were seeded in 6-well plates. The experimental design to pursue strategy 4 incorporated the following treatment groups (**Table 3.10**):

Table 3.10: Treatment groups for Rho kinase pathway experiments

| # | Treatment Groups |
|---|---|
| 1 | The control group (only SFM and no treatment) |
| 2 | Oxytocin 100 nM |
| 3 | Ang II 100 nM |
| 4 | Oxytocin (100 nM) + Ang II (100 nM) |
| 5 | L-371,257 [oxytocin receptor antagonist (OXTA)] 1 μ M |
| 6 | OXTA 1 μ M + Oxytocin (100 nM) + Ang II (100 nM) |

To determine the effect of oxytocin, and its interaction with Ang II on the phosphorylation of ROCK-1 and ROCK-2, cells were treated with oxytocin for ten minutes, and with Ang II also for ten minutes. In the combination group, cells were pre-treated with oxytocin for ten minutes before the treatment with Ang II. OXTA was added 30 minutes before the treatment with oxytocin.

3.12. Specific aim 3, strategy, and experimental design

Specific aim 3: To characterize the effect of oxytocin and its interaction with Ang II on proliferation, inflammation, oxidative, and vasocontraction pathway in primary aortic VSMCs obtained from SHRs.

Strategies and experimental design for specific aim 3:

To pursue specific aim 3, the approaches similar to specific aims 1 and 2 were adopted, and identical experimental designs were employed in aortic VSMCs obtained from hypertensive rats (SHRs).

Chapter 4: Results

4.1. Normotensive Wistar rats

(Specific aims 1 and 2)

4.1.1. Effects of oxytocin and Ang II on proliferation of aortic VSMCs isolated from normotensive Wistar rats

4.1.1.1. Cell counting by Hemocytometer method in Wistar VSMCs

Oxytocin (100 nM) significantly reduced the proliferation of aortic VSMCs compared to the control group in normotensive Wistar rats. Oxytocin-induced reduction of VSMCs proliferation rate of VSMCs was time-dependent, with a $50.9\% \pm 8.3\%$ ($P < 0.05$) reduction in proliferation at 24 hours, $89.2\% \pm 1.2\%$ ($P < 0.001$) reduction at 48 hours and $94.7\% \pm 5.3\%$ ($P < 0.001$) at 72 hours compared to control (**Figures 4.1 and 4.2**).

Contrary to oxytocin, Ang II (100 nM) significantly increased the proliferation of VSMCs by $354\% \pm 46\%$ ($P < 0.001$) at 24 hours, $217\% \pm 13\%$ ($P < 0.001$) at 48 hours, and $118\% \pm 73\%$ ($P < 0.001$) at 72 hours when compared to control (**Figures 4.1 and 4.2**).

Pre-treatment with oxytocin (100 nM) for ten minutes remarkably reduced the Ang II-induced increase in VSMCs proliferation by $62.9\% \pm 8.8\%$ ($P < 0.01$) at 24 hours, $82.9\% \pm 21.5\%$ ($P < 0.001$) at 48 hours and $86.9\% \pm 5.5\%$ ($P < 0.001$)

at 72 hours compared to Ang II alone. These effects of oxytocin were prevented by pre-treatment with the OXTA– L-371,257 (1 μ M), (**Figures 4.1** and **4.2**).

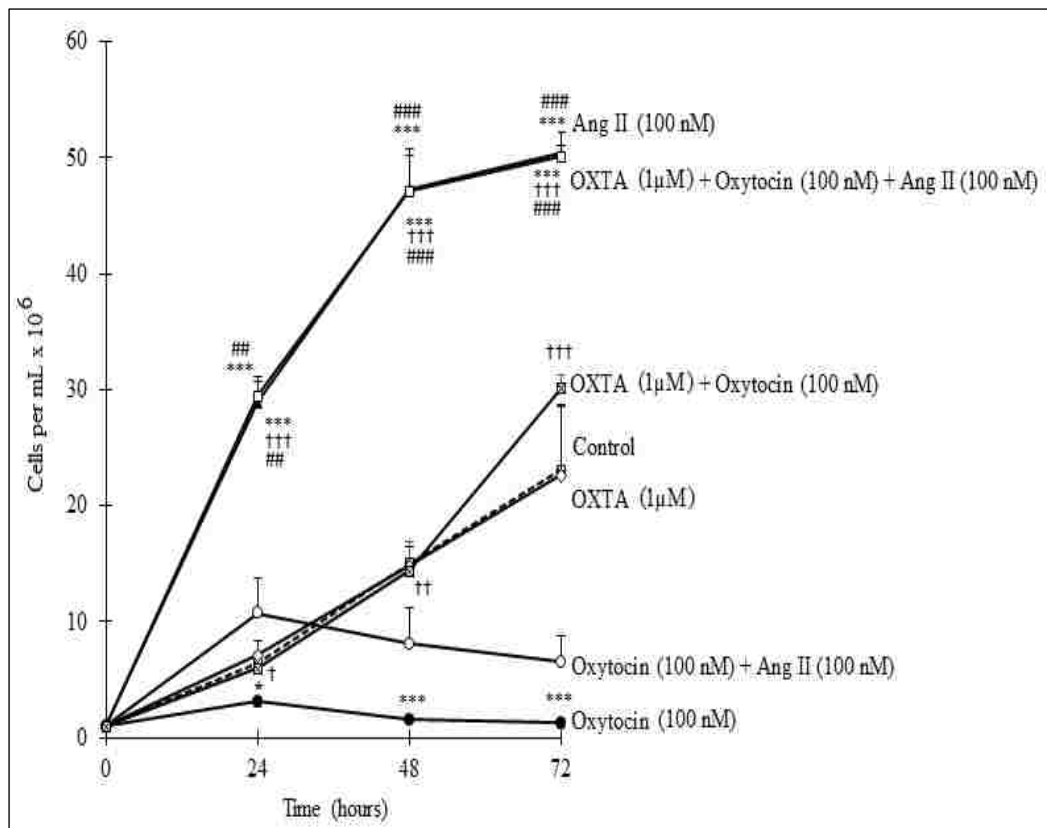


Figure 4.1. The effect of oxytocin and different treatment groups on aortic VSMCs proliferation in Wistar rats as assessed by the Hemocytometer method. Cells were seeded at 1×10^6 cells/well in 6-well plates. Cell proliferation was measured by counting the number of cells per milliliter (mL). The data are represented as the mean \pm SEM from three independent experiments performed in duplicates using aortic VSMCs from three Wistar rats. Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.

* $P < 0.05$ and *** $P < 0.001$: All groups versus Control

† $P < 0.05$, †† $P < 0.01$, and ††† $P < 0.001$: Oxytocin versus (OXTA + Oxytocin); and Oxytocin versus Ang II

$P < 0.01$ and ### $P < 0.001$: (Oxytocin + Ang II) versus Ang II alone; and (Oxytocin + Ang II) versus (OXTA + Oxytocin + Ang II)

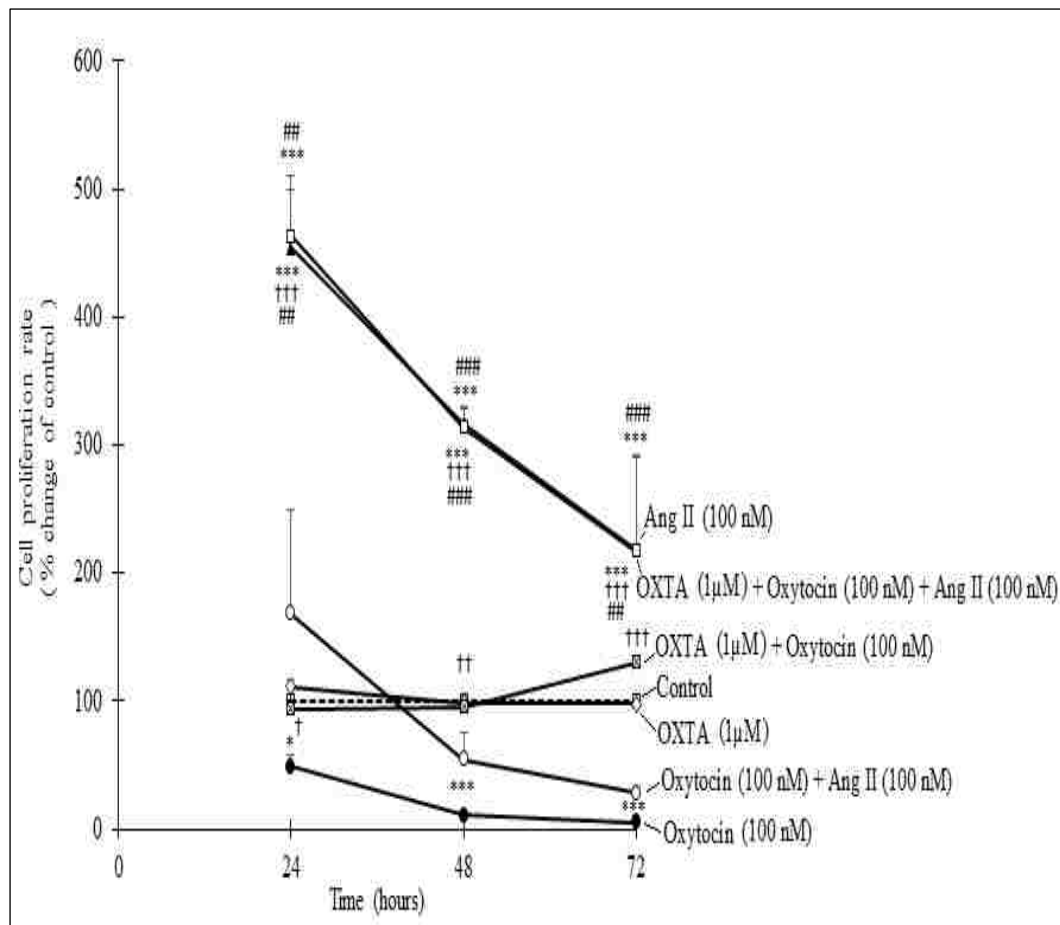


Figure 4.2. The effect of oxytocin and different treatment groups on the proliferation rate of aortic VSMCs in Wistar rats as assessed by the Hemocytometer method. Cells were seeded at 1×10^6 cells/well in 6-well plates. Cell proliferation rate was assessed as percent (%) change of control. The data are represented as the mean \pm SEM from three independent experiments performed using aortic VSMCs from three Wistar rats. Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.

* $P < 0.05$ and *** $P < 0.001$: All groups versus Control

† $P < 0.05$, †† $P < 0.01$, and ††† $P < 0.001$: Oxytocin versus (OXTA + Oxytocin); and Oxytocin versus Ang II

$P < 0.01$ and ### $P < 0.001$: (Oxytocin + Ang II) versus Ang II alone; and (Oxytocin + Ang II) versus (OXTA + Oxytocin + Ang II)

4.1.1.2. Assessment of cell viability with the MTT-assay in Wistar VSMCs

The effect of oxytocin on cell viability in aortic VSMCs was evaluated with the MTT assay. Oxytocin (100 nM) induced a significant decrease in cell viability of VSMCs in a time-dependent manner with $4.4\% \pm 3\%$ ($P < 0.001$), $22.6\% \pm 7\%$ ($P < 0.001$), and $21.4\% \pm 6\%$ ($P < 0.001$) reduction at 24, 48 and 72 hours, respectively compared to control (**Figures 4.3 and 4.4**).

Contrary to oxytocin's effect, Ang II (100 nM) treatment showed a significant increase in cell viability of VSMCs with $112\% \pm 15\%$ ($P < 0.001$), $119\% \pm 15\%$ ($P < 0.001$), and $79.6\% \pm 10\%$ ($P < 0.001$) increases in proliferation of VSMCs at 24, 48, and 72 hours, respectively compared to control (**Figures 4.3 and 4.4**).

The pre-treatment with oxytocin (100 nM) also significantly reduced Ang II-induced VSMCs viability with $53.8\% \pm 4.5\%$ ($P < 0.001$) decrease at 24 hours, $63.8\% \pm 7.7\%$ ($P < 0.001$) decrease at 48 hours and a $51.6\% \pm 5.8\%$ ($P < 0.001$) decrease at 72 hours compared to Ang II alone. Both these effects of oxytocin were significantly antagonized by the OXTA (**Figures 4.3 and 4.4**).

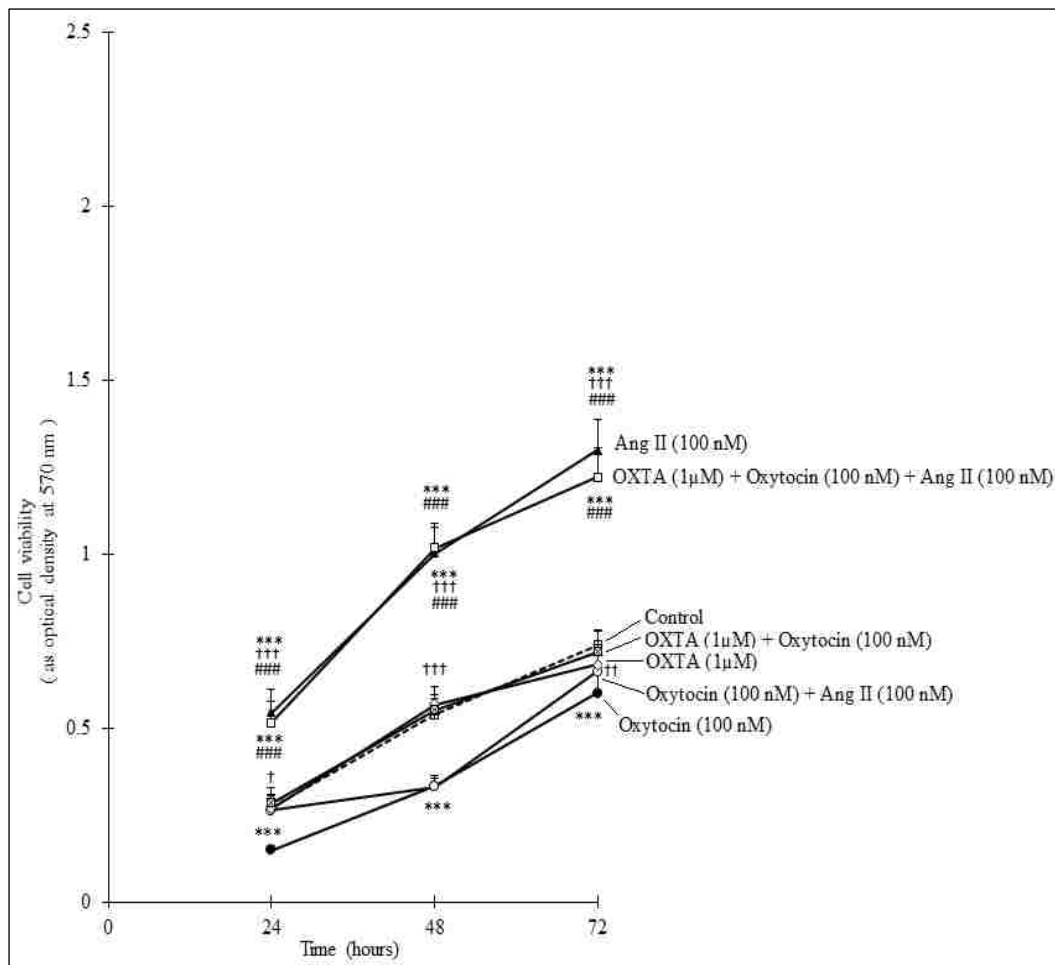


Figure 4.3. The effect of oxytocin and different treatment groups on Wistar aortic VSMCs viability as assessed by the MTT assay. Cells were seeded at 1×10^4 cells/well in 96-well plates. Cell viability was determined as the function of an optical density (OD) measured at 570 nm. The data are shown as the mean \pm SEM of three independent experiments performed using aortic VSMCs isolated from three Wistar rats. Each experiment was performed with 12 replicates. Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.

*** $P < 0.001$: All groups versus Control

† $P < 0.05$, †† $P < 0.01$, and ††† $P < 0.001$: Oxytocin versus (OXTA + Oxytocin); and Oxytocin versus Ang II

$P < 0.001$: (Oxytocin + Ang II) versus Ang II alone; and (Oxytocin + Ang II) versus (OXTA + Oxytocin + Ang II)

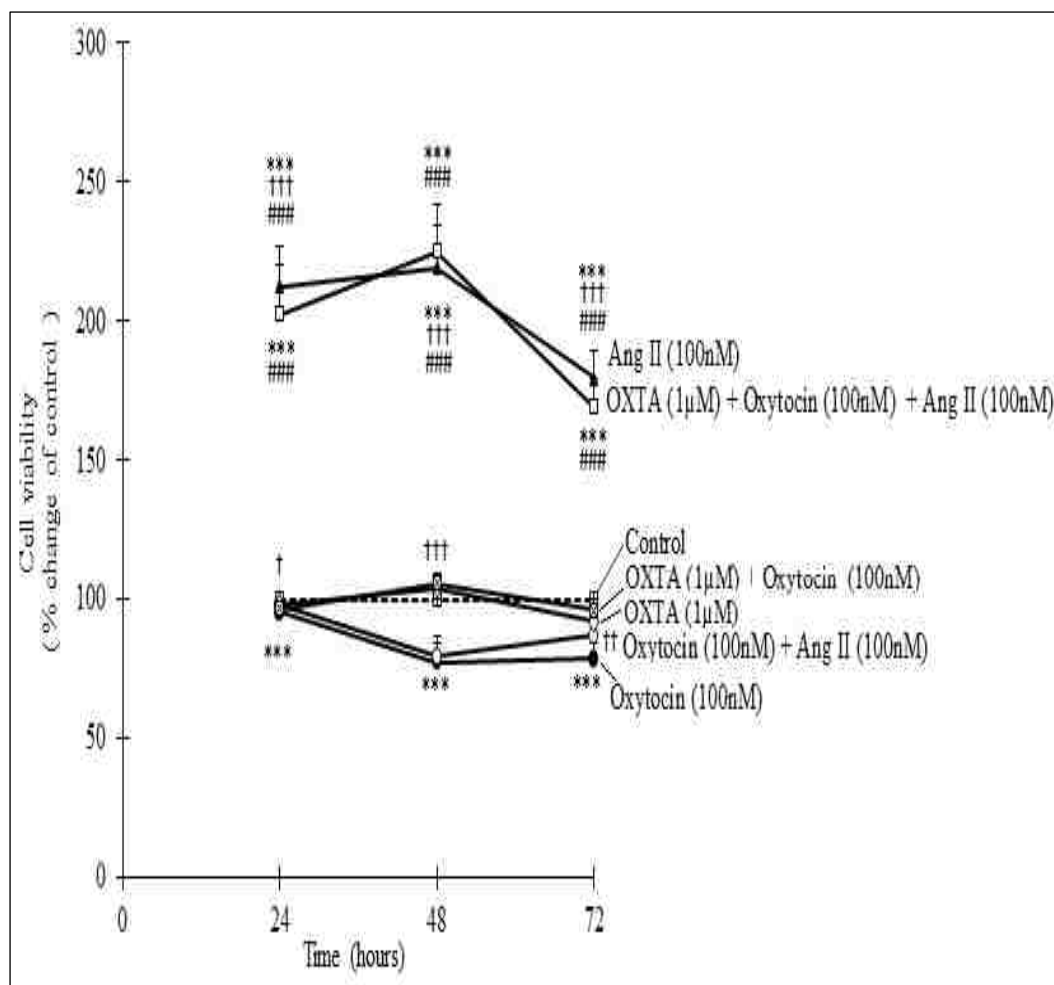


Figure 4.4. The effect of oxytocin and different treatment groups on Wistar aortic VSMCs viability rate as assessed by the MTT assay. Cells were seeded at 1×10^4 cells/well in 96-well plates. Cell viability was measured as the percent (%) change of control. The data are the shown as the mean \pm SEM of three independent experiments performed using aortic VSMCs isolated from three Wistar rats. Each experiment was performed with 12 replicates. Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.

*** $P < 0.001$: All groups versus Control

† $P < 0.05$, †† $P < 0.01$, and ††† $P < 0.001$: Oxytocin versus (OXTA + Oxytocin); and Oxytocin versus Ang II

$P < 0.001$: (Oxytocin + Ang II) versus Ang II alone; and (Oxytocin + Ang II) versus (OXTA + Oxytocin + Ang II)

4.1.1.3. Assessment of cell growth with ^3H -Thymidine incorporation assay in Wistar VSMCs

The growth assay allowed an indirect measurement of cell division/growth – as the function of the incorporation of the radioisotope ^3H -Thymidine into deoxyribonucleic acid (DNA) of the rapidly dividing cells. The radioactivity recovered from the cells was measured in terms of counts per minute (cpm). A higher count indicated a higher growth rate and vice-versa.

Growth assay results validated that oxytocin (100 nM) significantly reduced the ^3H -Thymidine incorporation in aortic VSMCs from $5,857 \pm 3,825$ cpm (control) to $2,171 \pm 1,446$ cpm [44% \pm 12% decrease ($P < 0.01$), **Figure 4.5**].

On the other hand, treatment with Ang II (100 nM) significantly increased ^3H -Thymidine incorporation in VSMCs from $5,857 \pm 3,825$ cpm (control) to $11,194 \pm 7,874$ cpm [70% \pm 15% increase ($P < 0.01$), **Figure 4.5**].

Ang II-induced growth of VSMCs was significantly decreased by pre-treatment with oxytocin [4,092 cpm \pm 2,257 cpm (43% \pm 15% decrease), $P < 0.01$] compared to Ang II alone (11,194 cpm \pm 7,874 cpm). Both these effects of oxytocin were antagonized by OXTA (Figure 4.5).

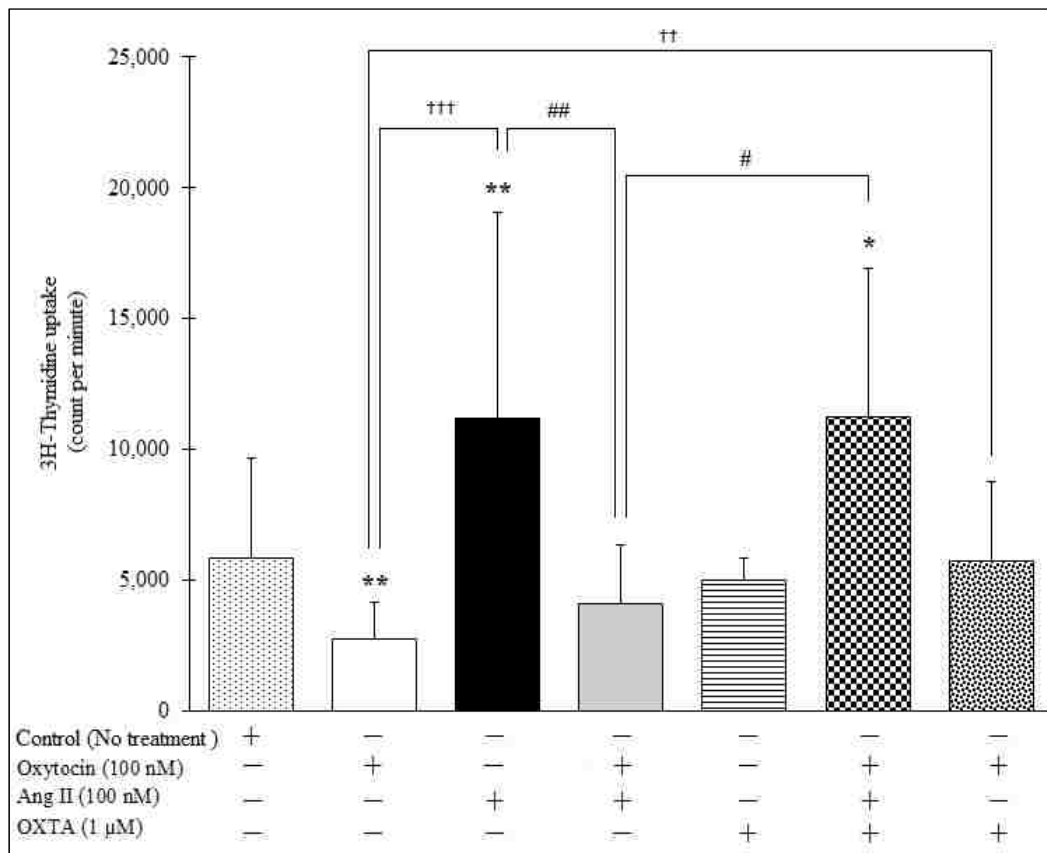


Figure 4.5. The effect of oxytocin and various treatment groups on aortic Wistar VSMCs growth as assessed by the ^3H -Thymidine incorporation assay. VSMCs were seeded at 5×10^4 cells/well in 24-well plates. Cell growth was measured as the function of the intake of the radioisotope ^3H -Thymidine into the deoxyribonucleic acid (DNA) of the rapidly dividing cells and counted by the scintillation counter. The data are represented as the counts per minute (cpm) and are shown as the mean \pm SEM of four independent experiments, each performed with triplicates, using aortic VSMCs from four Wistar rats. Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.

* $P < 0.05$ and ** $P < 0.01$: versus Control

†† $P < 0.01$ and ††† $P < 0.001$: Oxytocin versus (OXTA + Oxytocin); and Oxytocin versus Ang II

$P < 0.05$ and ### $P < 0.01$: (Oxytocin + Ang II) versus (OXTA + Oxytocin + Ang II); and (Oxytocin + Ang II) versus and Ang II alone

4.1.1.4. Assessment of cell viability by flow cytometry in Wistar VSMCs

The outcomes of the flow cytometry experiments revealed that treatment with oxytocin decreased cell viability, and induced apoptosis and necrosis in aortic VSMCs isolated from Wistar rats.

The flow experiments utilized two different concentrations of oxytocin: 10 nM and 100 nM. When compared to the control group ($95.5\% \pm 0.7\%$), the live cell percentage in aortic VSMCs significantly decreased to $91.7\% \pm 0.4\%$ ($P < 0.05$) in the 10 nM oxytocin group and further plummeted to $67.5\% \pm 2.3\%$ ($P < 0.01$) in the 100 nM oxytocin group, respectively (**Figure 4.6**).

Oxytocin significantly induced apoptosis in aortic VSMCs. The treatment with 100 nM oxytocin significantly increased apoptosis up to $26.1\% \pm 3\%$ ($P < 0.05$) compared to apoptosis in the control group ($2.6\% \pm 1.1\%$). However, there were no significant differences in the percentage of apoptotic cells between the 10 nM oxytocin group ($4.2\% \pm 1.6\%$) and the control group [$2.7\% \pm 1.1\%$ (**Figure 4.6**)].

In addition to inducing apoptosis, the flow results demonstrated that oxytocin considerably induced necrosis in aortic VSMCs. When compared to control ($0.7\% \pm 0.1\%$), the percentage of cells undergoing necrosis significantly increased to $3.6\% \pm 1.4\%$ ($P < 0.05$) and $3.9\% \pm 0.3\%$ ($P < 0.01$) in the 10 nM oxytocin group and the 100 nM oxytocin group, respectively. However, there was no statistically significant difference between the percentage of necrotic cells between oxytocin 10 nM and oxytocin 100 nM treatment groups (**Figure 4.6**).

The percentage of aortic VSMCs double positive for both necrosis and apoptosis significantly increased from $0.9\% \pm 0.1\%$ (in control group) to $2.5\% \pm 0.1\%$ [in 100 nM oxytocin group ($P < 0.01$)]. Conversely, there were no significant differences between the 10 nM oxytocin group ($0.5\% \pm 0.1\%$) and the control group [$0.9\% \pm 0.1\%$ (**Figure 4.6**)].

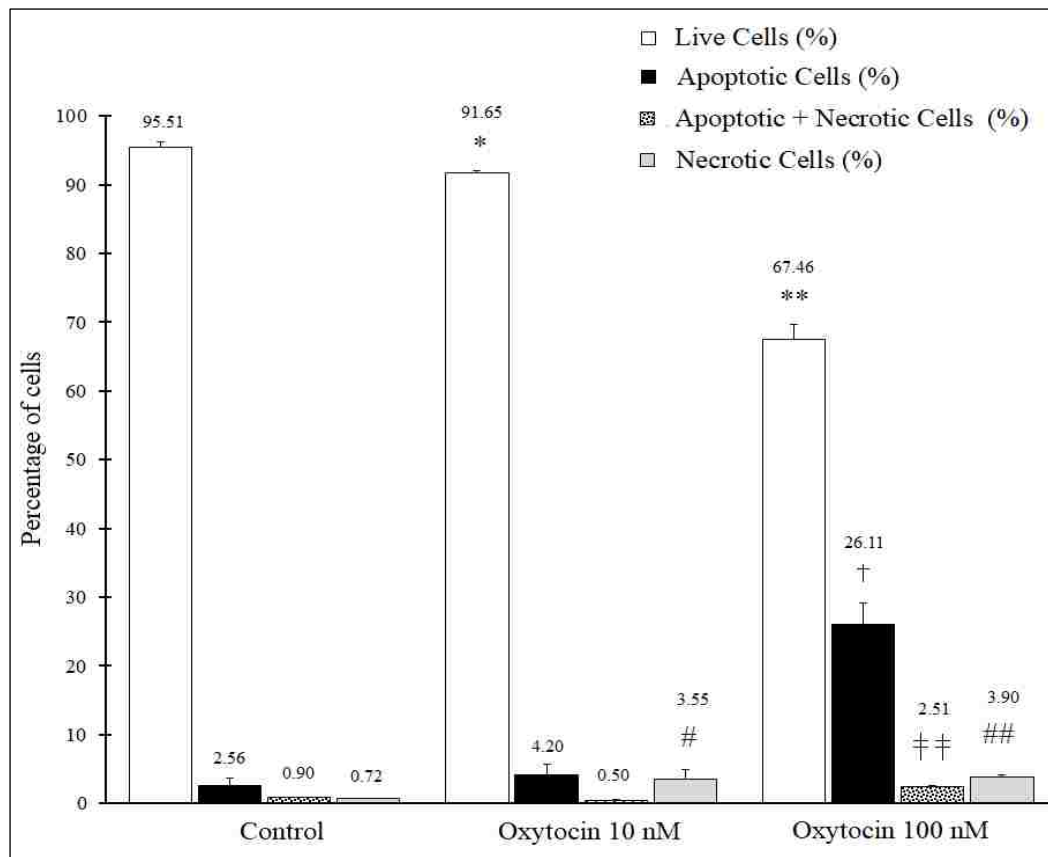


Figure 4.6. The effect of oxytocin on apoptosis and necrosis in aortic Wistar VSMCs as assessed by flow cytometry. VSMCs were seeded at 1×10^6 cells/well in 6-well plates. The data are shown as the percentage of cells positive for apoptosis, necrosis and apoptosis + necrosis across different treatment groups. The data are represented as the mean \pm SEM from three independent experiments performed using aortic VSMCs from three Wistar rats. The treatment was carried out for 24 hours with the two different concentrations of oxytocin: 10 nM and 100 nM. Cells in the control group were not treated with oxytocin (only SFM). Cells were stained with fluorescent dyes: Annexin V (for apoptosis) and 7-amino actinomycin D [(7-AAD) for necrosis] and assessed with the flow cytometer.

* $P < 0.05$: Live cells in Oxytocin 10 nM group versus Live cells in Control group

** $P < 0.01$: Live cells in Oxytocin 100 nM group versus Live cells in Control group

‡ $P < 0.05$: Apoptotic cells in Oxytocin 100 nM group versus Apoptotic cells in Control group

$P < 0.05$: Necrotic cells in Oxytocin 10 nM group versus Necrotic cells in Control group

$P < 0.01$: Necrotic cells in Oxytocin 100 nM group versus Necrotic cells in Control group

‡‡ $P < 0.01$: (Apoptotic + Necrotic cells) in Oxytocin 100 nM group versus (Apoptotic + Necrotic cells) in Control group

4.1.2. Effect of oxytocin and its pre-treatment with Ang II on the phosphorylation of ERK1/2 in aortic Wistar VSMCs

In aortic Wistar VSMCs, oxytocin (100 nM) significantly decreased the active phosphorylated form of ERK1/2 (pERK1/2) after ten minutes of treatment compared to the control group (basal levels). While the treatment with Ang II (100 nM) significantly increased pERK1/2 after ten minutes of treatment compared to control (**Figure 4.7**).

The pre-treatment with oxytocin (100 nM) significantly reduced Ang II-induced activation of pERK1/2, and pre-treatment with OXTA (1 μ M) for 30 minutes significantly abrogated these effects of oxytocin (**Figure 4.7**).

The densitometric analysis of the western blot images showed that oxytocin significantly decreased pERK1/2 by $57.9\% \pm 14.2\%$ ($P < 0.001$) compared to control, Ang II significantly increased pERK1/2 by $223\% \pm 11\%$ ($P < 0.001$) compared to control, and the oxytocin pre-treatment significantly decreased pERK1/2 by $76.6\% \pm 1.4\%$ ($P < 0.01$) compared to Ang II alone (**Figure 4.7**).

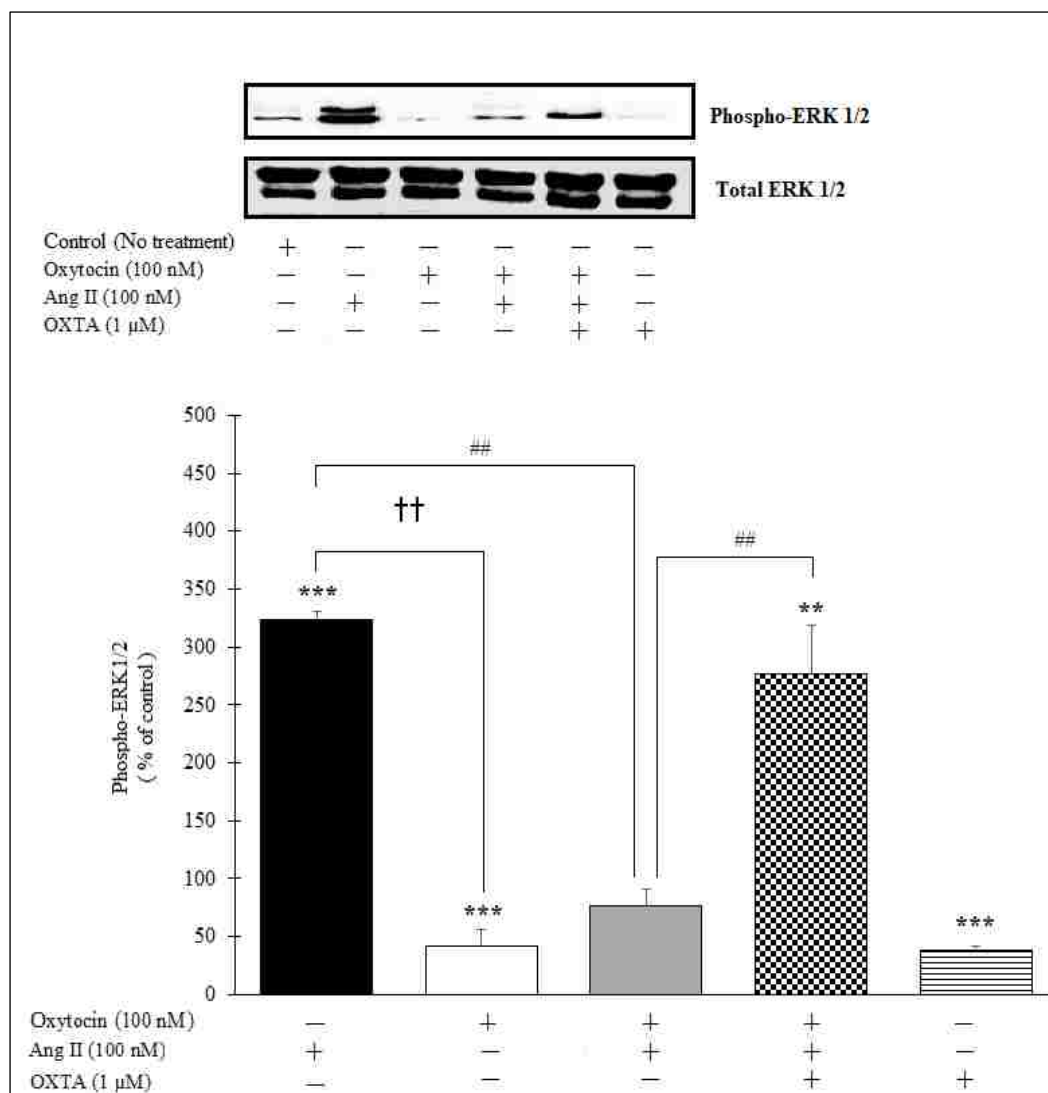


Figure 4.7. The effect of oxytocin and different treatment groups on the phosphorylation of ERK1/2 in aortic Wistar VSMCs. Western blotting was utilized to measure phospho-ERK1/2 levels, and the data were normalized with total ERK1/2. The effect of oxytocin and its combination with different treatment groups on the phosphorylation of ERK1/2 is shown as the percent (%) of control, with control as 100%. The data are shown as the mean \pm SEM of five independent experiments, performed using aortic VSMCs isolated from five Wistar rats. ** indicates $P < 0.01$ versus control; *** $P < 0.001$ versus control; †† $P < 0.01$ Oxytocin versus Ang II; ## $P < 0.01$ (Oxytocin + Ang II) versus Ang II alone; and (Oxytocin + Ang II) versus (OXTA + Oxytocin + Ang II). Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.

4.1.3. Effect of oxytocin and its pre-treatment with Ang II on the phosphorylation of PI3K p110 α and Akt in aortic Wistar VSMCs

In primary aortic VSMCs obtained from Wistar rats, the densitometric analysis of the western blot images showed that the treatment with oxytocin (100 nM) significantly decreased the active phosphorylated form of PI3K p110 α (phospho-PI3K p110 α) by $64.8\% \pm 2.8\%$ ($P < 0.01$) and the active phosphorylated form of Akt (phospho-Akt) by $46.2\% \pm 9.5\%$ ($P < 0.05$) compared to controls, respectively (**Figures 4.8 and 4.9**).

On the other hand, treatment with Ang II (100 nM) significantly increased the levels of phospho-PI3K p110 α by $184\% \pm 17\%$ ($P < 0.05$) and phospho-Akt by $44.4\% \pm 3.5\%$ ($P < 0.001$) compared to control respectively (**Figures 4.8 and 4.9**).

Pre-treatment with oxytocin (100 nM) significantly reduced Ang II-mediated increased levels of phospho-PI3K p110 α by $83.8\% \pm 8.6\%$ ($P < 0.01$) and phospho-Akt by $62.1\% \pm 10.9\%$ ($P < 0.001$) compared to Ang II alone, respectively. These effects of oxytocin were significantly abated by pre-treatment with OXTA (1 μ M) for 30 minutes (**Figures 4.8 and 4.9**).

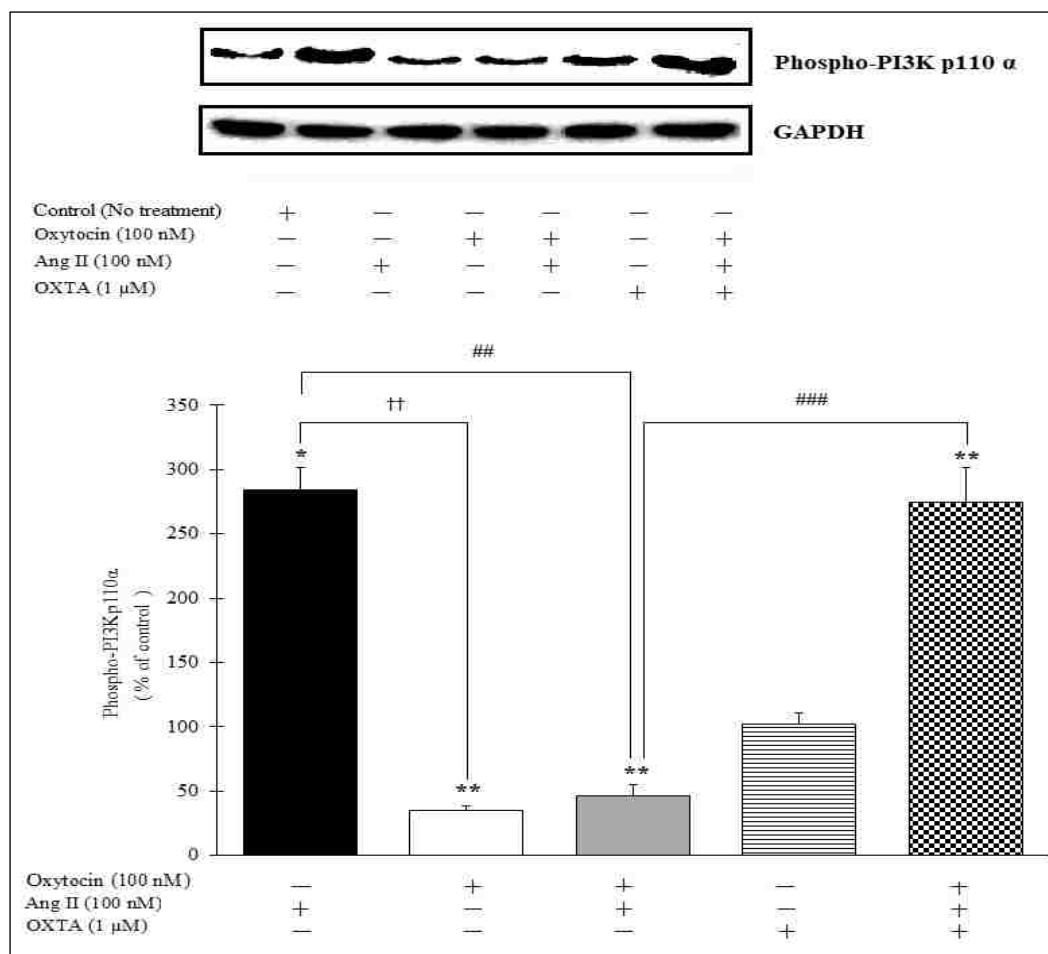


Figure 4.8. The effect of oxytocin and different treatment groups on the phosphorylation of PI3K p110 α in aortic Wistar VSMCs. Western blotting was utilized to measure the phosphorylation of PI3K p110 α , and the data were normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The effect of oxytocin and its combination with different treatment groups on the phosphorylation of PI3K p110 α is shown as the percent (%) of control, with control as 100 %. The data are shown as the mean \pm SEM of three independent experiments, performed using aortic VSMCs isolated from three Wistar rats. * indicates $P < 0.05$ versus control; ** $P < 0.01$ versus control; †† $P < 0.01$ Oxytocin versus Ang II; ## $P < 0.01$ (Oxytocin + Ang II) versus Ang II alone; ### $P < 0.001$ (Oxytocin + Ang II) versus (OXTA + Oxytocin + Ang II). Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.

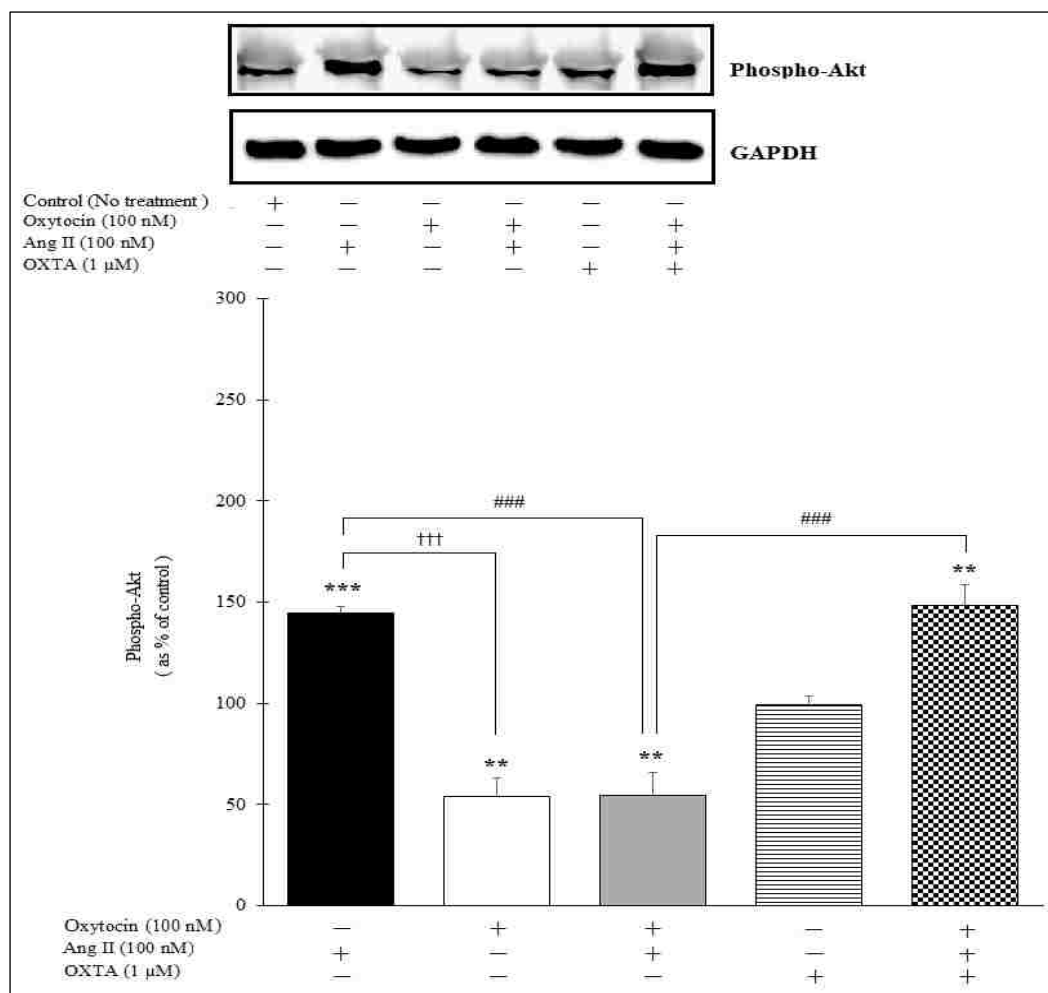


Figure 4.9. The effect of oxytocin and different treatment groups on the phosphorylation of Akt in aortic Wistar VSMCs. Western blotting was utilized to measure the phosphorylation of Akt, and the data were normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The effect of oxytocin and its combination with different treatment groups on the phosphorylation of Akt is shown as the percent (%) of control, with control as 100%. The data are shown as the mean \pm SEM of four independent experiments, performed using aortic VSMCs isolated from four Wistar rats. ** indicates $P < 0.01$ versus control; *** $P < 0.001$ versus control; ††† $P < 0.001$ Oxytocin versus Ang II; ### $P < 0.001$ (Oxytocin + Ang II) versus Ang II alone, and (Oxytocin + Ang II) versus (OXTA + Oxytocin + Ang II). Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.

4.1.4. Effect of oxytocin and its pre-treatment with Ang II on the activation of ROCK -1 and ROCK-2 in aortic Wistar VSMCs

Densitometric analysis of the western blot images showed that in Wistar rats, oxytocin (100 nM) significantly reduced the activation of ROCK-1 by 60.4% \pm 7.6% ($P < 0.001$) and ROCK-2 by 38.7% \pm 13.1% ($P < 0.01$) compared to controls, respectively (**Figures 4.10 and 4.11**).

Ang II (100 nM) significantly enhanced the activation of ROCK-1 by 147% \pm 50% ($P < 0.05$) and ROCK-2 by 83.2% \pm 14.6% ($P < 0.001$) compared to controls, respectively (**Figures 4.10 and 4.11**).

Pre-treatment with oxytocin (100 nM) significantly reduced Ang II-induced activation of ROCK-1 by 85.2% \pm 10.6% ($P < 0.01$) and ROCK-2 by 65.9% \pm 13.2% ($P < 0.001$) compared to Ang II alone, respectively. These effects of oxytocin were significantly reduced by pre-treatment with OXTA (1 μ M) (**Figures 4.10 and 4.11**).

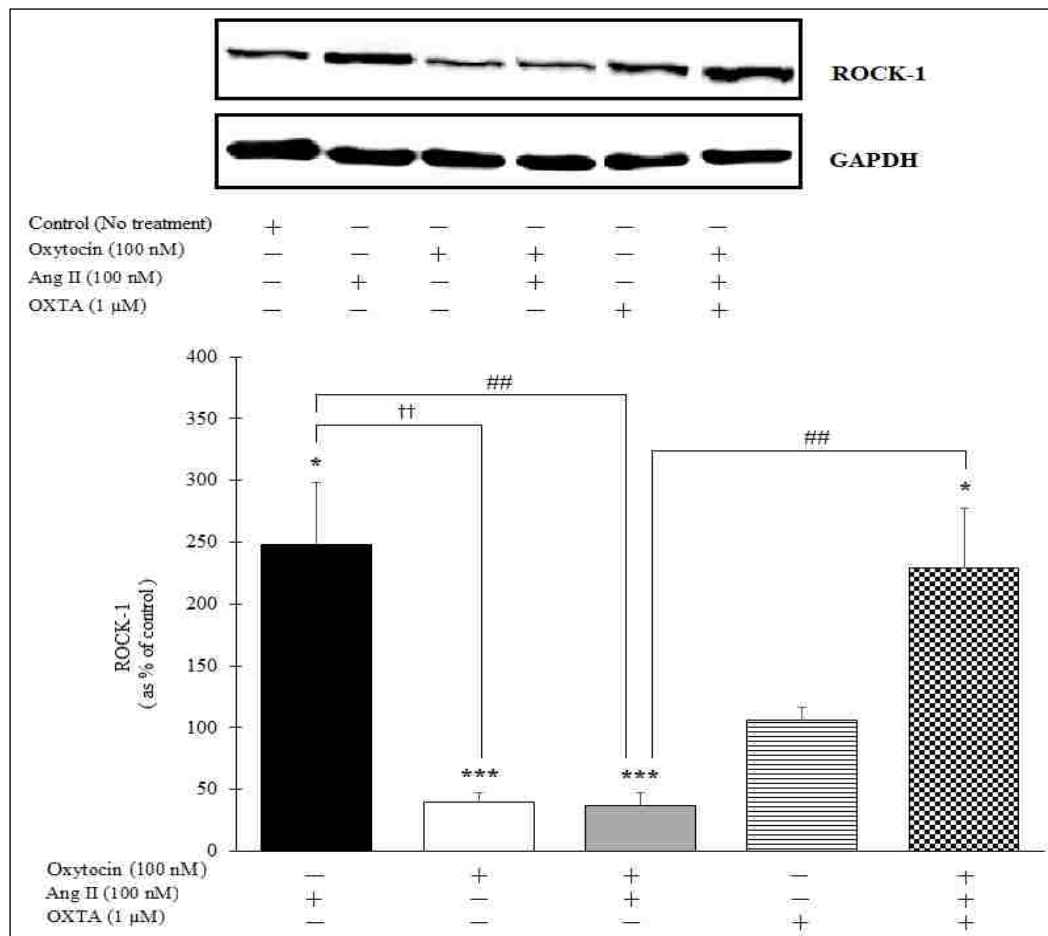


Figure 4.10. The effect of oxytocin and different treatment groups on the activation of ROCK-1 in aortic Wistar VSMCs. Western blotting was utilized to measure the ROCK-1 levels, and the data were normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The effect of oxytocin and its combination with different treatment groups on the activation of ROCK-1 is shown as the percent (%) of control, with control as 100%. The data are shown as the mean \pm SEM of four independent experiments, performed using aortic VSMCs isolated from four Wistar rats. * indicates $P < 0.05$ versus control; *** $P < 0.001$ versus control; †† $P < 0.01$ Oxytocin versus Ang II; ††† $P < 0.01$ (Oxytocin + Ang II) versus Ang II alone, and (Oxytocin + Ang II) versus (OXTA + Oxytocin + Ang II). Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist; ROCK, Rho-associated coiled-coil containing protein kinase.

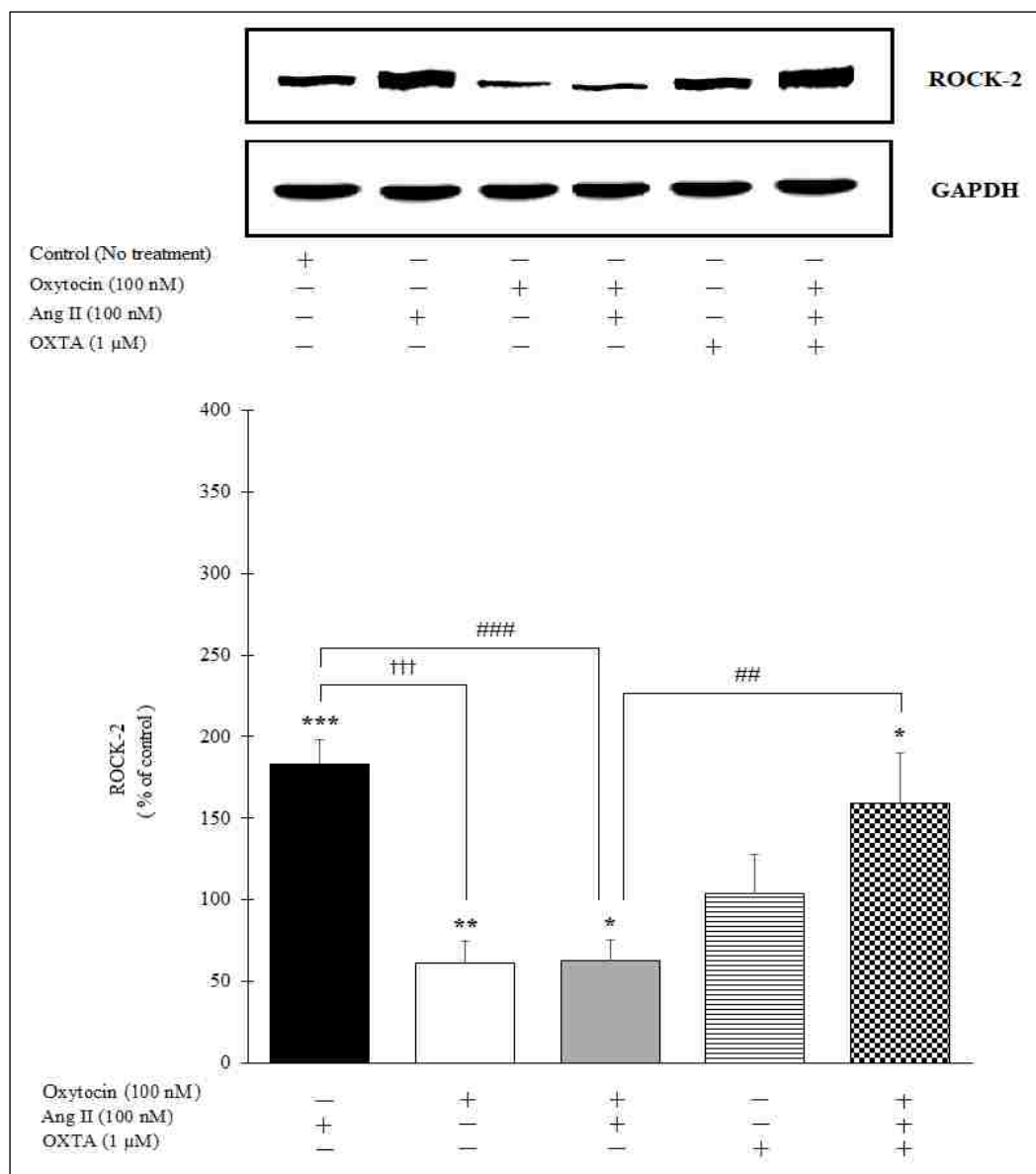


Figure 4.11. The effect of oxytocin and different treatment groups on the activation of ROCK-2 in aortic Wistar VSMCs. Western blotting was utilized to measure the ROCK-2 levels, and the data were normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The effect of oxytocin and its combination with different treatment groups on the activation of ROCK-2 is shown as the percent (%) of control, with control as 100%. The data are shown as the mean \pm SEM of five independent experiments, performed using aortic VSMCs isolated from five Wistar rats. * indicates $P < 0.05$ versus control; ** $P < 0.01$ versus control; *** $P < 0.001$ versus control; ††† $P < 0.001$ Oxytocin versus Ang II; ### $P < 0.001$

(Oxytocin + Ang II) versus Ang II alone; ^{##} P < 0.01 (Oxytocin + Ang II) versus (OXTA + Oxytocin + Ang II). Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist; ROCK, Rho-associated coiled-coil containing protein kinase.

4.1.5. Effect of oxytocin and its pre-treatment with Ang II on IL-6 secretion in aortic Wistar VSMCs

The IL-6 ELISA assay was carried out as per the manufacturer's protocol. The concentration of IL-6 secreted by aortic VSMCs in the culture media was measured in 'picogram per milliliter' (pg/mL). In Wistar rats, the outcomes of the IL-6 assay showed that oxytocin treatment resulted in a significant increase in IL-6 secretion compared to the control group. Three different concentrations of oxytocin were used: 10 nM, 100 nM, 1,000 nM. The treatment with oxytocin at 100 nM and 1,000 nM resulted in significant increases in IL-6 with $1,033 \pm 24$ pg/mL ($P < 0.01$) at 100 nM and 959 ± 52 pg/mL ($P < 0.05$) at 1,000 nM, respectively compared to control group [614 ± 104 pg/mL (**Figure 4.12**)].

However, there was no significant difference between oxytocin 10 nM (695 ± 101 pg/mL) and control group (614 ± 104 pg/mL). There was no significant difference between oxytocin 100 nM ($1,033 \pm 24$ pg/mL) and oxytocin 1,000 nM [959 ± 52 pg/mL (**Figure 4.12**)].

Ang II (100 nM) treatment showed a significant increase in IL-6 [$1,481 \pm 91$ pg/mL ($P < 0.01$)] compared to control group (614 ± 104 pg/mL). Ang II (100 nM) treatment resulted in more IL-6 secretion ($1,481 \pm 91$ pg/mL) compared to treatment with oxytocin [100 nM ($1,033 \pm 24$ pg/mL), $P < 0.01$] when compared to control [614 ± 104 pg/mL (**Figure 4.12**)].

Interestingly, in contrast to its singular effect, oxytocin's pre-treatment (100 nM) with Ang II (100 nM) significantly decreased IL-6 secretion (735 ± 77 pg/mL)

compared to Ang II 100 nM alone [$1,481 \pm 91$ pg/mL ($P < 0.01$)] and oxytocin 100 nM alone [$1,033 \pm 24$ pg/mL ($P < 0.01$)], respectively (**Figure 4.12**).

The effect of oxytocin pre-treatment (100 nM) on Ang II (100 nM) was significantly abolished by pre-treatment with 1 μ M OXTA. IL-6 secretion was significantly higher [$1,487 \pm 64$ pg/mL ($P < 0.001$)] in the OXTA-pre-treatment group [OXTA (1 μ M) + Oxytocin (100 nM) + Ang II (100 nM)] than Oxytocin 100 nM + Ang II 100 nM group [735 ± 77 pg/mL (**Figure 4.12**)].

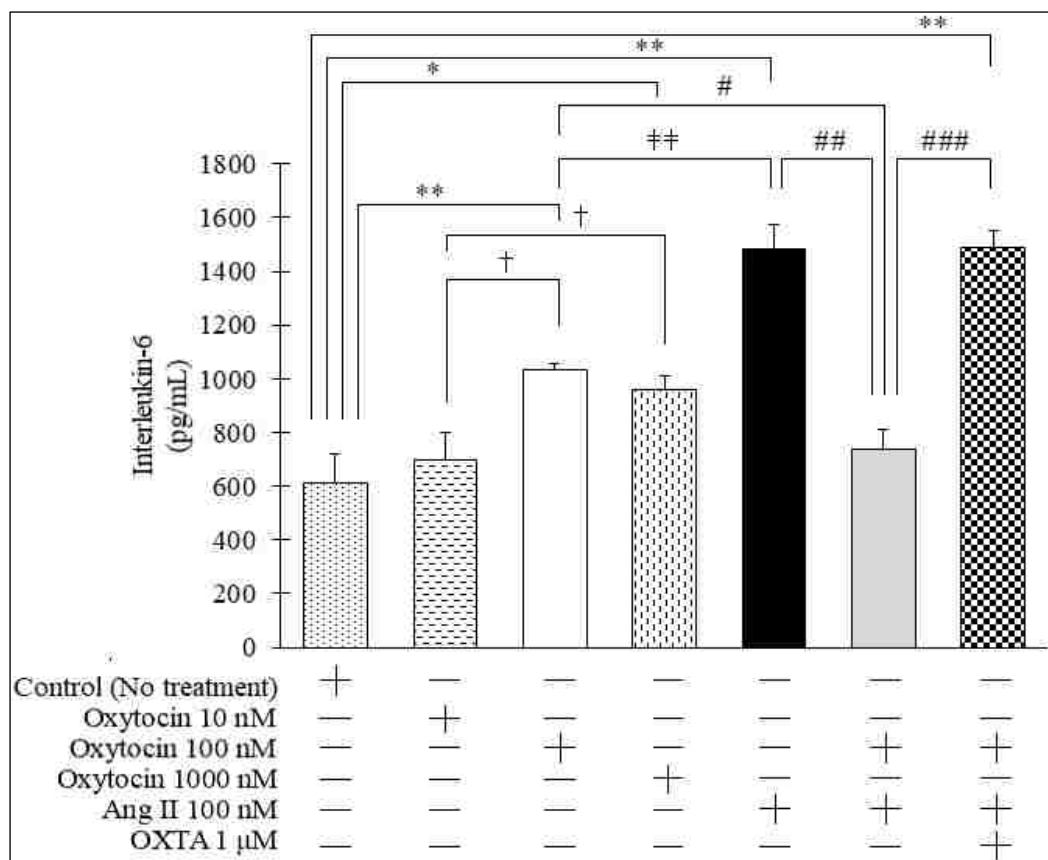


Figure 4.12. The effect of oxytocin and different treatment groups on IL-6 secretion in aortic Wistar VSMCs. The concentration of IL-6 secreted by aortic VSMCs in the culture media was measured in picogram per milliliter (pg/mL). The data are represented as the mean \pm SEM from three independent experiments performed using aortic VSMCs from three Wistar rats. In each experiment each treatment group had duplicates. * indicates $P < 0.05$ versus Control group; ** $P < 0.01$ versus Control group; † $P < 0.05$ Oxytocin 10 nM versus Oxytocin 100 nM and Oxytocin 1000 nM respectively. # $P < 0.05$ (Oxytocin 100 nM + Ang II 100 nM) versus Oxytocin 100 nM alone; ## $P < 0.01$ (Oxytocin 100 nM + Ang II 100 nM) versus Ang II 100 nM alone; ### $P < 0.001$ (Oxytocin 100 nM + Ang II 100 nM) versus (OXTA 1 μ M + Oxytocin 100 nM + Ang II 100 nM); †† $P < 0.01$ Oxytocin 100 nM versus Ang II 100 nM. Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.

4.1.6. Effect of oxytocin and its pre-treatment with Ang II on ROS in aortic Wistar VSMCs

ROS was assessed as the function of fluorescent units. The outcomes of the ROS assay in aortic Wistar VSMCs demonstrated that the treatment with oxytocin (100 nM) significantly increased ROS production [$8,584 \pm 114$ ($P < 0.001$)] compared to the control group ($7,542 \pm 128$). Ang II (100 nM) significantly increased ROS production [$8,336 \pm 204$ ($P < 0.001$)] compared to the control group ($7,542 \pm 128$). There was no significant difference in the ROS production between oxytocin [100 nM ($8,584 \pm 114$)] and Ang II [100 nM ($8,336 \pm 204$)] treatment groups (**Figure 4.13**).

The pre-treatment of oxytocin (100 nM) with Ang II (100 nM) significantly increased ROS production [$9,346 \pm 143$ ($P < 0.001$)] compared to the control group [$7,542 \pm 128$ (**Figure 4.13**)].

Interestingly, the pre-treatment of oxytocin (100 nM) followed by Ang II (100 nM) resulted in significantly higher ($P < 0.001$) ROS production ($9,346 \pm 143$) than oxytocin (100 nM) alone ($8,584 \pm 114$) and Ang II (100 nM) alone ($8,336 \pm 204$), respectively, indicating an additive effect (**Figure 4.13**).

OXTA (1 μ M) on its own significantly increased ROS production [$8,530 \pm 267$ ($P < 0.001$)] compared to the control group ($7,542 \pm 128$). In addition, pre-treatment of OXTA (1 μ M) with oxytocin (100 nM) resulted in significantly increased ROS production [$9,594 \pm 177$ ($P < 0.001$)] when compared with oxytocin (100 nM) alone ($8,584 \pm 114$). This suggests an additive effect of OXTA on oxytocin-stimulated ROS production (**Figure 4.13**).

The combination of OXTA (1 μ M), oxytocin (100 nM), and Ang II (100 nM) significantly increased ROS production [$10,511 \pm 179$ ($P < 0.001$)] compared to control ($7,542 \pm 128$) in aortic Wistar VSMCs (**Figure 4.13**).

ROS production in the combination group of OXTA (1 μ M), oxytocin (100 nM), and Ang II (100 nM) was significantly higher [$10,511 \pm 179$ ($P < 0.001$)] than Oxytocin (100 nM) alone ($8,584 \pm 114$), Ang II (100 nM) alone ($8,336 \pm 204$), and OXTA (1 μ M) alone ($8,530 \pm 267$), respectively, thus indicating an additive effect (**Figure 4.13**).

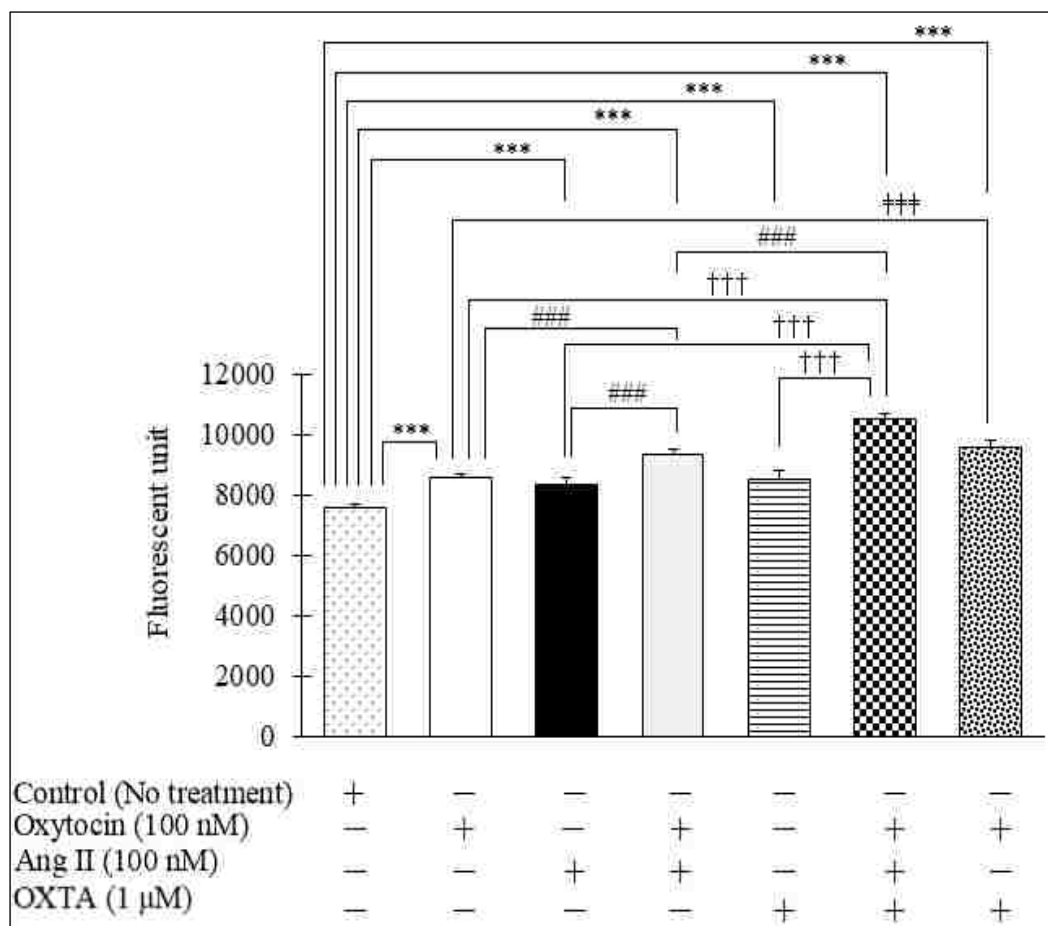


Figure 4.13. The effect of oxytocin and different treatment groups on ROS production in aortic Wistar VSMCs. VSMCs were seeded at 1×10^4 cells/well in 96-well plates. The ROS levels were measured as the function of the fluorescent unit. The data are represented as the mean \pm SEM from three independent experiments performed using aortic VSMCs from three Wistar rats. In each experiment each treatment group had 12 replicates. *** indicates $P < 0.001$ versus Control group; ### $P < 0.01$ (Oxytocin + Ang II) versus Oxytocin alone, Ang II alone, and (OXTA + Oxytocin + Ang II); ††† $P < 0.001$ (OXTA + Oxytocin + Ang II) versus Oxytocin alone, Ang II alone, and OXTA alone; ‡‡‡ $P < 0.001$ Oxytocin versus (OXTA + Oxytocin). Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.

4.2. Spontaneously hypertensive rats [Specific Aim 3]

4.2.1. Effect of oxytocin and its pre-treatment with Ang II on aortic SHR VSMCs proliferation

4.2.1.1. Cell counting by Hemocytometer method in SHR VSMCs

Similar to its effects on VSMCs proliferation from the normotensive rats, oxytocin (100 nM) significantly reduced aortic VSMCs proliferation in SHRs compared to the control group (**Figures 4.14 and 4.15**).

The hemocytometer cell counting revealed that oxytocin-induced reduction in SHR VSMCs proliferation rate was time-dependent, with $44.8\% \pm 3\%$ reduction ($P < 0.001$) in proliferation at 24 hours, $84.4\% \pm 0.9\%$ reduction ($P < 0.001$) reduction at 48 hours and $88.2\% \pm 4.0\%$ reduction ($P < 0.001$) reduction at 72 hours compared to control (**Figures 4.14 and 4.15**).

On the other hand, the treatment with Ang II (100 nM) significantly increased SHR VSMCs proliferation, with $190\% \pm 18\%$ ($P < 0.001$) increase in proliferation rate at 24 hours, $112\% \pm 15\%$ ($P < 0.001$) increase at 48 hours, and $207\% \pm 56\%$ ($P < 0.001$) increase at 72 hours compared to control (**Figures 4.14 and 4.15**).

It is noteworthy that even in the SHRs, the oxytocin (100 nM) pre-treatment significantly reduced Ang II-induced VSMCs proliferation in a time-dependent manner, with $66\% \pm 24\%$ ($P < 0.001$) decrease at 24 hours, $93\% \pm 3\%$ ($P < 0.001$) decrease at 48 hours and $97\% \pm 4\%$ ($P < 0.001$) decrease at 72 hours compared to Ang II alone (**Figures 4.14 and 4.15**). Pre-treatment with OXTA (1 μ M) significantly diminished these effects of oxytocin (**Figures 4.14 and 4.15**).

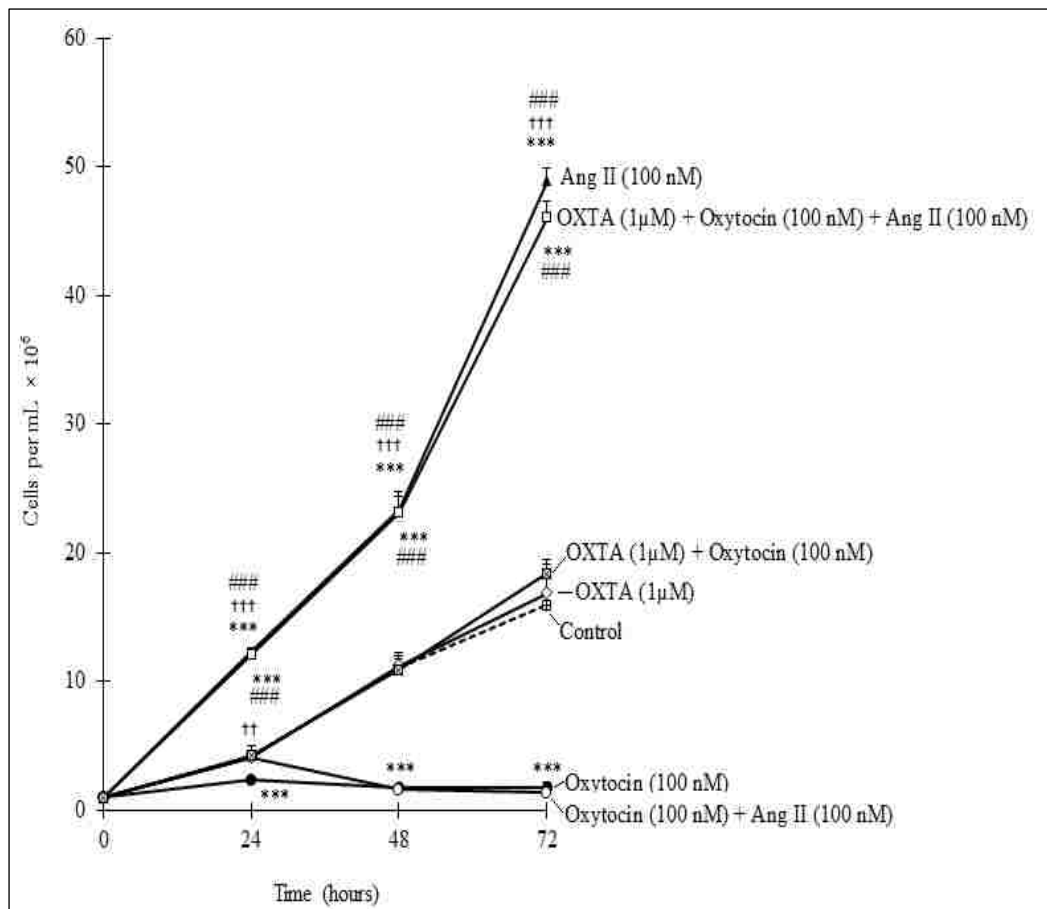


Figure 4.14. The effect of oxytocin and different treatment groups on aortic VSMCs proliferation in SHR as assessed by the Hemocytometer method. SHR VSMCs were seeded at 1×10^6 cells/well in 6-well plates. Cell proliferation was measured by counting the number of cells per milliliter (mL). The data are represented as the mean \pm SEM from three independent experiments performed using aortic VSMCs from three SHR. Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.

*** $P < 0.001$: All groups versus Control

** $P < 0.01$ and *** $P < 0.001$: Oxytocin versus (OXTA + Oxytocin); and Oxytocin versus Ang II

$P < 0.001$: (Oxytocin + Ang II) versus Ang II alone; and (Oxytocin + Ang II) versus (OXTA + Oxytocin + Ang II)

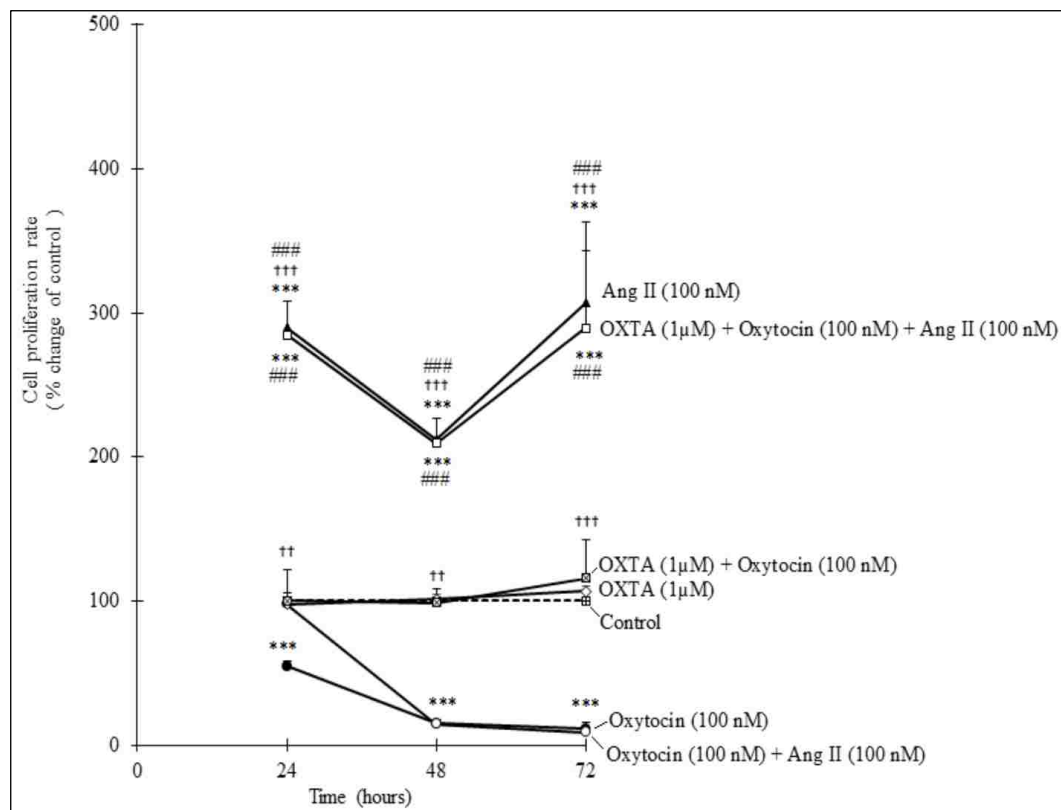


Figure 4.15. The effect of oxytocin and different treatment groups on aortic VSMCs proliferation rate in SHR assessed by the Hemocytometer method. SHR VSMCs were seeded at 1×10^6 cells/well in 6-well plates. Cell proliferation rate was assessed as the percent (%) change of control. The data are represented as the mean \pm SEM from three independent experiments performed using aortic VSMCs from three SHR. Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.

*** $P < 0.001$: All groups versus Control

** $P < 0.01$ and *** $P < 0.001$: Oxytocin versus (OXTA + Oxytocin); and Oxytocin versus Ang II

$P < 0.001$: (Oxytocin + Ang II) versus Ang II alone; and (Oxytocin + Ang II) versus (OXTA + Oxytocin + Ang II)

4.2.1.2. Assessment of cell viability with MTT-assay in SHR VSMCs

The findings from the MTT assay in aortic SHRs VSMCs were similar to those in Wistar rats. Oxytocin (100 nM) significantly reduced cell viability of aortic SHR VSMCs (**Figures 4.16 and 4.17**).

The MTT assay demonstrated that oxytocin caused a reduction in the cell viability of SHR VSMCs by $37.5\% \pm 3.9\%$ ($P < 0.001$) and $32\% \pm 3.8\%$ ($P < 0.001$) at 48 hours and 72 hours, respectively compared to control. However, at 24 hours oxytocin did not show any significant reduction in VSMCs viability rate (**Figures 4.16 and 4.17**).

The treatment with Ang II (100 nM) showed an increase in the VSMCs viability with $145\% \pm 20\%$ ($P < 0.001$), $121\% \pm 14\%$ ($P < 0.001$), and $166\% \pm 13\%$ ($P < 0.001$) increase at 24, 48 and 72 hours, respectively compared to control (**Figures 4.16 and 4.17**).

The pre-treatment with oxytocin (100 nM) prominently reduced Ang II-induced VSMC viability by $51.8\% \pm 8.3\%$ ($P < 0.001$) at 24 hours, $63.7\% \pm 5.7\%$ ($P < 0.001$) at 48 hours, and $71.9\% \pm 3.9\%$ ($P < 0.001$) at 72 hours compared to Ang II alone, and the pre-treatment with OXTA (1 μ M) significantly blocked the effects of oxytocin (**Figures 4.16 and 4.17**).

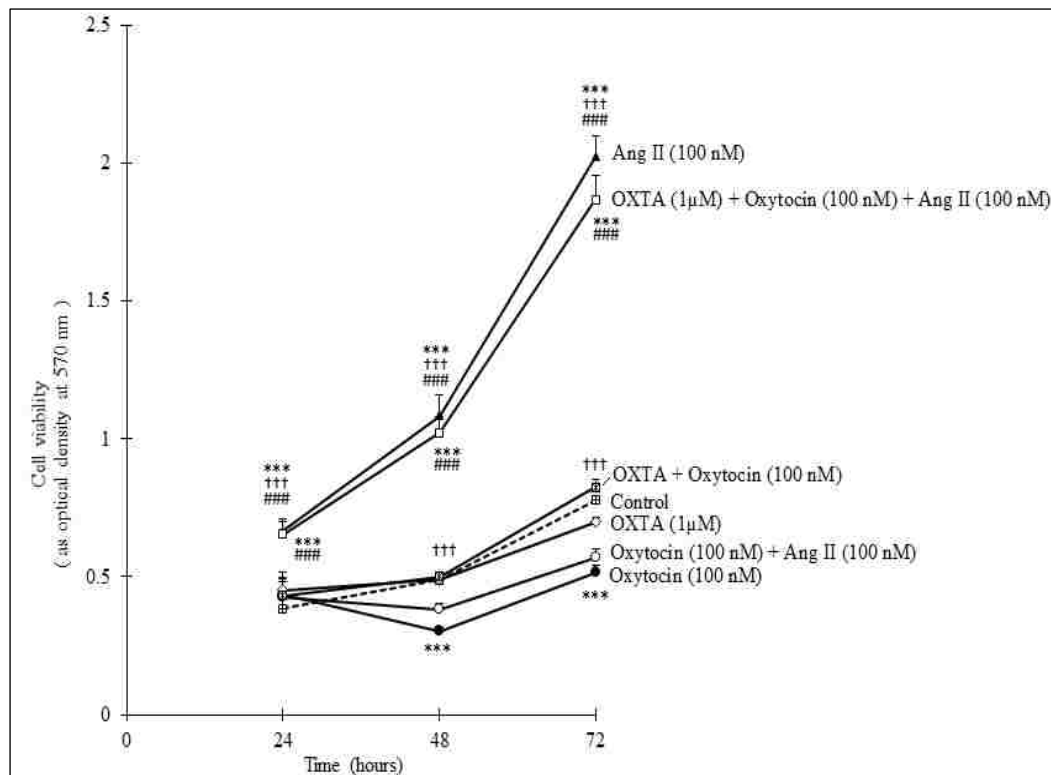


Figure 4.16. The effect of oxytocin and different treatment groups on aortic SHR VSMCs viability as assessed by the MTT assay. SHR VSMCs were seeded at 1×10^4 cells/well in 96-well plates. Cell viability was determined as the function of an optical density (OD) measured at 570nm. The data are shown as the mean \pm SEM of three independent experiments performed using aortic VSMCs isolated from three SHRs. Each experiment was performed with 12 replicates. Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.

*** $P < 0.001$: All groups versus Control

††† $P < 0.001$: Oxytocin versus Ang II; and Oxytocin versus (OXTA + Oxytocin)

$P < 0.001$: (Oxytocin + Ang II) versus Ang II alone; and (Oxytocin + Ang II) versus (OXTA + Oxytocin + Ang II)

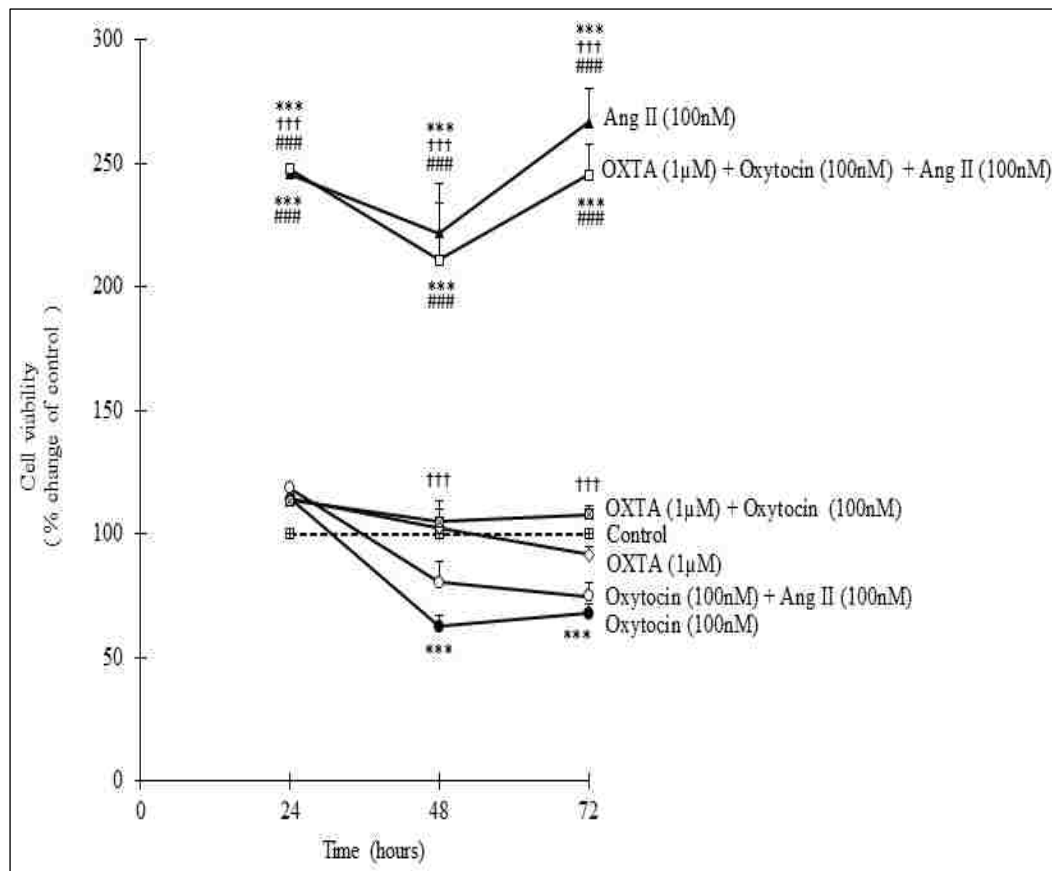


Figure 4.17. The effect of oxytocin and different treatment groups on aortic SHR VSMCs viability rate as assessed by the MTT assay. SHR VSMCs were seeded at 1×10^4 cells/well in 96-well plates. Cell viability was measured as the percent (%) change of control. The data are shown as the mean \pm SEM of three independent experiments performed using aortic VSMCs isolated from three SHRs. Each experiment was performed with 12 replicates. Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.

*** $P < 0.001$: All groups versus Control

††† $P < 0.001$: Oxytocin versus Ang II; and Oxytocin versus (OXTA + Oxytocin)

$P < 0.001$: (Oxytocin + Ang II) versus Ang II alone; and (Oxytocin + Ang II) versus (OXTA + Oxytocin + Ang II)

4.2.1.3. Assessment of cell growth with ³H-Thymidine incorporation assay in SHR VSMCs

The growth assay results corroborated that oxytocin (100 nM) significantly reduced ³H-Thymidine incorporation in aortic SHR VSMCs from 2,668 ± 1,419 cpm (control) to 1,958 ± 2,473 cpm [36% ± 6% decrease (P < 0.01), **Figure 4.18**].

In contrast, treatment with Ang II (100 nM) significantly increased the ³H-Thymidine incorporation into aortic SHR VSMCs from 2,668 ± 1,419 cpm (control) to 5,245 cpm ± 1,108 cpm [46% ± 71% increase (P < 0.001), **Figure 4.18**].

Pre-treatment with oxytocin (100 nM) followed by Ang II (100 nM) significantly decreased Ang II-induced growth of SHR VSMCs [1,385 cpm ± 1,170 cpm (73% ± 25% decrease), P < 0.001] compared to Ang II alone (5,245 ± 1,108 cpm), and these effects were antagonized by OXTA [1 μM (**Figure 4.18**)].

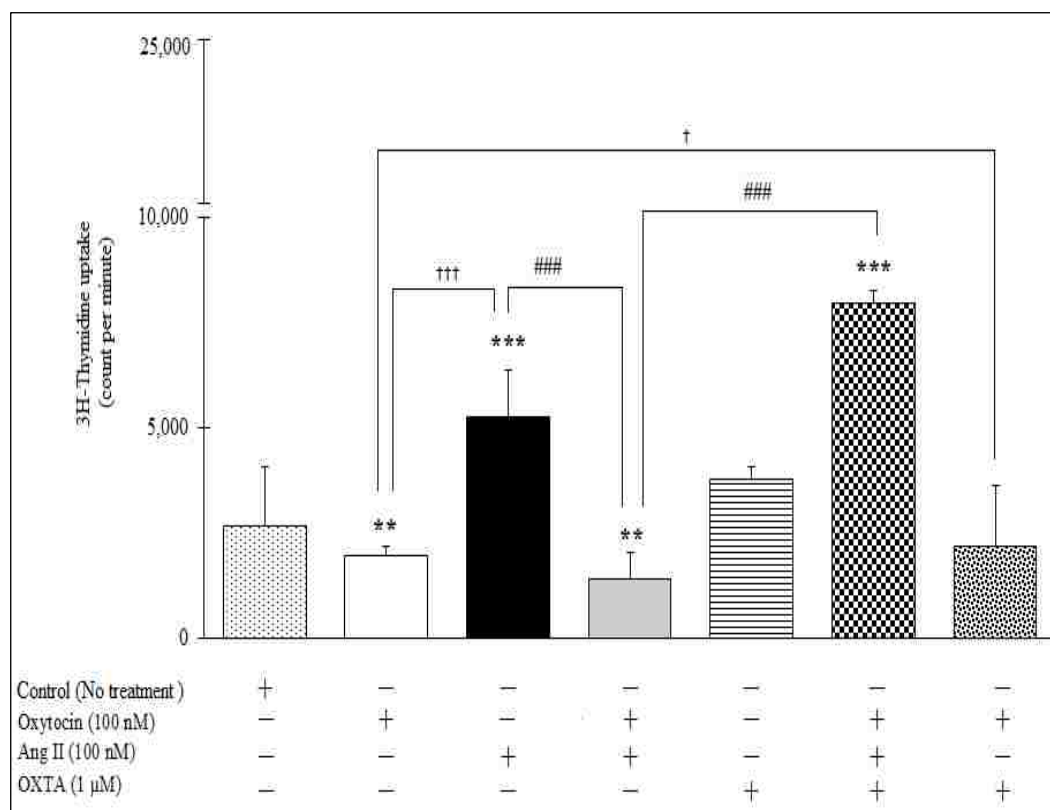


Figure 4.18. The effect of oxytocin and various treatments groups on aortic SHR VSMCs growth as assessed by the ^3H -Thymidine incorporation assay. SHR VSMCs were seeded at 5×10^4 cells/well in 24-well plates. Cell growth was measured as the function of the uptake of the radioisotope ^3H -Thymidine into the deoxyribonucleic acid (DNA) of the rapidly dividing cells and counted by the scintillation counter. The data are represented as the count per minute (cpm) and are shown as the mean \pm SEM of four independent experiments, each performed with triplicates, using aortic VSMCs isolated from four SHRs. Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.

** $P < 0.01$ and *** $P < 0.001$: versus Control

††† $P < 0.001$ and † $P < 0.05$: Oxytocin versus (OXTA + Oxytocin); and Oxytocin versus Ang II

$P < 0.01$: (Oxytocin + Ang II) versus (OXTA + Oxytocin + Ang II); and (Oxytocin + Ang II) versus Ang II alone

4.2.1.4. Assessment of cell viability by flow cytometry in SHR VSMCs

In aortic SHR VSMCs, the flow cytometry results showed oxytocin treatment decreased the cell viability and induced apoptosis in a concentration-dependent manner, also caused necrosis (**Figure 4.19**).

The flow experiments utilized two different concentrations of oxytocin: 10 nM and 100 nM. When compared to the control group ($93.4\% \pm 1.10\%$), the live cell percentage in aortic VSMCs significantly decreased to $80.9\% \pm 0.7\%$ ($P < 0.01$) in 10 nM oxytocin group and further declined to $65\% \pm 0.9\%$ ($P < 0.01$) in 100 nM oxytocin group, respectively (**Figure 4.19**).

Oxytocin significantly induced apoptosis in aortic SHR VSMCs. The treatment with 10 nM and 100 nM oxytocin significantly increased apoptosis up to $13.9\% \pm 0.4\%$ ($P < 0.001$) and $23.9\% \pm 0.1\%$ ($P < 0.001$), respectively compared to apoptosis in the control group [$2.6\% \pm 1.1\%$ (**Figure 4.19**)].

In addition to inducing apoptosis, oxytocin considerably induced necrosis in aortic VSMCs. The percentage of aortic VSMCs undergoing necrosis significantly increased from $2.2\% \pm 0\%$ (in control group) to $8.6\% \pm 1.3\%$ ($P < 0.05$) in 100 nM oxytocin group. However, there was no significant difference between 10 nM oxytocin group ($2.6\% \pm 0.3\%$) and the control group [$2.2\% \pm 0\%$ (**Figure 4.19**)].

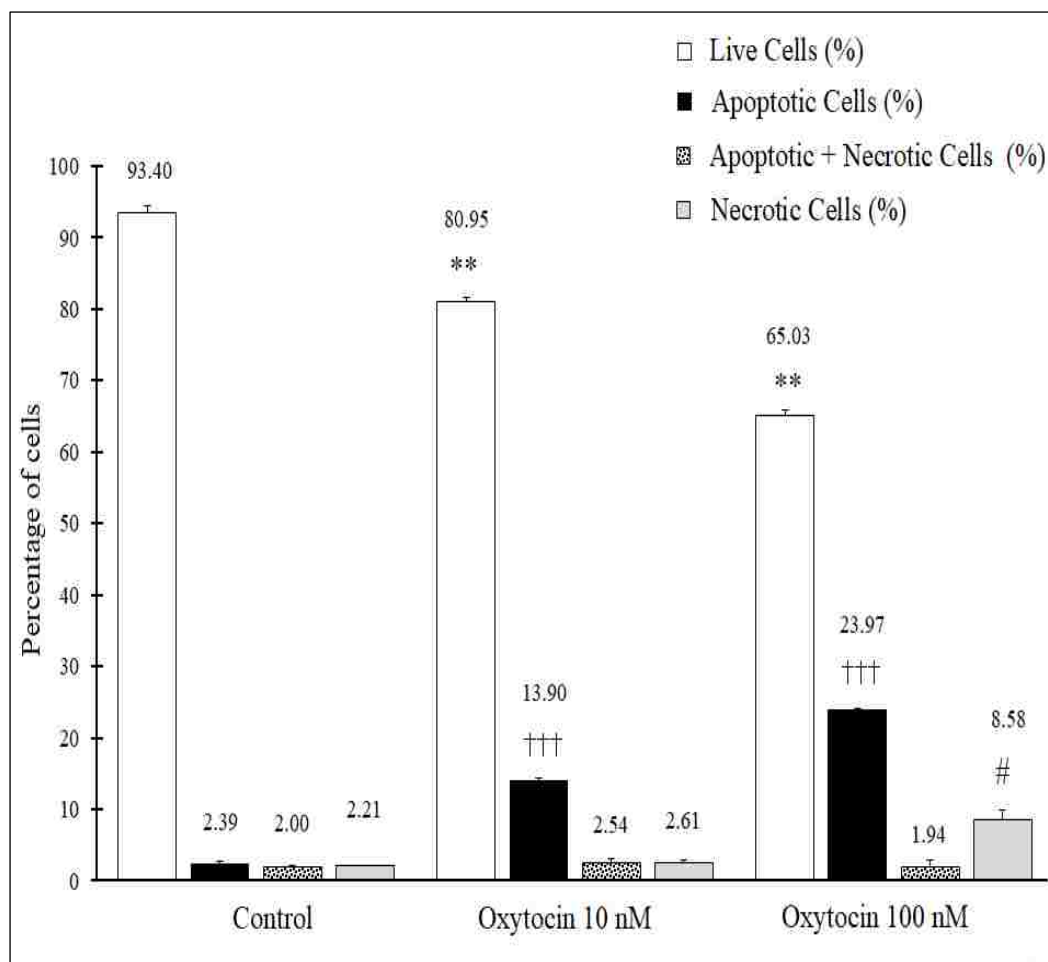


Figure 4.19. *The effect of oxytocin on apoptosis and necrosis in aortic SHR VSMCs as assessed by flow cytometry. SHR VSMCs were seeded at 1×10^6 cells/well in 6-well plates. The data are shown as the percentage of cells positive for apoptosis, necrosis and apoptosis + necrosis across different treatment groups. The data are represented as the mean \pm SEM from three independent experiments performed using aortic VSMCs from three SHRs. The treatment was carried out for 24 hours with the two different concentrations of oxytocin: 10 nM and 100 nM. The cells in the control group were not treated with oxytocin (only SFM). The cells were stained with fluorescent dyes: Annexin V (for apoptosis) and 7-amino actinomycin D [(7-AAD) for necrosis] and assessed with flow cytometer.*

** $P < 0.01$: Live cells in Oxytocin 10 nM group versus Live cells in Control group

** $P < 0.01$ Live cells in Oxytocin 100 nM group versus Live cells in Control group

††† $P < 0.001$ Apoptotic cells in Oxytocin 10 nM group versus Apoptotic cells in Control group

††† $P < 0.001$ Apoptotic cells in Oxytocin 100 nM group versus Apoptotic cells in Control group

$P < 0.05$ Necrotic cells in Oxytocin 100 nM group versus Necrotic cells in Control group.

4.2.2. Effect of oxytocin and its pre-treatment with Ang II on the phosphorylation of ERK1/2 in aortic SHR VSMCs

In aortic VSMCs isolated and cultured from SHRs, the densitometric analysis of the western blot images showed that treatment with oxytocin (100 nM) significantly decreased the active phosphorylated form of ERK1/2 (pERK1/2) by $35.4\% \pm 4\%$ ($P < 0.001$) compared to control (**Figure 4.20**).

Contrary to oxytocin's effect on pERK1/2 in SHR VSMCs, treatment with Ang II (100 nM) significantly increased phosphorylation of ERK1/2 by $157\% \pm 21\%$ ($P < 0.001$) compared to control (**Figure 4.20**).

Interestingly, the oxytocin (100 nM) pre-treatment significantly reduced Ang II-induced pERK1/2 levels by $93.3\% \pm 1.4\%$ ($P < 0.001$) compared to Ang II alone. The effects of oxytocin were significantly antagonized by the pre-treatment with $1 \mu\text{M}$ OXTA (**Figure 4.20**).

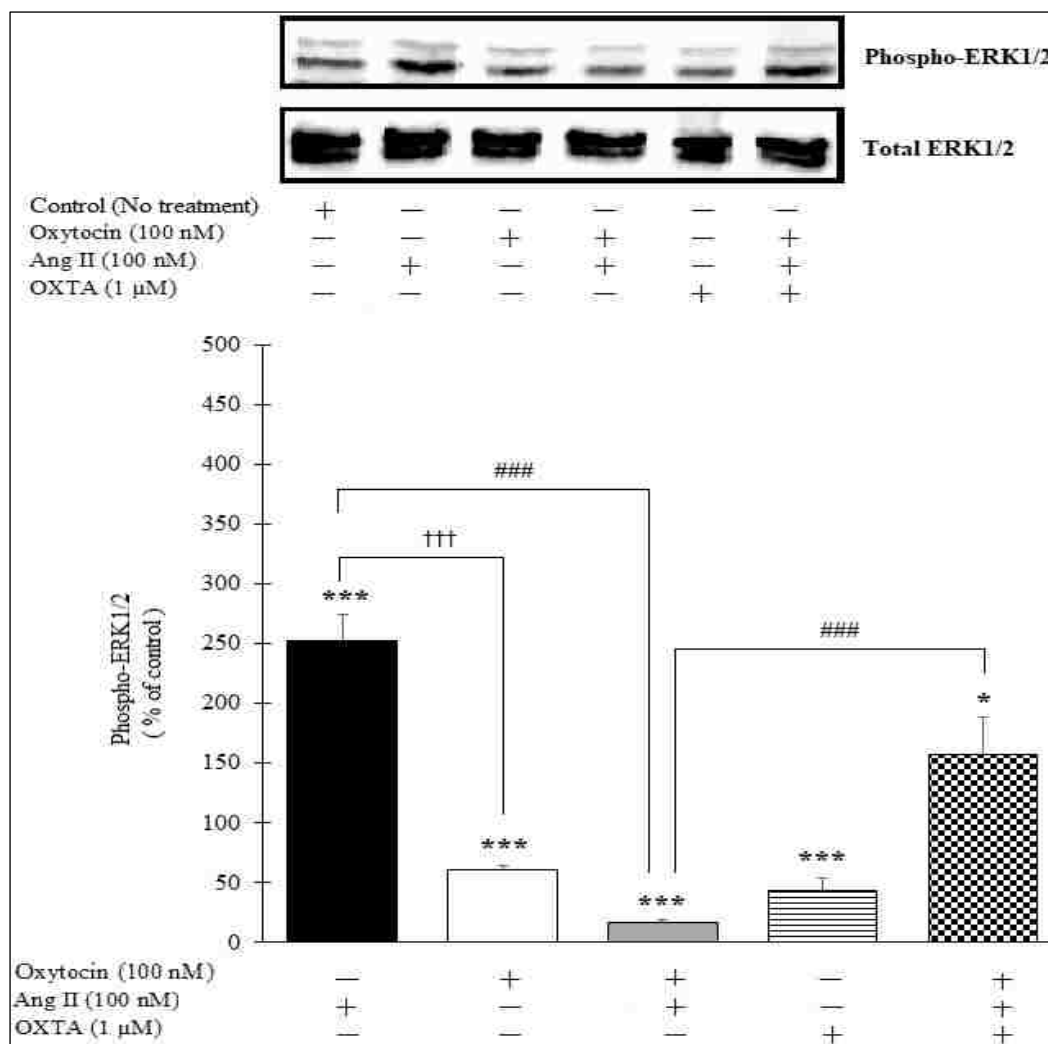


Figure 4.20. The effect of oxytocin and different treatment groups on the phosphorylation of ERK1/2 in aortic SHR VSMCs. Western blotting was utilized to measure phospho-ERK1/2 levels, and the data were normalized with total ERK1/2. The effect of oxytocin and its combination with different treatment groups on the phosphorylation of ERK1/2 is shown as the percent (%) of control, with control as 100%. The data are represented as the mean \pm SEM of five independent experiments, performed using aortic VSMCs isolated from five SHRs. *** indicates $P < 0.001$ versus control; ††† $P < 0.001$ Oxytocin versus Ang II; ### $P < 0.001$ (Oxytocin + Ang II) versus Ang II alone, and (Oxytocin + Ang II) versus (OXTA + Oxytocin + Ang II). Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.

4.2.3. Effect of oxytocin and its pre-treatment with Ang II on the phosphorylation of PI3K p110 α and Akt in aortic SHRs VSMCs

In aortic VSMCs cultured from SHRs, the densitometric analysis of western blot images showed that treatment with oxytocin (100 nM) significantly decreased the phosphorylated forms of PI3K p110 α by $31\% \pm 5.1\%$ ($P < 0.01$) and Akt by $45.5\% \pm 8.6\%$ ($P < 0.01$) compared to control, respectively (**Figures 4.21 and 4.22**).

On the other hand, treatment with Ang II (100 nM) increased phosphorylated forms of PI3K p110 α by $79.8\% \pm 10.3\%$ ($P < 0.001$) and Akt by $70.2\% \pm 20.4\%$ ($P < 0.05$) compared to control, respectively (**Figures 4.21 and 4.22**).

It is noteworthy that the oxytocin (100 nM) pre-treatment significantly reduced Ang II-induced higher levels of phospho-PI3K p110 α by $61.3\% \pm 4.8\%$ ($P < 0.01$) and phospho-Akt by $60.4\% \pm 2.6\%$ ($P < 0.001$) compared to Ang II alone, respectively. And these effects of oxytocin were antagonized by pre-treatment with $1 \mu\text{M}$ OXTA (**Figures 4.21 and 4.22**).

Western blots depicting effect of oxytocin and various treatments on the phosphorylation of PI3K p110 α and Akt in SHR VSMCs are shown in **Figures 4.21 and 4.22**, respectively.

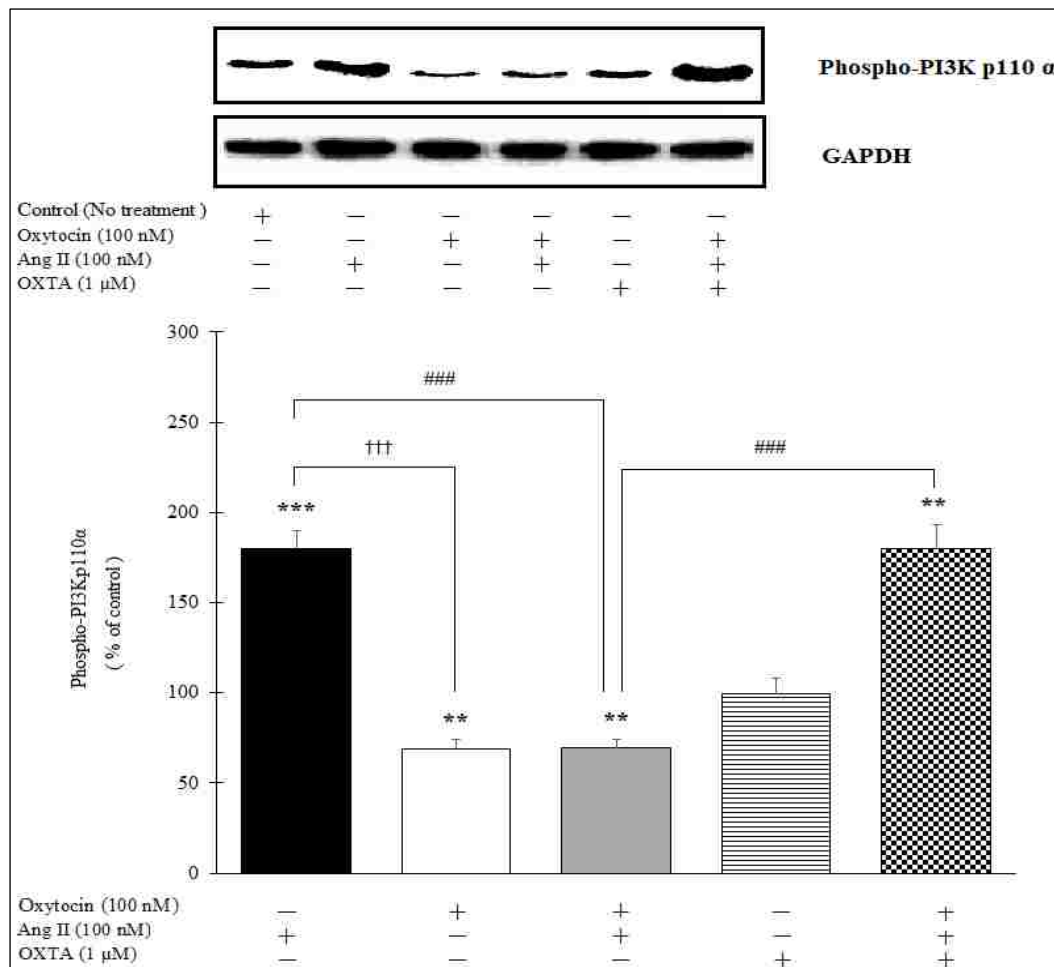


Figure 4.21. The effect of oxytocin and different treatment groups on the phosphorylation of PI3K p110α in aortic SHR VSMCs. Western blotting was utilized to measure the phosphorylation of PI3K p110α, and the data were normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The effect of oxytocin and its combination with different treatment groups on the phosphorylation of PI3K p110α is shown as the percent (%) of control, with control as 100%. The data are shown as the mean ± SEM of three independent experiments, performed using aortic VSMCs isolated from three SHRs. ** indicates $P < 0.01$ versus control; *** $P < 0.001$ versus control; ††† $P < 0.001$ Oxytocin versus Ang II; ### $P < 0.001$ (Oxytocin + Ang II) versus Ang II alone, and (Oxytocin + Ang II) versus (OXTA + Oxytocin + Ang II). Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.

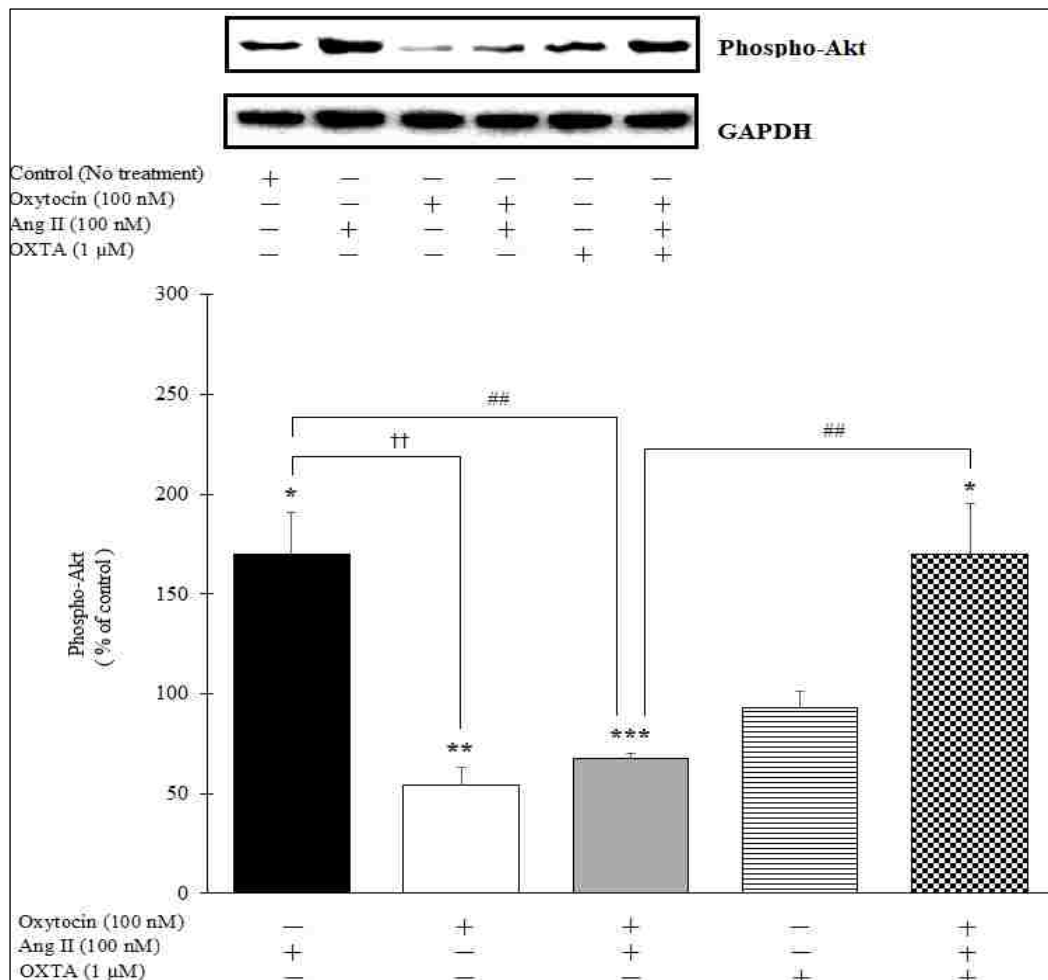


Figure 4.22. The effect of oxytocin and different treatment groups on the phosphorylation of Akt in aortic SHR VSMCs. Western blotting was utilized to measure the phosphorylation of Akt, and the data were normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The effect of oxytocin and its combination with different treatment groups on the phosphorylation of Akt is shown as the percent (%) of control, with control as 100%. The data are shown as the mean \pm SEM of four independent experiments, performed using aortic VSMCs isolated from four SHRs. * indicates $P < 0.05$ versus control; ** $P < 0.01$ versus control; *** $P < 0.001$ versus control; †† $P < 0.01$ Oxytocin versus Ang II; ## $P < 0.01$ (Oxytocin + Ang II) versus Ang II alone, and (Oxytocin + Ang II) versus (OXTA + Oxytocin + Ang II). Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.

4.2.4. Effect of oxytocin and its pre-treatment with Ang II on the activation of ROCK -1 and ROCK-2 in aortic SHR VSMCs

In aortic SHR VSMCs, the densitometric analysis of western blot images showed treatment with oxytocin (100 nM) significantly reduced the activation of ROCK-1 by $58.9\% \pm 5.4\%$ ($P < 0.001$) and ROCK-2 by $41.5\% \pm 12.8\%$ ($P < 0.01$) compared to control, respectively (**Figures 4.23** and **4.24**).

Treatment with Ang II (100 nM) prominently increased the activation of ROCK-1 by $102\% \pm 43\%$ ($P < 0.05$) and ROCK-2 by $81.2\% \pm 14.8\%$ ($P < 0.001$) compared to control, respectively (**Figures 4.23** and **4.24**).

Pre-treatment with oxytocin (100 nM) significantly reduced Ang II-induced activation of ROCK-1 by $79\% \pm 6.9\%$ ($P < 0.01$) and ROCK-2 by $69.5\% \pm 13.2\%$ ($P < 0.001$) compared to Ang II alone, respectively. These effects of oxytocin were significantly blocked by pre-treatment with $1\mu\text{M}$ OXTA (**Figures 4.23** and **4.24**).

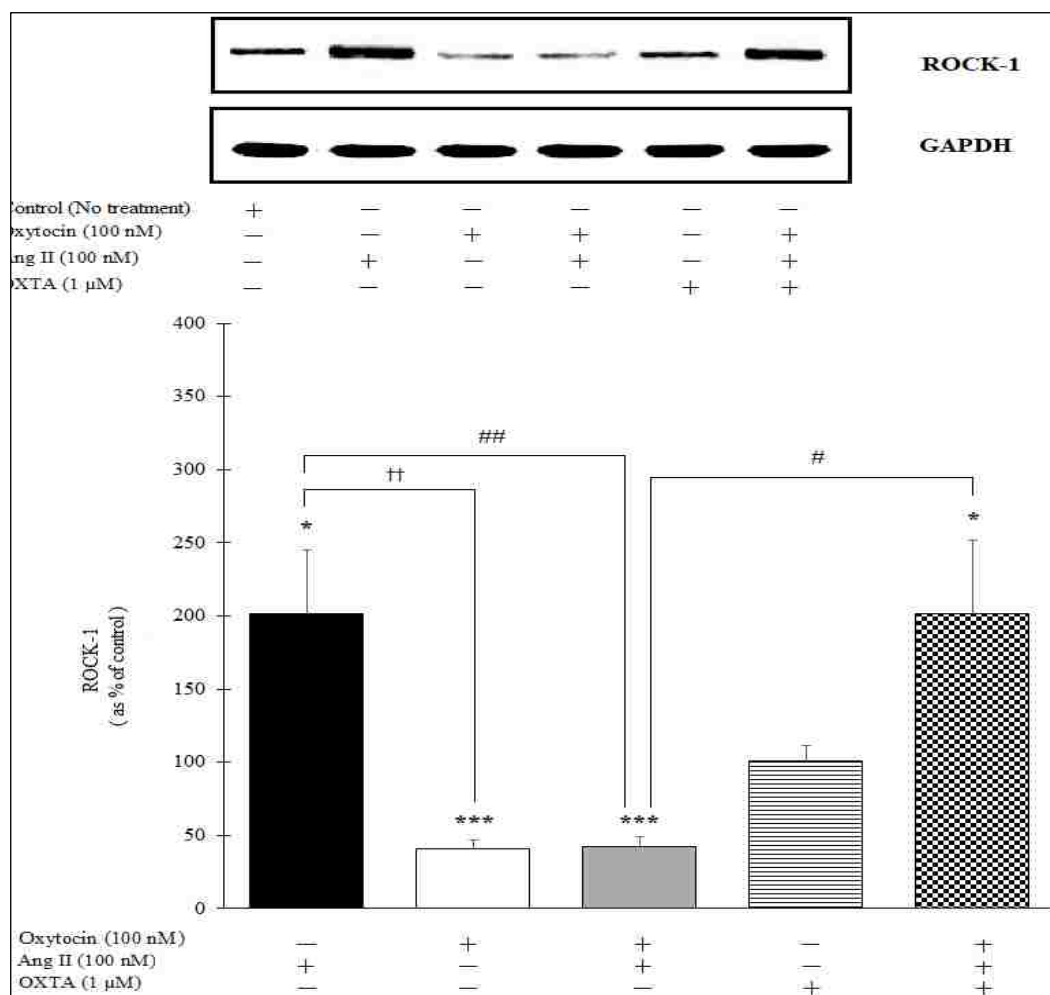


Figure 4.23. The effect of oxytocin and different treatment groups on the activation of ROCK-1 in aortic SHR VSMCs. Western blotting was utilized to measure the ROCK-1 levels, and the data were normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The effect of oxytocin and its combination with different treatment groups on the activation of ROCK-1 is shown as the percent (%) of control, with control as 100%. The data are shown as the mean \pm SEM of four independent experiments, performed using aortic VSMCs isolated from four SHRs. * indicates $P < 0.05$ versus control; *** $P < 0.001$ versus control; ** $P < 0.01$ Oxytocin versus Ang II; ## $P < 0.01$ Oxytocin + Ang II versus Ang II alone; # $P < 0.05$ Oxytocin + Ang II versus OXTA + Oxytocin + Ang II. Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist; ROCK, Rho-associated coiled-coil-containing protein kinase.

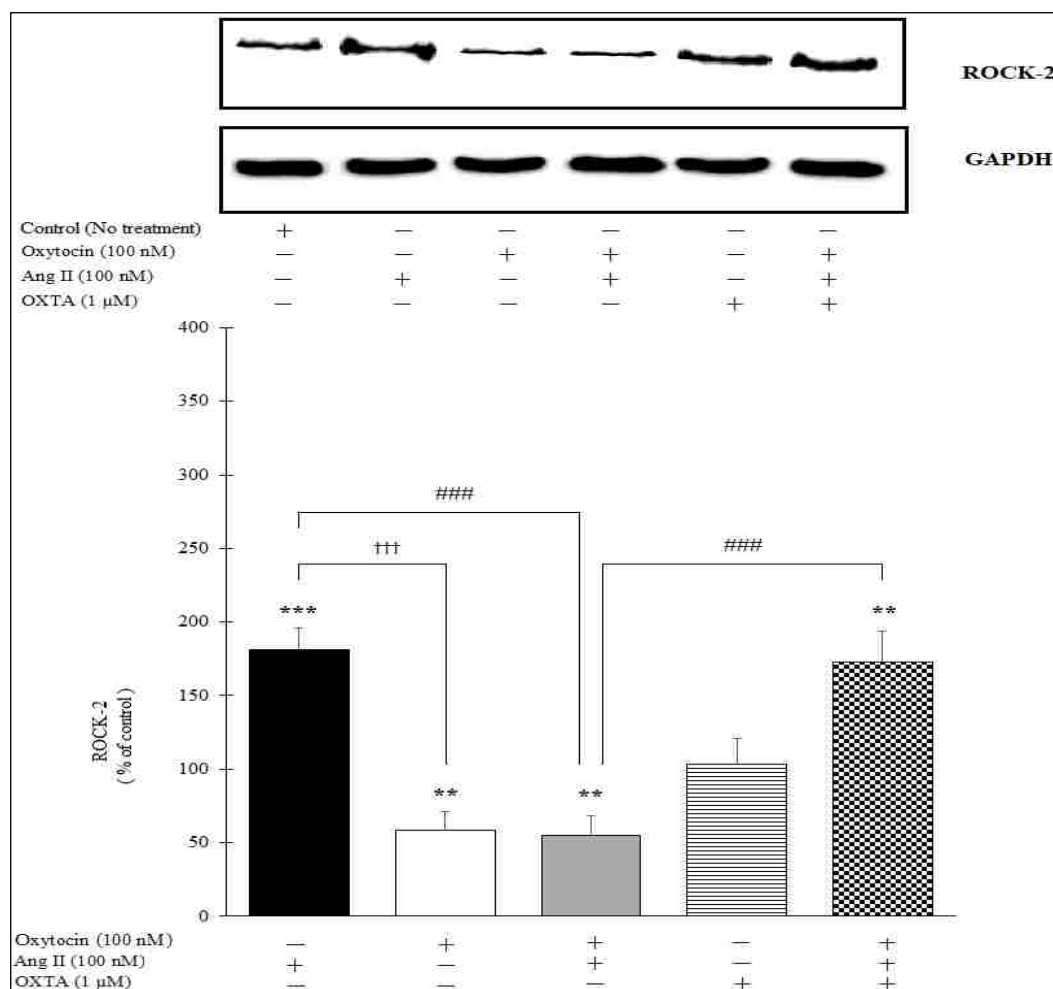


Figure 4.24. The effect of oxytocin and different treatment groups on the activation of ROCK-2 in aortic SHR VSMCs. Western blotting was utilized to measure the ROCK-2 levels, and the data were normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The effect of oxytocin and its combination with different treatment groups on the activation of ROCK-2 as the percent (%) of control, with control as 100%. The data are shown as the mean \pm SEM of five independent experiments, performed using aortic VSMCs isolated from five SHRs. ** indicates $P < 0.01$ versus control; *** $P < 0.001$ versus control; ††† $P < 0.01$ Oxytocin versus Ang II; ### $P < 0.01$ Oxytocin + Ang II versus Ang II alone, and Oxytocin + Ang II versus OXTA + Oxytocin + Ang II. Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist; ROCK, Rho-associated coiled-coil containing protein kinase.

4.2.5. Effect of oxytocin and its pre-treatment with Ang II on IL-6 secretion in aortic SHR VSMCs

As per the manufacturer's protocol, the IL-6ELISA assay was carried out and the concentration of IL-6 secreted by the aortic VSMCs in the culture media was measured as 'picogram per milliliter' (pg/mL). In spontaneously hypertensive rats (SHRs), the findings of the IL-6 assay demonstrated that oxytocin significantly increased IL-6 secretion in culture media compared to the control group (**Figure 4.25**).

The assay employed three different concentrations of oxytocin: 10 nM, 100 nM, 1,000 nM. When compared with control group (848 ± 9 pg/mL), oxytocin significantly increased IL-6 secretion in a concentration-dependent manner: 985 ± 25 pg/mL ($P < 0.01$) at 10 nM; $1,289 \pm 8$ pg/mL ($P < 0.001$) at 100 nM; and $1,468 \pm 19$ pg/mL ($P < 0.01$) at 1,000 nM, respectively (**Figure 4.25**).

The treatment with Ang II (100 nM) showed a significant increase in IL-6 secretion [$1,620 \pm 66$ pg/mL ($P < 0.001$)] compared to the control group [$(848 \pm 9$ pg/mL), **Figure 4.25**].

When oxytocin (100 nM) and Ang II (100 nM) groups were compared to the control group, Ang II (100 nM) showed significantly more IL-6 [$1,620 \pm 66$ pg/mL ($P < 0.01$)] than oxytocin [100 nM ($1,289 \pm 8$ pg/mL)] compared to the control group [$(848 \pm 9$ pg/mL), **Figure 4.25**].

Although oxytocin on its own significantly increased IL-6 secretion, interestingly, oxytocin's pre-treatment (100 nM) with Ang II (100 nM) significantly decreased IL-6 secretion [891 ± 59 pg/mL ($P < 0.001$)] compared to

Ang II 100 nM alone ($1,620 \pm 66$ pg/mL) and oxytocin 100 nM alone [$1,289 \pm 8$ pg/mL ($P < 0.001$)], respectively (**Figure 4.25**).

The effect of oxytocin's pre-treatment (100 nM) on Ang II (100 nM) was significantly inhibited by pre-treatment with 1 μ M OXTA. IL-6 secretion was significantly higher [$1,476 \pm 58$ pg/mL ($P < 0.01$)] in the OXTA-pre-treatment group [OXTA (1 μ M) + Oxytocin (100 nM) + Ang II (100 nM)] than Oxytocin 100 nM + Ang II 100 nM group [$(891 \pm 59$ pg/mL), **Figure 4.25**].

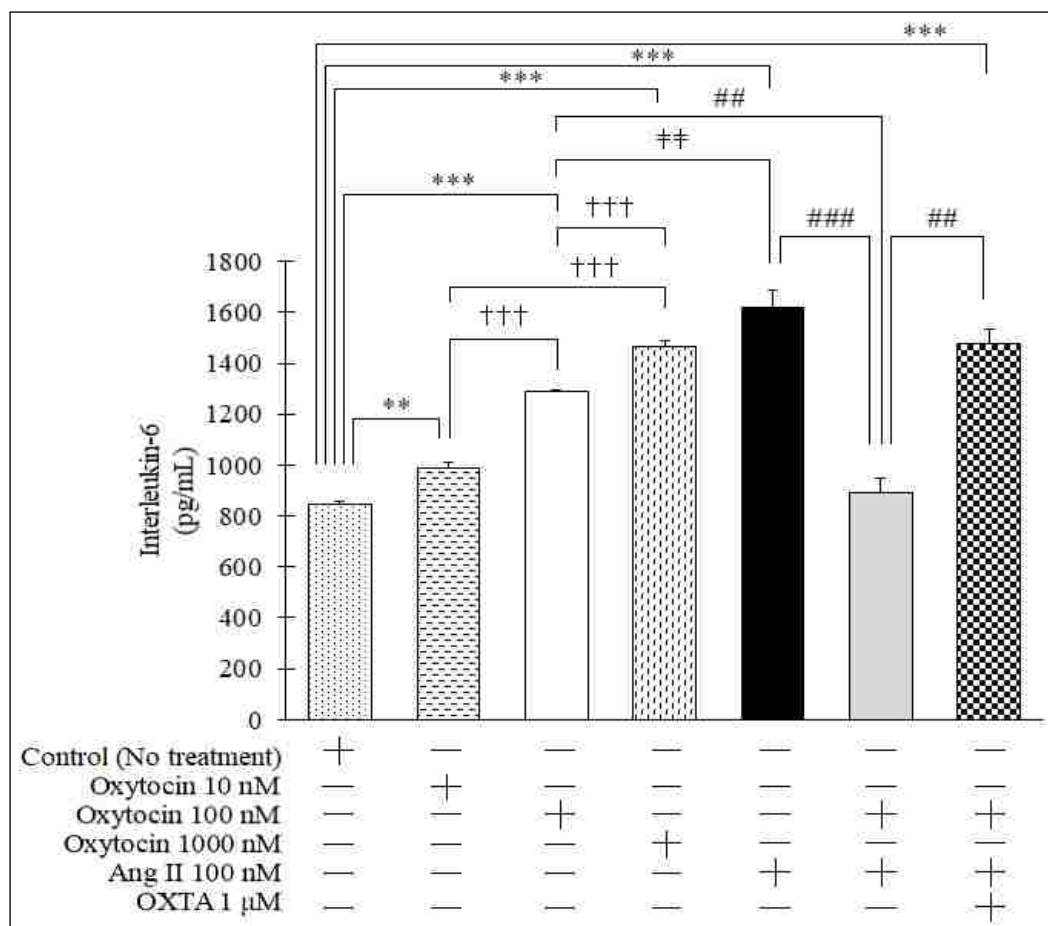


Figure 4.25. The effect of oxytocin and different treatment groups on IL-6 secretion in aortic SHR VSMCs. The concentration of IL-6 secreted by aortic SHR VSMCs in culture media was measured in picogram per mL (pg/mL). The data are represented as the mean \pm SEM from three independent experiments performed using aortic VSMCs from three SHRs. In each experiment each treatment group had duplicates. ** indicates $P < 0.01$ versus Control group; *** $P < 0.001$ versus Control group; ††† $P < 0.001$ Oxytocin 10 nM versus Oxytocin 100 nM and Oxytocin 1000 nM respectively, and Oxytocin 100 nM versus Oxytocin 1000 nM. ## $P < 0.01$ Oxytocin 100 nM + Ang II 100 nM versus Oxytocin 100 nM alone and OXTA 1 μ M + Oxytocin 100 nM + Ang II 100 nM; #### $P < 0.001$ Oxytocin 100 nM + Ang II 100 nM versus Ang II 100 nM alone; †† $P < 0.01$ Oxytocin 100 nM versus Ang II 100 nM. Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.

4.2.6. Effect of oxytocin and its pre-treatment with Ang II on ROS production in aortic SHR VSMCs

The outcomes of the ROS assay in aortic SHR VSMCs revealed that oxytocin significantly increased ROS production and its pre-treatment with Ang II also considerably increased ROS production (**Figure 4.26**).

Measured as the function of fluorescent units, the ROS assay findings showed that treatment with oxytocin (100 nM) significantly increased ROS production [$6,657 \pm 202$ ($P < 0.001$)] compared to the control group ($5,411 \pm 259$) in aortic SHR VSMCs. The treatment with Ang II (100 nM) significantly increased ROS production [$6,286 \pm 208$ ($P < 0.001$)] compared to the control group ($5,411 \pm 259$) (**Figure 4.26**).

There was no significant difference in ROS production between oxytocin [100 nM ($6,657 \pm 202$)] and Ang II [100 nM ($6,286 \pm 208$)] treatment groups (**Figure 4.26**).

The pre-treatment of oxytocin (100 nM) followed by Ang II (100 nM) significantly increased ROS production [$7,481 \pm 162$ ($P < 0.001$)] compared to the control group [$5,411 \pm 259$ (**Figure 4.26**)].

Additionally, pre-treatment with Oxytocin (100 nM) followed by Ang II (100 nM) significantly increased the ROS production ($7,481 \pm 162$) compared to oxytocin (100 nM) alone [$6,657 \pm 202$ ($P < 0.01$)] and Ang II (100 nM) alone [$6,286 \pm 208$ ($P < 0.001$)], respectively, indicating an additive effect (**Figure 4.26**).

Interestingly, OXTA (1 μ M) on its own significantly increased the ROS production [$6,703 \pm 173$ ($P < 0.001$)] compared to the control group ($5,411 \pm 259$).

In addition, pre-treatment with OXTA (1 μ M) followed by Oxytocin (100 nM) also significantly increased ROS production [$6,978 \pm 223$ ($P < 0.001$)] compared to control group [$5,411 \pm 259$ (**Figure 4.26**)].

There was no significant difference between Oxytocin (100 nM) group ($6,657 \pm 202$) and OXTA (1 μ M) + Oxytocin (100 nM) group [$6,978 \pm 223$ (**Figure 4.26**)].

The combination of OXTA (1 μ M), Oxytocin (100 nM), and Ang II significantly increased ROS production [$7,705 \pm 211$ ($P < 0.001$)] compared to control ($5,411 \pm 259$) in aortic SHR VSMCs (**Figure 4.26**).

The combination of OXTA, Oxytocin, and Ang II (100 nM) significantly increased ROS production [$7,705 \pm 211$ ($P < 0.001$)] compared to Oxytocin (100 nM) alone ($6,657 \pm 202$), Ang II (100 nM) alone ($6,286 \pm 208$) and OXTA (1 μ M) alone ($6,703 \pm 173$), respectively, indicating an additive effect (**Figure 4.26**).

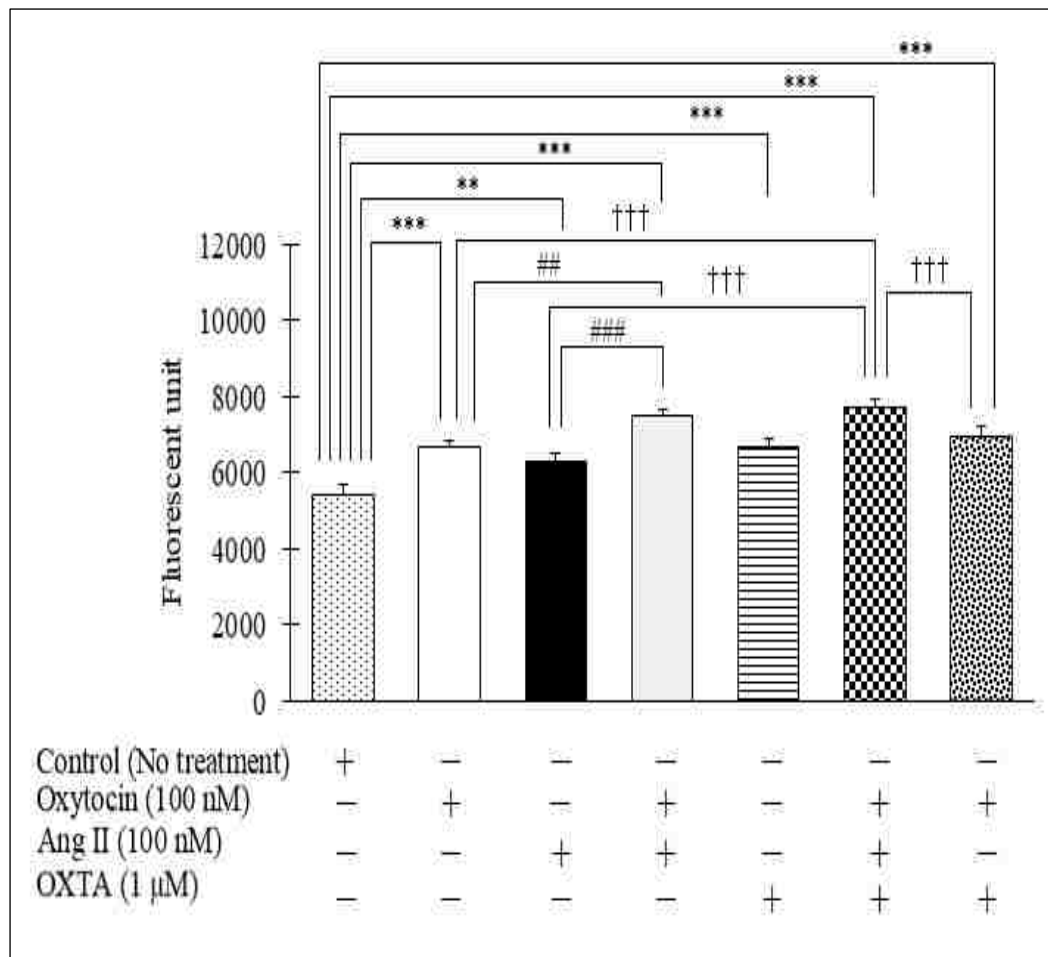


Figure 4.26. The effect of oxytocin and different treatment groups on ROS production in aortic SHR VSMCs. SHR VSMCs were seeded at 1×10^4 cells/well in 96-well plates. The ROS levels were measured as the function of the fluorescent unit. The data are represented as the mean \pm SEM from three independent experiments performed using aortic VSMCs from three SHRs. In each experiment each treatment group had 12 replicates. ** indicates $P < 0.01$ Ang II versus Control; *** $P < 0.001$ versus Control group; ## $P < 0.01$ Oxytocin + Ang II versus Oxytocin alone; ### Oxytocin + Ang II versus Ang II alone; +++ $P < 0.001$ OXTA + Oxytocin + Ang II versus Oxytocin alone, Ang II alone, and OXTA alone. Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.

4.3. Comparison of the effects of oxytocin and its pre-treatment with Ang II on aortic Wistar VSMCs versus SHR VSMCs

The effects of oxytocin on aortic Wistar VSMCs and SHR VSMCs alone and after pre-treatment with Ang II were analyzed and compared, and are explained under the following sub-headings:

4.3.1. Comparison between Wistar VSMCs and SHR VSMCs regarding the effect of oxytocin and its pre-treatment with Ang II on aortic VSMCs proliferation

In control group, the proliferation of Wistar VSMCs was significantly higher at 24 hours [6.37×10^6 cells/mL \pm 9.20×10^5 cells/mL ($P < 0.05$)] and 48 hours [1.50×10^7 cells/mL \pm 1.47×10^6 cells/mL ($P < 0.05$)] compared to SHR VSMCs (4.24×10^6 cells/mL \pm 1.51×10^5 cells/mL at 24 hours and 1.10×10^7 cells/mL \pm 7.04×10^5 at 48 hours). However, at 72 hours there was no significant difference in proliferation between Wistar VSMCs (2.30×10^7 cells/mL \pm 5.53×10^6 cells/mL) and SHR VSMCs [1.59×10^7 cells/mL 2.72×10^6 cells/mL (**Figure 4.27**)].

Oxytocin significantly decreased proliferation in Wistar VSMCs: 3.12×10^6 cells/mL \pm 4.14×10^5 cells/mL at 24 hours; 1.64×10^6 cells/mL \pm 3.07×10^5 cells/mL at 48 hours; and 1.23×10^6 cells/mL \pm 4.45×10^5 cells/mL at 72 hours post-treatment compared to their respective control. Similar to the results in Wistar VSMCs, oxytocin remarkably decreased proliferation in SHR VSMCs: 2.34×10^6 cells/mL \pm 1.98×10^5 cells/mL at 24 hours; 1.72×10^6 cells/mL \pm 1.04×10^5 cells/mL at 48 hours; and 1.71×10^6 cells/mL \pm 4.29×10^5 cells/mL at 72 hours

post-treatment compared to their respective control. It is noteworthy that there was no significant difference in the pattern and magnitude of oxytocin-induced inhibition of proliferation in Wistar VSMCs and SHR VSMCs (**Figure 4.27**).

Ang II showed significantly more proliferation in Wistar VSMCs [2.88×10^7 cells/mL $\pm 1.74 \times 10^6$ cells/mL ($P < 0.001$) at 24 hours; 4.73×10^7 cells/mL $\pm 3.45 \times 10^6$ cells/mL ($P < 0.01$) at 48 hours; and 5.03×10^7 cells/mL $\pm 1.82 \times 10^6$ cells/mL ($P < 0.05$) at 72 hours post-treatment] than SHR VSMCs (1.22×10^7 cells/mL $\pm 2.98 \times 10^5$ cells/mL at 24 hours; 2.33×10^7 cells/mL $\pm 1.03 \times 10^6$ cells/mL at 48 hours; and 4.88×10^7 cells/mL $\pm 9.99 \times 10^5$ cells/mL at 72 hours post-treatment) compared to their respective controls (**Figure 4.27**).

Oxytocin pre-treatment with Ang II significantly reduced Ang II-mediated proliferation of VSMCs isolated from both Wistar rats (1.07×10^7 cells/mL $\pm 2.99 \times 10^6$ cells/mL at 24 hours; 8.06×10^6 cells/mL $\pm 3.13 \times 10^6$ cells/mL at 48 hours; and 6.55×10^6 cells/mL $\pm 2.23 \times 10^6$ cells/mL at 72 hours post-treatment) and SHRs (4.14×10^6 cells/mL $\pm 8.47 \times 10^5$ cells/mL at 24 hours; 1.60×10^6 cells/mL $\pm 2.80 \times 10^5$ cells/mL at 48 hours; and 1.37×10^6 cells/mL $\pm 2.32 \times 10^5$ cells/mL at 72 hours post-treatment) compared to their respective Ang II (alone) groups. However, this effect was significantly higher in SHRs than Wistar Rats at 24 hours ($P < 0.05$), 48 hours ($P < 0.05$), and 72 hours ($P < 0.05$) post-treatment (**Figure 4.27**).

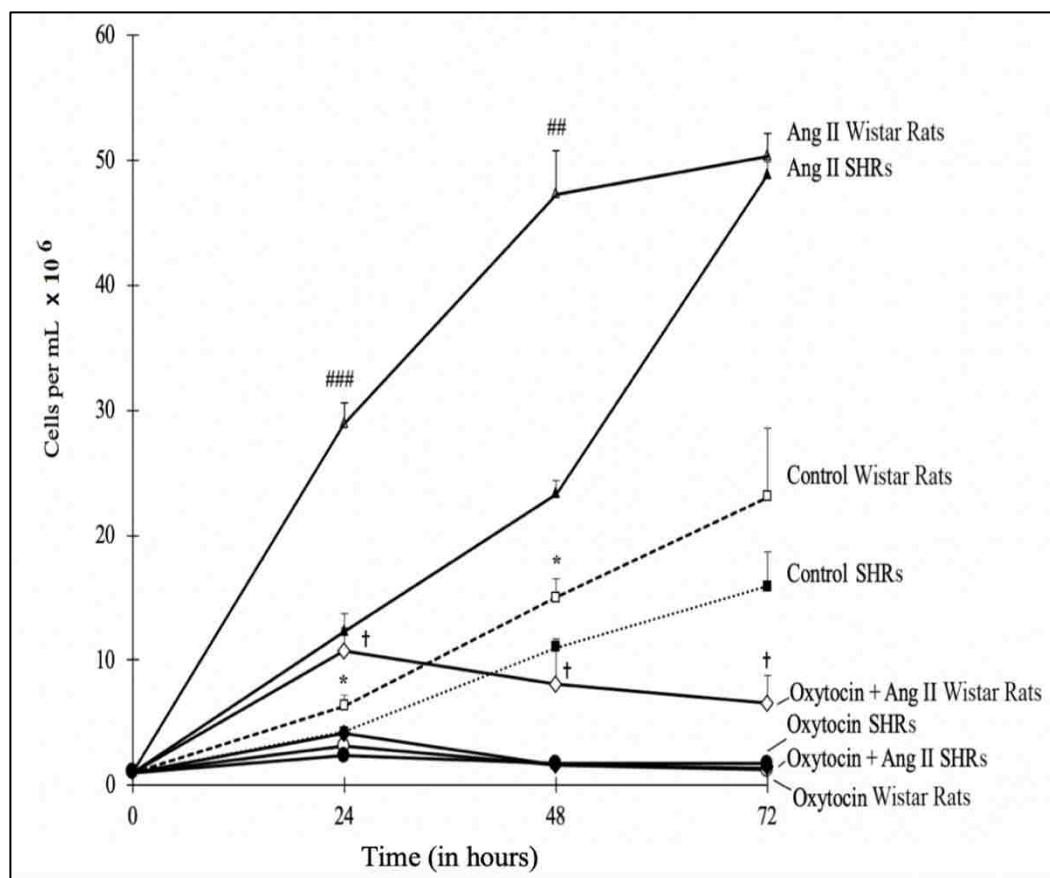


Figure 4.27. The comparison of the effects of oxytocin, Ang II, and the pre-treatment of oxytocin with Ang II on proliferation of aortic VSMCs between Wistar VSMCs and SHR VSMCs as assessed by the Hemocytometer method. VSMCs isolated from both Wistar rats and SHRs were seeded at 1×10^6 cells/well in 6-well plates. Cell proliferation was measured by counting the number of cells per milliliter (mL). The data are represented as the mean \pm SEM from three independent experiments performed using aortic VSMCs from three Wistar rats and three SHRs, respectively. * indicates $P < 0.05$ versus Control Wistar rats versus Control SHRs; † $P < 0.05$ (Oxytocin + Ang II) Wistar rats versus (Oxytocin + Ang II) SHRs; ## $P < 0.01$ Ang II Wistar rats vs Ang II SHRs; ### $P < 0.001$ Ang II Wistar rats vs Ang II SHRs. Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.

4.3.2. Comparison between Wistar VSMCs and SHR VSMCs regarding the effect of oxytocin and its pre-treatment with Ang II on aortic VSMCs viability

In MTT assay, the cell viability was determined as the function of the optical density (OD) measured at 570 nm. On comparing the cell viability of aortic VSMCs isolated from both Wistar rats and SHRs, significantly higher cell viability was observed in the control group SHR VSMCs [$0.4 \text{ OD} \pm 0.1$ ($P < 0.05$)] OD at 24 hours than Wistar VSMCs ($0.3 \text{ OD} \pm 0.0 \text{ OD}$). However, at 48 hours and 72 hours there was no significant difference in the cell viability between Wistar VSMCs ($0.5 \text{ OD} \pm 0.0 \text{ OD}$ at 48 hours and $0.7 \text{ OD} \pm 0.0 \text{ OD}$ at 72 hours) and SHR VSMCs [$0.5 \text{ OD} \pm 0.0 \text{ OD}$ at 48 hours and $0.8 \text{ OD} \pm 0.0 \text{ OD}$ at 72 hours] (**Figure 4.28**).

Oxytocin significantly decreased the cell viability of aortic VSMCs isolated from both Wistar rats and SHRs compared to the control groups, respectively. At 24 hours post-treatment, Oxytocin significantly decreased more cell viability of Wistar VSMCs [$0.2 \text{ OD} \pm 0.0 \text{ OD}$ ($P < 0.05$)] than SHR VSMCs ($0.4 \text{ OD} \pm 0.1 \text{ OD}$). However, at 48 hours and 72 hours there was no significant difference in the oxytocin-induced decrement in viability between the Wistar VSMCs ($0.3 \text{ OD} \pm 0.0 \text{ OD}$ at 48 hours and $0.6 \text{ OD} \pm 0.1 \text{ OD}$ at 72 hours) and SHR VSMCs ($0.3 \text{ OD} \pm 0.0 \text{ OD}$ at 48 hours and $0.5 \text{ OD} \pm 0.0 \text{ OD}$ at 72 hours). Oxytocin showed a similar pattern and magnitude of inhibition in cell viability in both Wistar VSMCs and SHR VSMCs at 48 hours and 72 hours post-treatment (**Figure 4.28**).

Ang II significantly increased the cell viability of aortic VSMCs isolated from both Wistar rats (0.5 OD \pm 0.1 OD at 24 hours, 1.0 OD \pm 0.1 OD at 48 hours, and 1.3 OD \pm 0.1 OD at 72 hours) and SHR (0.7 OD \pm 0.0 OD at 24 hours, 1.1 OD \pm 0.1 OD at 48 hours, and 2.0 OD \pm 0.1 OD at 72 hours) compared to the respective control groups. However, at 72 hours post-treatment, Ang II showed a more significant increase in cell viability in SHR VSMCs [2.0 OD \pm 0.1 OD ($P < 0.001$)] than Wistar VSMCs [1.3 OD \pm 0.1 OD (**Figure 4.28**)].

The pre-treatment of oxytocin with Ang II significantly decreased Ang II-induced cell viability in both Wistar VSMCs (0.2 OD \pm 0.0 OD at 24 hours, 0.3 OD \pm 0.0 OD at 48 hours, and 0.6 OD \pm 0.1 OD at 72 hours) and SHR VSMCs (0.4 OD \pm 0.1 OD at 24 hours, 0.3 OD \pm 0.0 OD at 48 hours, and 0.6 OD \pm 0.0 OD at 72 hours) compared to the respective Ang II (alone) groups (**Figure 4.28**).

At 24 hours post-treatment, the oxytocin-pretreatment showed more significant decrease viability in Wistar VSMCs [0.2 OD \pm 0.0 OD ($P < 0.01$)] than SHR VSMCs (0.4 OD \pm 0.1 OD). Although, at 48 and 72 hours, respectively, there was no significant difference in the cell viability between Wistar VSMCs and SHR VSMCs in the oxytocin-pre-treatment with Ang II group (**Figure 4.28**).

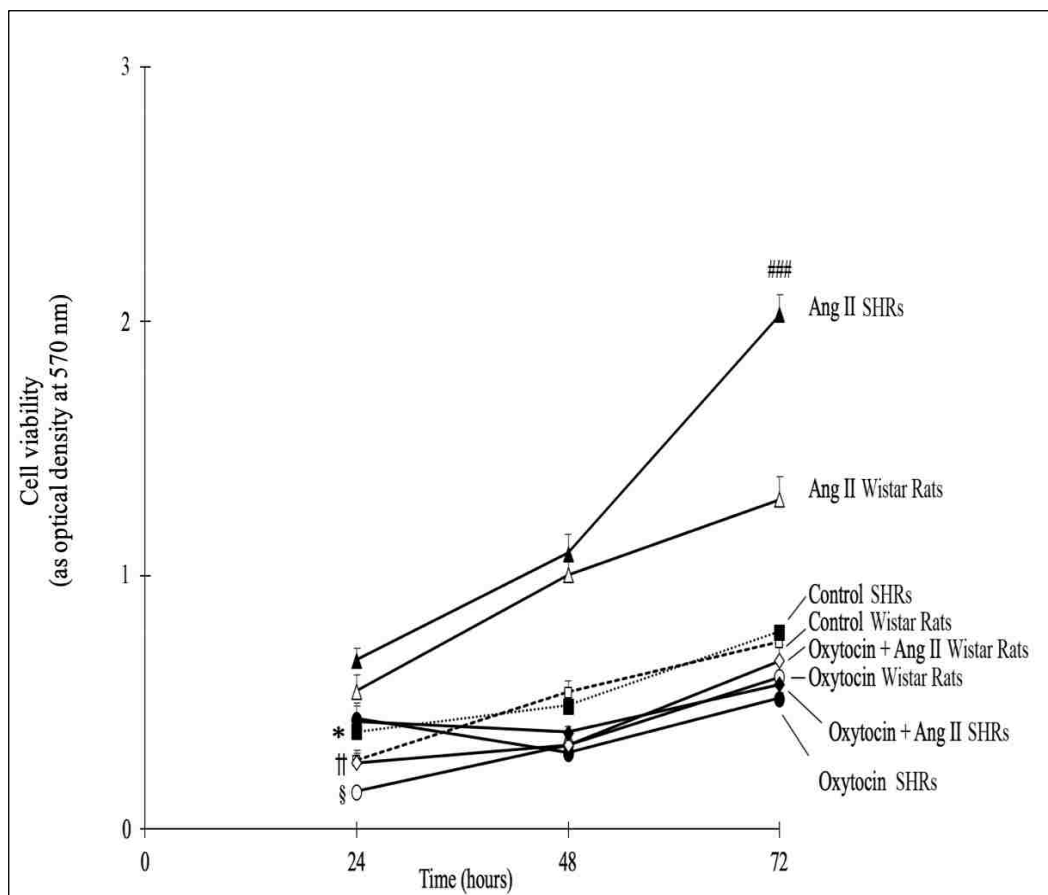


Figure 4.28. The comparison of the effects of oxytocin, Ang II, and the pre-treatment of oxytocin with Ang II on viability of aortic Wistar VSMCs and SHR VSMCs as assessed by the MTT assay. VSMCs isolated from both Wistar rats and SHRs were seeded at 1×10^4 cells/well in 96-well plates. Cell viability was measured as the function of an optical density (OD) at 570 nm. The data are shown as the mean \pm SEM from three independent experiments performed using aortic VSMCs from three Wistar rats and three SHRs, respectively. Each experiment was performed with 12 replicates. * indicates $P < 0.05$ Control Wistar rats versus Control SHRs; § $P < 0.05$ Oxytocin Wistar rats versus Oxytocin SHRs; †† $P < 0.01$ (Oxytocin + Ang II) Wistar rats versus (Oxytocin + Ang II) SHRs; $^{\#\#\#}$ $P < 0.001$ Ang II Wistar rats versus Ang II SHRs. Ang II indicates Angiotensin II; OXTA, Oxytocin receptor antagonist.

4.3.3. Comparison of the effect of oxytocin on apoptosis and necrosis between Wistar VSMCs and SHR VSMCs

In aortic VSMCs isolated from both Wistar rats and SHRs, the flow cytometry results showed that oxytocin significantly reduced live cells percentage, prominently induced apoptosis (in a concentration-dependent manner) and induced necrosis.

The flow experiments employed two different concentrations of oxytocin: 10 nM and 100 nM. In the control group, there was no significant difference between: live cells in Wistar VSMCs ($95.5 \pm 0.7\%$) and SHR VSMCs ($93.4\% \pm 1.1\%$ in SHRs); and apoptotic cells in Wistar VSMCs ($2.7\% \pm 1.1\%$) and SHR VSMCs ($2.4\% \pm 0.3\%$). However, the SHR VSMCs control group showed a significantly higher percentage of necrotic cells [$1.5\% \pm 0.0\%$ ($P < 0.01$)] compared to the Wistar VSMCs control group [$0.7\% \pm 0.1\%$ (**Figure 4.29**)].

The treatment with oxytocin 10 nM resulted in a more significant reduction in live cells percentage in SHR VSMCs [$80.9\% \pm 0.7\%$ ($P < 0.01$)] than Wistar VSMCs ($91.6\% \pm 0.4\%$) compared to their respective controls. In addition, oxytocin 10 nM induced significantly more apoptosis in SHR VSMCs [$13.9\% \pm 0.4\%$ ($P < 0.05$)] than Wistar VSMCs ($4.2\% \pm 1.6\%$) compared to their respective controls (**Figure 4.29**).

Treatment with oxytocin 100 nM showed a similar pattern and magnitude in reduction of the percentage of live cells and induction of apoptosis in between Wistar VSMCs ($67.5\% \pm 2.3\%$ live cells and $26.1\% \pm 3.0\%$ apoptotic cells) and SHR VSMCs ($65.0\% \pm 0.9\%$ live cells and $23.9\% \pm 0.1\%$ apoptotic cells) compared

to their respective controls. Interestingly, oxytocin 100 nM resulted in a significantly higher percentage of cells undergoing necrosis [$8.6\% \pm 1.3\%$ ($P < 0.05$)] in SHR VSMCs than Wistar VSMCs [$3.9\% \pm 0.3\%$ (**Figure 4.29**)].

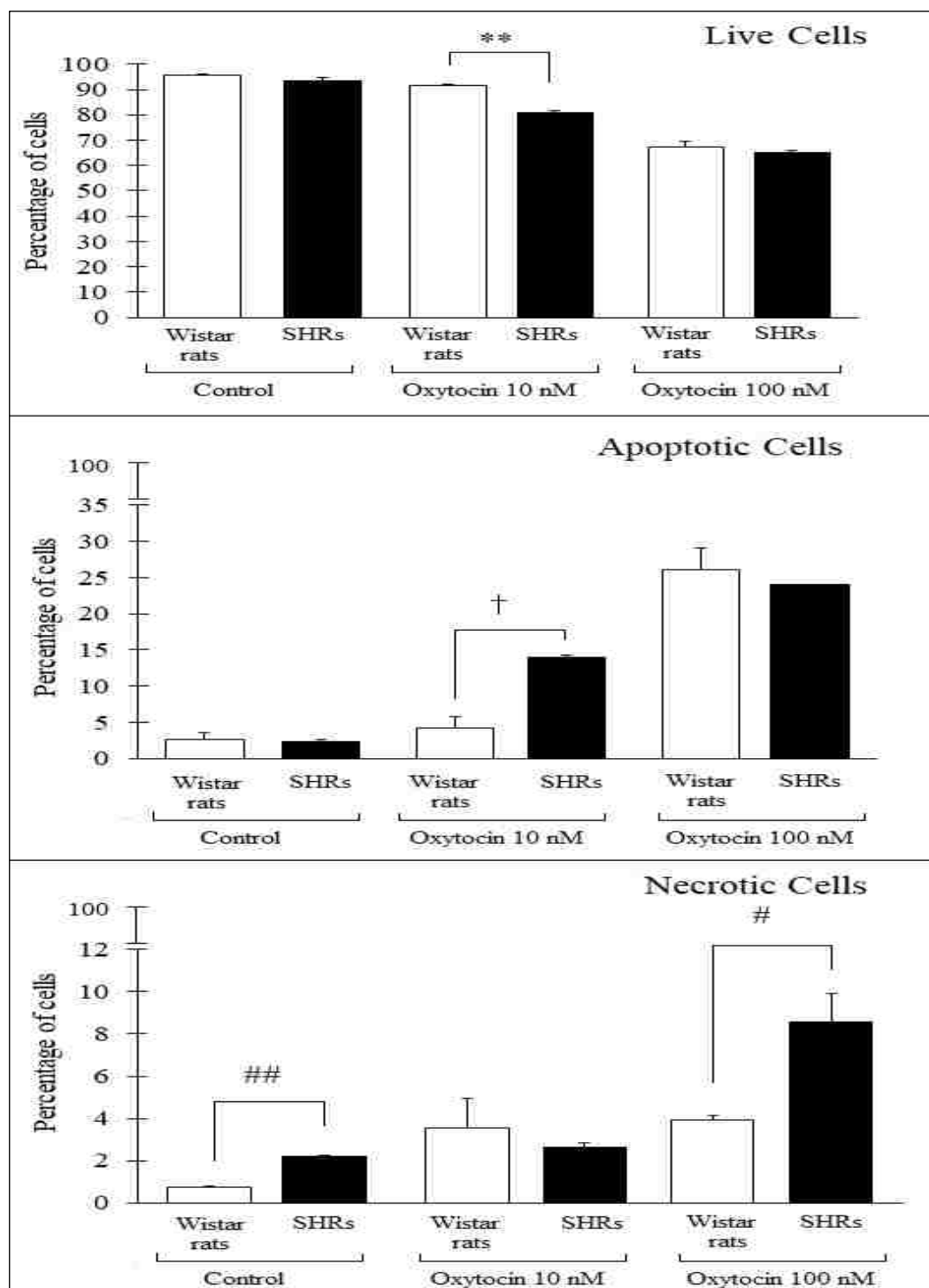


Figure 4.29. The comparison of the effects of oxytocin on the live cells, apoptosis, and necrosis between Wistar VSMCs and SHR VSMCs as assessed by flow cytometry. Aortic VSMCs isolated from both Wistar rats and SHRs were seeded at 1×10^6 cells/well in 6-well plates. The data are shown as the percentage of cells and

are represented as the mean \pm SEM from three independent experiments performed using aortic VSMCs from three Wistar rats and three SHRs, respectively. The treatment was carried out 24 hours with two different concentrations of oxytocin: 10nM and 100nM. The cells were stained with fluorescent dyes: Annexin V (for apoptosis) and 7-amino actinomycin D [(7-ADD) for necrosis]. ** indicates $P < 0.01$ Live Cells in Oxytocin 10 nM group in Wistar rats versus Live Cells in Oxytocin 100 nM in group SHRs; † $P < 0.05$ Apoptotic cells in Oxytocin 10 nM group in Wistar rats versus Apoptotic cells in Oxytocin 10 nM group in SHRs; # $P < 0.05$ Necrotic cells in Oxytocin 100 nM group in Wistar rats vs Necrotic cells in Oxytocin 100 nM group in SHRs; ### $P < 0.01$ Necrotic cells in Control group in Wistar rats vs Necrotic cells in Control group in SHRs.

4.3.4. Comparison of the effect of oxytocin and its pre-treatment with Ang II on the phosphorylation of ERK1/2, PI3K p110 α , and AKT in aortic Wistar VSMCs and SHR VSMCs

Western blotting was utilized to study the effect of oxytocin, Ang II, and its pre-treatment with Ang II on the phosphorylation of ERK1/2, PI3K p110 α , and Akt. The densitometric analysis of western blot images demonstrated that oxytocin and its pre-treatment with Ang II significantly decreased the phosphorylation of ERK1/2, PI3K p110 α , and Akt in aortic Wistar VSMCs and SHR VSMCs.

On the phosphorylation of ERK1/2

There was no significant difference between Wistar VSMCs and SHR VSMCs regarding oxytocin-mediated decrease in the levels of the phosphorylated forms of (active form) ERK1/2: oxytocin decreased the phosphorylation of ERK1/2 by $57.9\% \pm 14.2\%$ in Wistar VSMCs and by $35.4\% \pm 4\%$ in SHR VSMCs compared to respective controls (**Figure 4.30 b**).

Although, Ang II-induced phosphorylation of ERK1/2 was significantly higher in Wistar VSMCs ($P < 0.05$) than SHR VSMCs. In Wistar VSMCs, Ang II increased the phosphorylation of ERK1/2 by $223\% \pm 11\%$ compared to control, whereas, in SHR VSMCs, Ang II increased the phosphorylation of ERK1/2 by $157\% \pm 21\%$ compared to control (**Figure 4.30 a**).

The pre-treatment of oxytocin with Ang II caused more significant decrease in the phosphorylation of ERK1/2 in SHR VSMCs [$93.3\% \pm 1.4\%$ decrease ($P < 0.001$) than in Wistar VSMCs ($76.6\% \pm 1.4\%$ decrease) compared to their respective Ang II (alone) groups (**Figure 4.30 c**).

On the phosphorylation of PI3K p110 α

There was a significant difference between oxytocin-mediated reduction in the phosphorylated form of (active form) of PI3K p110 α between Wistar VSMCs and SHR VSMCs. Oxytocin showed a more substantial decrease of phospho-PI3K p110 α in Wistar VSMCs ($P < 0.01$) than SHR VSMCs. In Wistar VSMCs, oxytocin reduced phospho-PI3K p110 α by $64.8\% \pm 2.8\%$ compared to control. On the other hand, in SHR VSMCs, oxytocin reduced phospho-PI3K p110 α by $31\% \pm 5.1\%$ compared to control (**Figure 4.30 e**).

Ang II-induced phosphorylation of PI3K p110 α was significantly higher in Wistar VSMCs ($P < 0.01$) than SHR VSMCs. In Wistar VSMCs, Ang II increased phospho-PI3K p110 α by $184\% \pm 17\%$ compared to control, whereas, in SHR VSMCs, Ang II increased phospho-PI3K p110 α by $79.8\% \pm 10.3\%$ compared to control (**Figure 4.30 d**).

The oxytocin's pre-treatment with Ang II showed significantly more decrease in phospho-PI3K p110 α levels in Wistar VSMCs ($P < 0.05$) than SHR VSMCs. The oxytocin's pre-treatment decreased the phosphorylation of PI3K p110 α by $83.8\% \pm 8.6\%$ in Wistar VSMCs and by $61.3\% \pm 4.8\%$ in SHR VSMCs, respectively, compared to their respective Ang II (alone) groups (**Figure 4.30 f**).

On the phosphorylation of Akt

There was no significant difference in oxytocin-mediated decrease in the phosphorylation of Akt between Wistar VSMCs ($46.2\% \pm 9.5\%$ decrease) and SHR VSMCs ($45.5\% \pm 8.6\%$ decrease) compared to the respective control groups (**Figure 4.30 h**).

Similarly, Ang II-induced increase in the phosphorylation of Akt was not markedly different between Wistar VSMCs ($44.4\% \pm 3.5\%$) rats and SHR VSMCs ($70.2\% \pm 20.4\%$ increase) compared to their respective control groups (**Figure 4.30 g**).

The pre-treatment of oxytocin with Ang II also didn't show any significant difference in inhibiting phospho-Akt between Wistar VSMCs ($62.1\% \pm 10.9\%$ decrease) and SHR VSMCs ($60.4\% \pm 2.6\%$ decrease) compared to the respective Ang II (alone) groups (**Figure 4.30 i**).

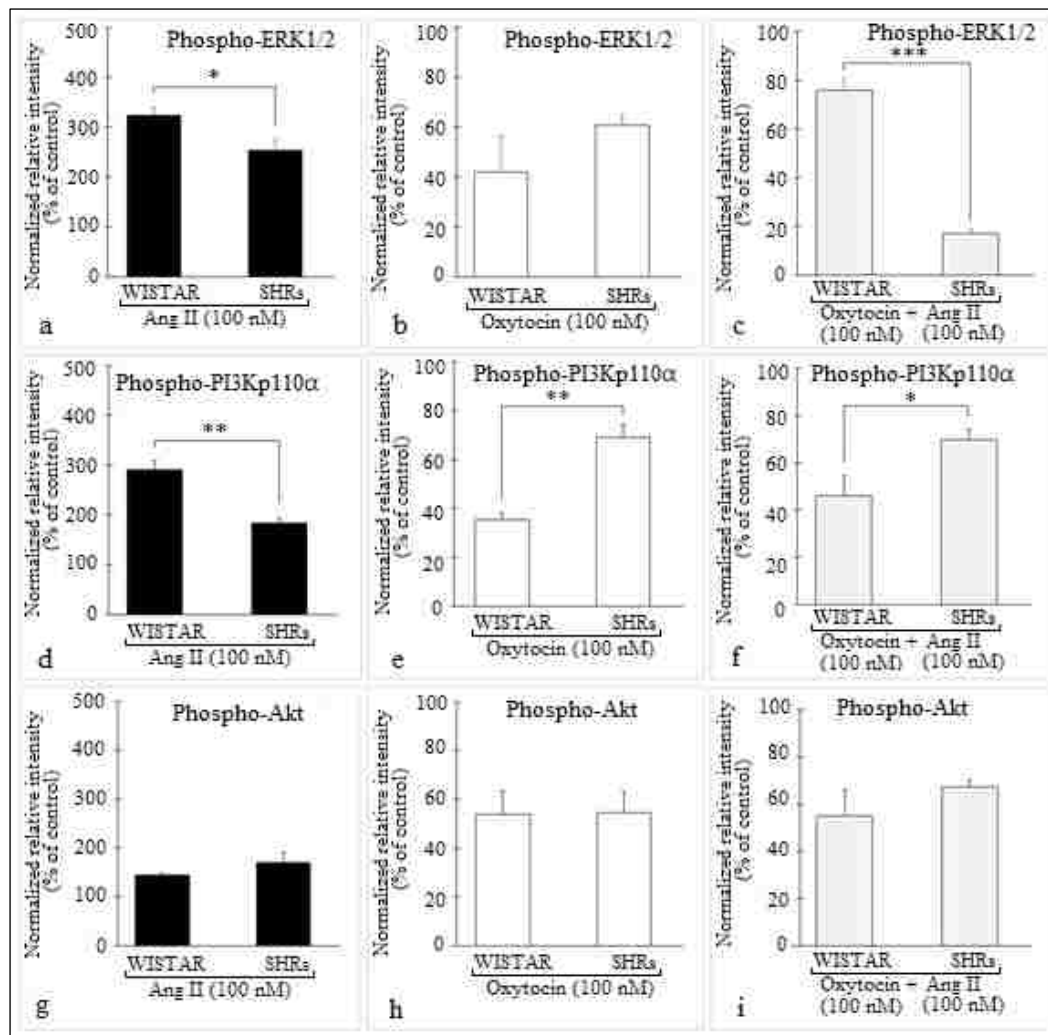


Figure 4.30. The comparison of the effects of oxytocin, Ang II, and pre-treatment of oxytocin with Ang II on the phosphorylation of ERK1/2 (figure a, b, and c), PI3K p110 α (figure d, e, and f), and Akt (figure g, h, and i) in aortic Wistar and SHR VSMCs. The data are shown as the normalized relative intensity of the bands and are represented as percent (%) of control, with control as 100%. The data are shown as the mean \pm SEM of five (five Wistar rats and five SHRs each), three (three Wistar rats and three SHRs each), and four (four Wistar rats and four SHRs each) independent experiments conducted for ERK1/2, PI3K p110 α , and Akt, respectively. * indicates $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Ang II indicates Angiotensin II; OXTA, oxytocin receptor antagonist; ERK, extracellular signal-regulated kinase; PI3K, phosphatidylinositol-3-kinase.

4.3.5. Comparison of the effect of oxytocin and its pre-treatment with Ang II on vasocontraction pathway: ROCK-1 and ROCK-2 in aortic VSMCs isolated from Wistar rats and SHRs

Western blotting was employed to investigate the effect of oxytocin, Ang II, and its pre-treatment with Ang II on the activation of ROCK-1 and ROCK-2. The densitometric analysis of western blot images demonstrated that oxytocin significantly decreased the activation of ROCK-1 and ROCK-2 in both aortic Wistar VSMCs and SHR VSMCs.

On ROCK-1

There was no significant difference between oxytocin-mediated reduced activation of ROCK-1 between Wistar VSMCs and SHR VSMCs, respectively (**Figure 4.31**).

Oxytocin reduced the ROCK-1 activation by $60.4\% \pm 7.6\%$ in Wistar VSMCs and by $58.9\% \pm 5.4\%$ in SHR VSMCs compared to their respective control groups (**Figure 4.31 b**).

Similarly, Ang II-induced activation of ROCK-1 was not markedly different between Wistar VSMCs ($147\% \pm 50\%$ increase) and SHR VSMCs ($102\% \pm 43\%$ increase) compared to their respective control groups (**Figure 4.31 a**).

The pre-treatment of oxytocin with Ang II also didn't show any significant difference in reducing the activation of ROCK-1 between Wistar VSMCs ($85.2\% \pm 10.6\%$ decrease) and SHR VSMCs ($79\% \pm 6.9\%$ decrease) compared to the respective Ang II (alone) groups (**Figure 4.31 c**).

On ROCK-2

Similar to ROCK-1, the impact of oxytocin in reducing the activation of ROCK-2 was not markedly different between Wistar VSMCs and SHR VSMCs (**Figure 4.31**).

Oxytocin-mediated reduced activation of ROCK-2 in Wistar VSMCs (38.7% \pm 13.1% decrease) was not statistically different from that in SHR VSMCs [(41.5% \pm 12.8% decrease (**Figure 4.31 e**)]. Ang II-induced increased activation of ROCK-1 in Wistar VSMCs (83.2% \pm 14.6% increase) was not prominently different from SHR VSMCs (81.2% \pm 14.8% increase) compared to their respective control groups (**Figure 4.31 d**).

The pre-treatment of oxytocin with Ang II also didn't show any substantial difference in reducing the activation of ROCK-1 between Wistar VSMCs (65.8% \pm 13.2% decrease) and SHR VSMCs (69.5% \pm 13.2% decrease) compared to their Ang II (alone) groups respectively (**Figure 4.31 f**).

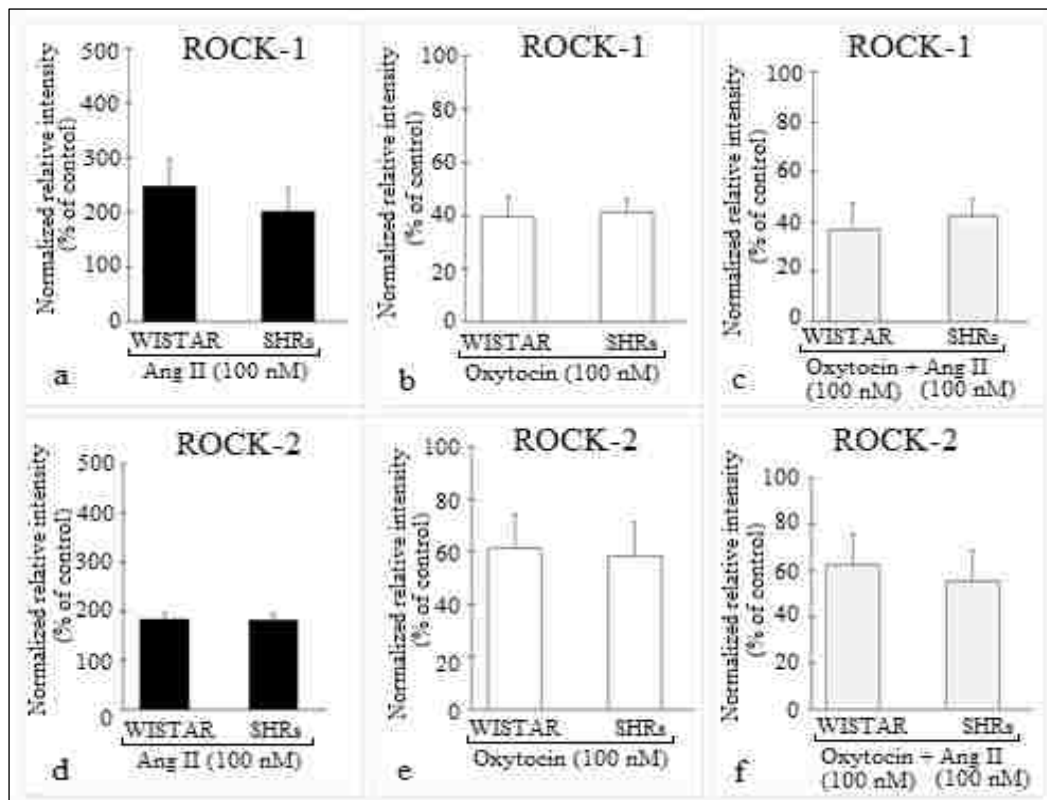


Figure 4.31. The comparison of the effects of oxytocin, Ang II, and the pretreatment of oxytocin with Ang II on the phosphorylation of Rho kinases – ROCK-1 (figure a, b, and c) and ROCK-2 (figure d, e, and f) in aortic Wistar and SHR VSMCs. The data are shown as the normalized relative intensity of the bands and are represented as percent (%) of control, with control as 100%. The data are shown as the mean \pm SEM of four (four Wistar rats and four SHRs each) and five (five Wistar rats and five SHRs each) independent experiments conducted for ROCK-1 and ROCK-2, respectively. Ang II indicates Angiotensin II; OXTA, oxytocin receptor antagonist; ROCK, rho-associated coiled-coil containing protein kinase.

4.3.6. Comparison of the effect of oxytocin and its pre-treatment with Ang II on IL-6 in aortic Wistar VSMCs and SHR VSMCs

The ELISA based IL-6 assay involved the measurement of the concentration of IL-6 released in the culture media. The findings of IL-6 assay established that oxytocin significantly increased IL-6 secretion from aortic VSMCs, but its pre-treatment with Ang II significantly decreased IL-6 in both Wistar VSMCs and SHR VSMCs.

There was a significant difference in IL-6 secretion levels in the control group between Wistar VSMCs and SHR VSMCs. The control group in SHR VSMCs showed more IL-6 [848 ± 9 pg/mL ($P < 0.05$)] than Wistar VSMCs [614 ± 104 pg/mL (**Figures 4.32 and 4.33**)].

The assay employed three different concentrations of oxytocin: 10 nM, 100 nM, 1000 nM. In response to different concentrations of oxytocin, aortic SHRs VSMCs showed significantly more IL-6 secretion [985 ± 25 pg/mL ($P < 0.05$) at 10 nM (**Figure 4.32**); $1,289 \pm 8$ pg/mL ($P < 0.001$) at 100 nM (**Figures 4.32 and 4.33**); and $1,468 \pm 19$ pg/mL ($P < 0.001$) at 1,000 nM (**Figure 4.32**)] than Wistar VSMCs (695 ± 101 pg/mL at 10 nM; 1033 ± 24 pg/mL at 100 nM; and 959 ± 52 pg/mL at 1,000 nM) compared to their respective controls.

There was no significant difference between Wistar VSMCs and SHR VSMCs with regards to Ang II-induced secretion of IL-6 ($1,481 \pm 91$ pg/mL in Wistar VSMCs and $1,620 \pm 66$ pg/mL in SHR VSMCs) compared to their respective control groups (**Figures 4.32 and 4.33**).

Although oxytocin on its own significantly increased IL-6 in both Wistar VSMCs and SHR VSMCs, interestingly, oxytocin's pre-treatment (100 nM) followed by Ang II (100 nM) significantly decreased IL-6 secretion in both Wistar VSMCs (735 ± 77 pg/mL) and SHR VSMCs (891 ± 59 pg/mL) compared to their respective Ang II 100 nM (alone) groups (**Figures 4.32** and **4.33**).

However, there was no significant difference between the Wistar VSMCs and SHR VSMCs concerning the effect of oxytocin's pre-treatment on Ang II-induced IL-6 secretion (**Figures 4.32** and **4.33**). The pattern and magnitude of the impact of oxytocin pre-treatment on Ang II-mediated IL-6 secretions were similar in both Wistar VSMCs and SHR VSMCs.

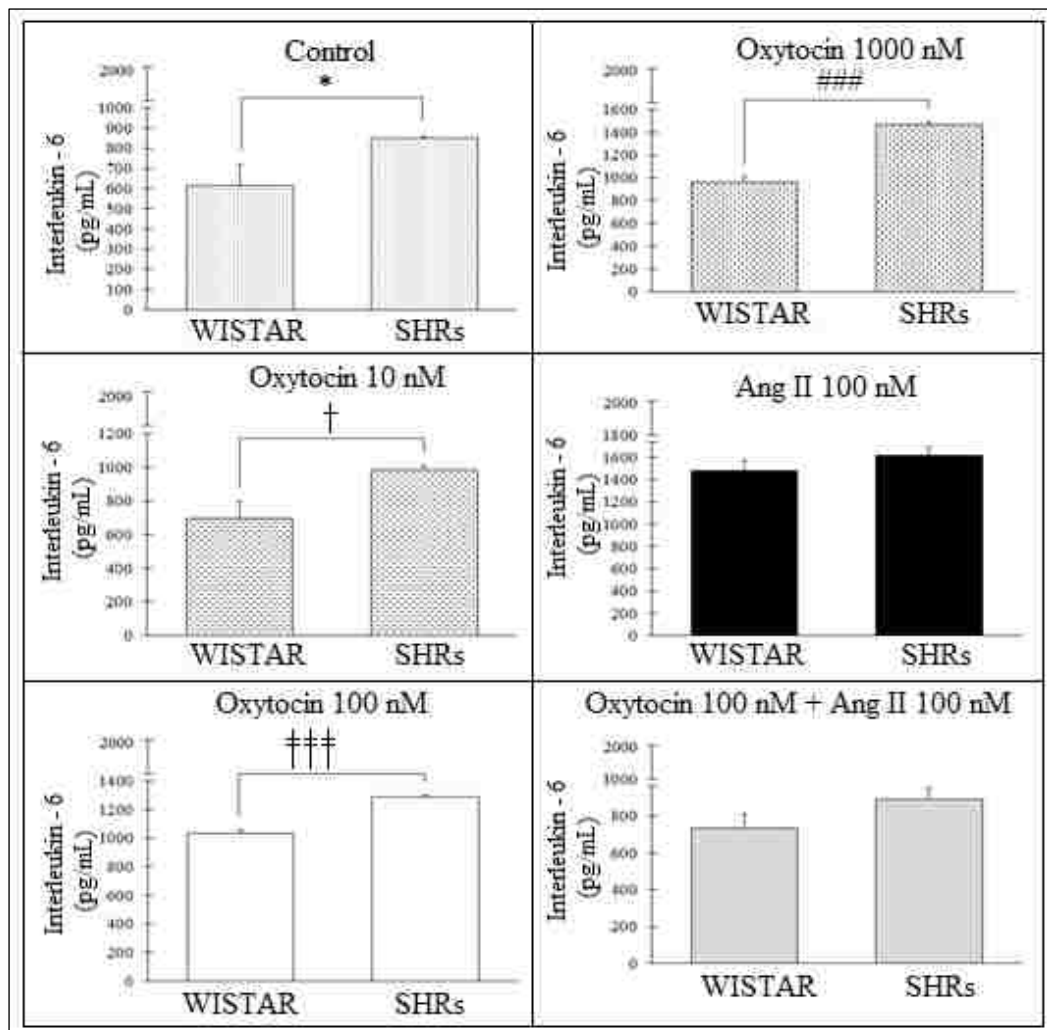


Figure 4.32. The comparison of the effect of oxytocin (10 nM, 100 nM, and 1,000nM), Ang II (100 nM), pre-treatment of oxytocin with Ang II on IL-6 secretion in aortic Wistar VSMCs and SHR VSMCs. The concentration of IL-6 secretion by the aortic VSMCs in the culture media was measured in picogram per mL (pg/mL). The data are represented as the mean \pm SEM from three independent experiments performed using aortic VSMCs from three Wistar rats and three SHRs, respectively. In each experiment each treatment group had duplicates. * indicates $P < 0.05$ Control Wistar rats versus Control SHRs; † $P < 0.05$ Oxytocin 10 nM Wistar rats versus Oxytocin 10 nM SHRs; ††† $P < 0.001$ Oxytocin 100 nM Wistar rats versus Oxytocin 100 nM SHRs; †††† $P < 0.001$ Oxytocin 1000 nM Wistar rats versus Oxytocin 1000 nM SHRs. Ang II indicates Angiotensin II.

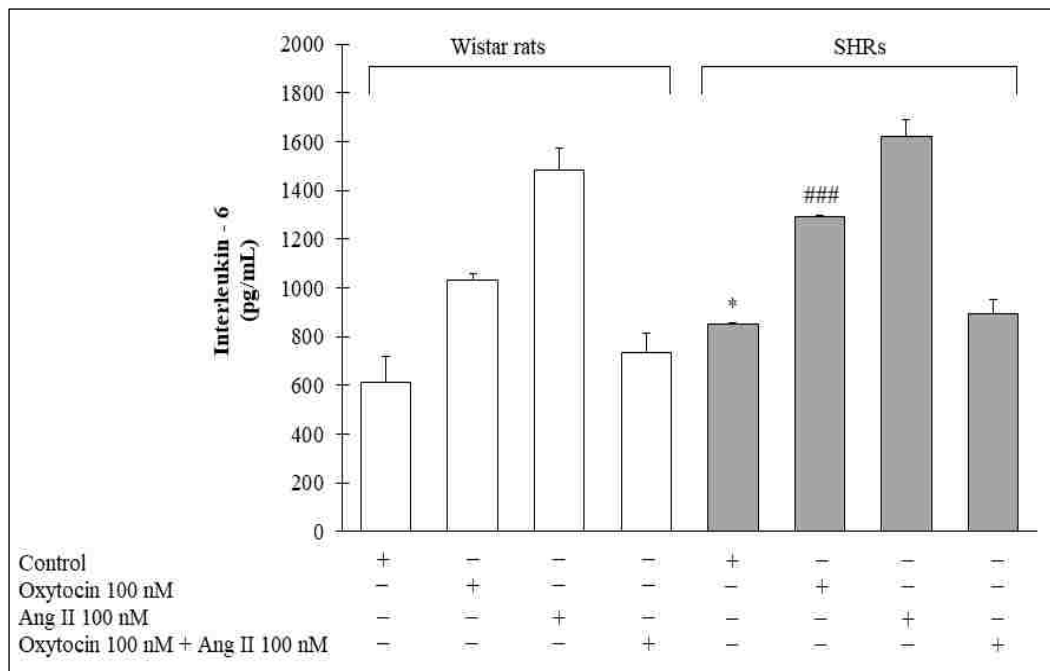


Figure 4.33. The comparison of the effect of oxytocin (100 nM), Ang II (100 nM), pre-treatment of oxytocin (100 nM) with Ang II (100 nM) on IL-6 secretion in aortic Wistar VSMCs and SHR VSMCs. The concentration of IL-6 secreted by the aortic VSMCs in the culture media was measured in picogram per mL (pg/mL). The data are represented as the mean \pm SEM from three independent experiments performed using aortic VSMCs from three Wistar rats and three SHRs, respectively. In each experiment each treatment group had duplicates. * indicates $P < 0.05$ Control group Wistar rats versus Control group SHRs; ### $P < 0.001$ Oxytocin 100 nM in Wistar rats versus Oxytocin 100 nM in SHRs. Ang II indicates Angiotensin II.

4.3.7. Comparison of the effect of oxytocin and its pre-treatment with Ang II on ROS production in aortic Wistar VSMCs and SHR VSMCs

The ROS assay findings demonstrated that oxytocin significantly increased the ROS production in aortic VSMCs isolated from both Wistar rats and SHRs.

In addition, the pre-treatment of oxytocin followed by Ang II significantly increased ROS production in both Wistar VSMCs and SHR VSMCs. However, there was a significant difference in the impact of oxytocin and its pre-treatment with Ang II on ROS production between Wistar VSMCs and SHR VSMCs.

The control group Wistar VSMCs showed more ROS levels [$7,542 \pm 128$ ($P < 0.001$)] compared to the control group SHR VSMCs [$5,411 \pm 259$ (**Figure 4.44**)].

The treatment with oxytocin (100 nM) resulted in a significantly more ROS production in Wistar VSMCs [$8,584 \pm 114$ ($P < 0.001$)] than SHR VSMCs ($6,657 \pm 202$) compared to their respective controls (**Figure 4.44**).

Ang II (100 nM)-induced ROS production was significantly higher in Wistar VSMCs [$8,336 \pm 204$ ($P < 0.001$)] than SHR VSMCs [$6,286 \pm 208$ (**Figure 4.44**)].

The pre-treatment of oxytocin (100 nM) followed by Ang II (100 nM) also showed significantly higher ROS production [$9,346 \pm 143$ ($P < 0.001$)] in Wistar VSMCs than SHR VSMCs ($7,481 \pm 162$) compared to their respective Ang II (alone) and Oxytocin groups (alone), respectively (**Figure 4.44**).

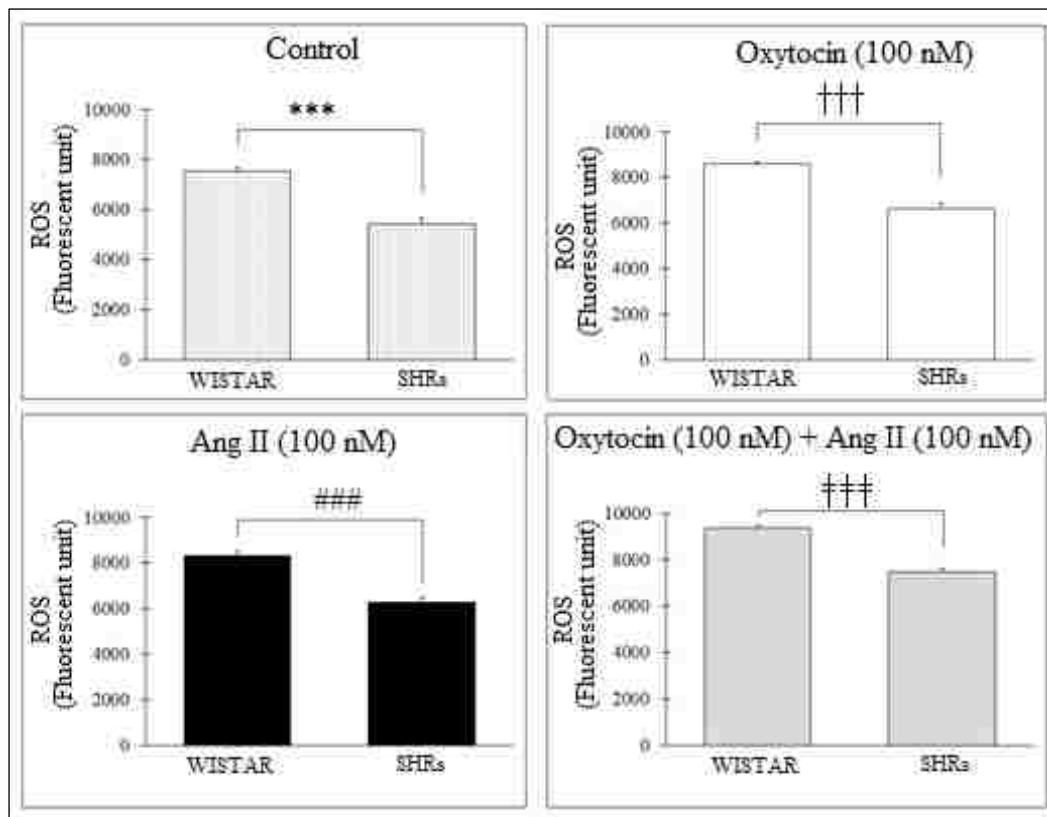


Figure 4.44. The comparison of the effect of oxytocin, Ang II, pre-treatment of oxytocin with Ang II on ROS production between aortic Wistar and SHR VSMCs. VSMCs were seeded at 1×10^4 cells/well in 96-well plates. The ROS levels were measured as the function of the fluorescent unit. The data are represented as the mean \pm SEM from three independent experiments performed using aortic VSMCs from three Wistar rats and three SHRs, respectively. In each experiment each treatment group had 12 replicates. *** indicates $P < 0.001$ Control group in Wistar rats versus Control group in SHRs; ††† $P < 0.001$ Oxytocin 100 nM in Wistar rats versus Oxytocin 100 nM in SHRs; ### $P < 0.001$ Ang II 100 nM in Wistar rats versus Ang II 100 nM in SHRs; ††† $P < 0.001$ Oxytocin 100 nM + Ang II 100 nM in Wistar rats versus Oxytocin 100 nM + Ang II 100 nM in SHRs. Ang II indicates Angiotensin II.

Chapter 5: Discussion

CVD is the leading cause of hospitalization in the United States and accounts for mortality more than other any other disease, including cancer and communicable diseases.²⁶³ Each year about 17 million deaths occur due to CVD, which is two times higher than deaths caused by cancer, and in all probability, this number is going to increase to about 23.3 million by 2030.²⁶⁴

Despite advancement in diagnosis and improvement in efforts aimed at prevention, control, and treatment, CVD remains a primary cause of premature death and disability across the globe.²⁶⁵ Consequentially, CVD healthcare management has imposed an enormous financial burden on patients.²⁶⁶

The current standard CVD management includes therapies aimed at controlling patients' lipid-profiles (statins), blood glucose levels (metformin), and prescription of anti-hypertensive (ACE inhibitors) and anti-platelet medications (aspirin).²⁶⁷ However, lesser favorable patient outcomes, as well as the rapidly rising rate of mortality due to CVD, paint a different picture. It reflects a growing need to expand CVD research so that new therapeutic options can be devised to effectively treat CVD. The present study was a step towards exploring the potential of oxytocin in CVS.

Over decade oxytocin, which is often referred to as a female reproductive hormone,^{1,9,268,269} has garnered the attention of the scientific community as a hormone with cardioprotective effects.^{23,24} Much of the research so far has focused on oxytocin-mediated

protective effects in cardiomyocytes, vascular endothelial cells, and cardiogenesis.^{247,30,32,40} Besides identifying the presence of the oxytocin receptors in VSMCs,²⁵ not much research has been done concerning the potential effect of oxytocin on VSMCs.

VSMCs play a crucial role in cardiovascular physiology and are often at the center of the events in the pathophysiology of CVD including hypertension.²⁷⁰ They undergo increased cell proliferation in response to hypertension-induced stress on the vasculature, leading to hypertrophy and an aberrant increase in vascular contraction.^{271,80} These changes are mediated by complex signaling pathways mainly the ERK1/2 pathway,⁹⁹ the PI3K/Akt pathway,¹⁰⁵ and ROCK pathway.¹⁴⁴ In addition, increased oxidative stress and prolonged inflammation add to and exacerbate injury to VSMCs, and manifest the long-term pathological changes in vasculature.^{272,273}

Interestingly, the interaction of oxytocin with other vital peptides playing crucial roles in CVD such as Ang II has not been well-researched. Ang II is the primary effector hormone and the central component of the RAAS. It causes vasoconstriction and maintains blood pressure in normal physiological conditions.⁵⁰

Ang II is also involved in CVD such as atherosclerosis, congestive heart failure, diabetes, hypertension, inflammation, and myocardial infarction.⁵¹ It mainly binds to AT1R in the CVS and executes its actions via activation of the ERK1/2 pathways (increasing phospho-ERK1/2),^{274,275} PI3K/Akt pathway (phosphorylating PI3K p110 α and increasing phospho-Akt)^{276,277} and ROCK pathway (activation of ROCK-1 and ROCK-2).^{278,279} These signaling pathways and kinases are involved in Ang II-induced hypercontraction of VSMCs, hypertrophy, hyperplasia, endothelial dysfunction, leading to

vascular diseases and their progression.^{53,54} In addition, in cardiovascular pathophysiological conditions Ang II precipitates inflammation and oxidative stress.^{280,59}

We hypothesized that oxytocin provides vascular protection in VSMCs via modulation of these pathways, inflammation, and reduction of oxidative stress, and antagonization of the Ang II effects on these pathways, inflammation and oxidative stress. We investigated oxytocin's effect on cell proliferation, inflammation, oxidative stress and on vasocontraction pathway in primary aortic VSMCs, isolated from the adult normotensive Wistar rats and SHRs.

5.1. Oxytocin reduced the proliferation in aortic VSMCs

The findings of the present study demonstrated that oxytocin significantly decreased cell proliferation of normotensive (Wistar) and hypertensive (SHR) VSMCs. This is in contrast with the proliferative effect of oxytocin on vascular endothelial cells derived from: immortalized human dermal microvasculature, breast-tumors, human umbilical vein; and cardiomyocytes.^{40,41}

It is noteworthy that the kind of effect oxytocin shows on cell proliferation depends on the type of cells involved. While previous studies have shown oxytocin exerting a proliferative influence on, for instance, trophoblast and astrocytes, others have shown oxytocin mediating anti-proliferative effects on cell lines obtained from breast cancer, ovarian cancer, and prostate cancer.^{281–283}

Even within the cell lines derived from a similar source but from different stages of differentiation, oxytocin has shown a differential effect on proliferation. For example, in osteosarcoma cell lines (SaOS-2, TE-85, and UMR-106) obtained from different stages of differentiation, oxytocin showed dual action on proliferation. Oxytocin increased cell proliferation in SaoS-2 cells and decreased cell proliferation in both TE-85 cells and UMR-106 cells.²⁸⁴

Although only one type of oxytocin receptor has been found across species,³ these dual effects of oxytocin on proliferation suggest the probability of another subtype of oxytocin receptor.

In addition to showing anti-proliferative effects of oxytocin on aortic VSMCs, the outcomes of the present study have revealed that oxytocin significantly reduced Ang II-induced cell proliferation of aortic VSMCs isolated from

normotensive Wistar rats. Importantly, this effect was consistent in hypertensive SHR VSMCs. Further, the oxytocin receptor antagonist significantly attenuated both these effects, suggesting that oxytocin mediated these effects via its receptors.

It is essential to understand the impact of oxytocin-induced inhibition of Ang II-mediated proliferation of SHR VSMCs in the broader context of CVD pathophysiology.

In hypertension induced by hyperactivation of Ang II signaling, VSMCs undergo extensive remodeling including hypertrophy and fibrosis. Moreover, cardiomyocytes in the left ventricle of the heart undergo hyper-proliferation leading to cardiac hypertrophy and ultimately, over time, compromise the function of heart and blood vessels.²⁸⁵ These events lay the foundation for a cascade of numerous other intra-vascular and intra-cardiac pathophysiological events worsening CVD.²⁸⁶

Evidence that oxytocin's anti-proliferative action on VSMCs and oxytocin-induced decrease in Ang II-mediated proliferation of SHR VSMCs generated from this study underline the potential oxytocin might have in vascular protection in CVD such as hypertension, and this merits further investigation.

5.2. Oxytocin deactivated the proliferation signal transduction pathways in aortic VSMCs

For greater understanding about how oxytocin reduces cell proliferation in aortic VSMCs, this study explored the two key downstream cell signaling pathways involved in the proliferation of VSMCs: the *ERK1/2 MAPK pathway* and the *PI3K/Akt pathway*.

This study results revealed that oxytocin significantly reduced the phosphorylation of ERK1/2 in normotensive and hypertensive VSMCs. This is in contrast with reports from previous studies, which reported that oxytocin treatment increased phosphorylation of ERK1/2 in cardiomyocytes and vascular endothelial cells.^{40,102}

The differential effects of oxytocin on the phosphorylation of ERK1/2 have been proposed to be based on the type of cells involved, location of oxytocin receptors, activation of the different G-proteins, and interaction with a phosphatase. For example, oxytocin demonstrated dual effects on phosphorylation of ERK1/2 in human embryonic kidney cells. These effects were based on the site of oxytocin receptors. Oxytocin decreased the phosphorylation of ERK1/2 when oxytocin receptors were present outside the caveolin-1-enriched microdomains and resulted in reduced cell proliferation. And, oxytocin increased the phosphorylation of ERK1/2 when oxytocin receptors were present inside the caveolin-1-enriched microdomains and henceforth, increased cell proliferation.¹⁰³

It is worth stating that caveolin-1 is vital in the trafficking of $G\alpha_s$ (stimulatory) protein, inhibition of $G\alpha_s$ /adenylyl cyclase pathway,¹⁰⁴ and is also thought to be involved in the regulation of cell proliferation and cell survival.²⁸⁷

In another study, oxytocin caused the dephosphorylation of eukaryotic elongation factor 2 (eEF2) and contributed to an increase in protein translation and synthesis in myometrial cells via PKC.²⁸⁸ The eEF2 kinase is responsible for the phosphorylation of eEF2. The phosphorylated form of eEF2 is inactive and causes downregulation of protein translation, whereas, the dephosphorylated form is the active form and leads to an increase in protein translation and synthesis. The eEF2 kinase is regulated by a phosphatase known as protein phosphatase 2 A (PP2A).²⁸⁹

In addition to oxytocin inducing dephosphorylation of eEF-2 through PKC,²⁸⁸ it could very well be that oxytocin might increase the phosphatase (PP2A) leading to reduced activity of eEF2 kinase and thus inducing dephosphorylation of eEF2. Furthermore, oxytocin reduced Ang II-induced phosphorylation of ERK1/2, and these effects were significantly abolished by pre-treatment with an oxytocin receptor antagonist. Interestingly, these effects of oxytocin were consistent in aortic VSMCs isolated from hypertensive rats.

The significance of the study results demonstrating oxytocin-induced inhibition of phosphorylation of ERK1/2 and oxytocin-mediated inhibition of Ang II-induced ERK 1/2 phosphorylation in SHR VSMCs can be understood by assessing the implication of the ERK1/2 signaling pathway in CVD pathophysiology.²⁹⁰

An upregulation of agonist (Ang II)-stimulated ERK1/2 signaling for prolonged duration has been shown to induce pathological hypertrophy in VSMCs and decompensated cardiac hypertrophy in the heart.²⁹¹ Thus, the ERK 1/2 signaling pathway plays a crucial role in the precipitation of CVD, for example, hypertension, myocardial infarction, and stroke.⁹⁹

The present study provided evidence, that by decreasing the phosphorylation of ERK1/2, oxytocin deactivated Ang II-stimulated ERK1/2 signaling in SHR VSMCs, and thus supports a protective role of oxytocin in pathophysiological conditions, for instance, hypertension. However, *in vivo* studies are required to further shed more light on oxytocin's protective effect on hypertension.

The PI3K/Akt signaling pathway has been shown to alter vascular function by increasing the myogenic tone of resistant arteries.¹⁰⁷ It also results in an aberrant increase in the activity of L-VGCC leading to increased intracellular calcium, increased cell volume in cardiomyocytes, and hyper-proliferation of VSMCs.^{120,110} Thus, the PI3K/Akt pathway leads to various cardiovascular pathophysiological conditions including hypertension, pathological cardiac hypertrophy, and cardiac arrhythmia.¹¹⁹ Ang II has been known to participate in and worsen these pathophysiological events via hyper-activation of the PI3K/Akt signaling.^{276,53}

The findings of this study showed that oxytocin remarkably reduced the phosphorylation of PI3K p110 α and Akt in aortic VSMCs isolated from normotensive and hypertensive rats. These findings are consistent with a previous

study, wherein oxytocin reduced the phosphorylation of PI3K p110 α and Akt, albeit in gut cells.²⁹²

Similar to the effect of oxytocin on Ang II-induced induced phosphorylation of ERK1/2, oxytocin prominently diminished Ang II-induced phosphorylation of PI3K p110 α and Akt, whereas, the oxytocin receptor antagonist prevented these effects of oxytocin. These findings were also consistent in aortic SHR VSMCs, which further suggests that oxytocin induced-deactivation of PI3K/Akt signaling pathway in SHR VSMCs might provide vascular protective effects in hypertension in vivo.

There is a possibility that in aortic VSMCs, oxytocin via its receptors, which belong to GPCRs superfamily,³ might be recruiting G α_i (inhibitory) protein, and thus inhibiting downstream cell signaling by reducing the levels of phospho-ERK1/2, phospho-PI3K p110 α , and phospho-Akt, leading to the deactivation of the ERK1/2 pathway and the PI3K/Akt pathway.

Alternatively, it could very well be that oxytocin might regulate the phosphatase and thereby reduce the phosphorylation of ERK/12, PI3K p110 α , and Akt.

5.3. Oxytocin deactivated the ROCK pathway in aortic VSMCs

The present study explored the effect of oxytocin on the ROCK pathway. The agonist-mediated phosphorylation of Rho kinases leads to the activation of both ROCK-1 and ROCK-2, along with concurrent calcium mobilization, causing the organization and remodeling of the actin cytoskeleton, extracellular matrix, and contraction of VSMCs.¹⁴⁶ The ROCK signaling pathway has been implicated in the pathogenesis of various CVD such as hypertension, pulmonary hypertension, angina pectoris, stroke, and heart failure.^{140–144} It is also established that Ang II activates ROCK signaling in these pathophysiological conditions.^{135,53}

Together with the reduction in cell proliferation and deactivation of the proliferation signal transduction pathways by oxytocin, the present research demonstrated for the first time that oxytocin significantly reduced Rho kinase-mediated vasocontraction in aortic VSMCs. The study results highlight that oxytocin reduced the activation of both ROCK-1 and ROCK-2 in aortic VSMCs isolated not only from normotensive rats but also from hypertensive rats. These findings suggest that oxytocin could provide vascular protective effects in pathophysiological conditions associated with hyper-signaling of ROCK pathways such as hypertension and stroke.

However, to state definitively about oxytocin's anti-vasocontraction effect on VSMCs, direct measurement of oxytocin's impact on vasocontraction would be needed. The study was limited in this regard as this study did not directly assess oxytocin's effect on vasocontraction. Instead, it investigated the impact of oxytocin on the vasocontraction pathway (ROCK pathway). Future studies could be carried

out by employing an ex vivo model such as the organ bath to directly measure vasocontraction.

The study outcomes further revealed that oxytocin reduced Ang II-mediated activation of both ROCK-1 and ROCK-2 in normotensive as well as in hypertensive VSMCs, and these effects were blocked by an oxytocin receptor antagonist, indicating that oxytocin causes these effects via its receptors. Based on the study results, the fact that oxytocin antagonized Ang II-stimulated ROCK pathway in aortic VSMCs suggests the potential cardioprotective effects oxytocin might have in conditions where Ang II-mediated hyper-signaling of ROCK causes vasoconstriction of blood vessels and disrupts the hemodynamics, culminating in the development and worsening of CVD such as hypertension, angina pectoris, and stroke.

This study was the first attempt to investigate the impact of oxytocin and its pre-treatment with Ang II on the ROCK pathway. However, future in vivo studies would be needed to further explore oxytocin's effect on the contraction of blood vessels in normal and pathophysiological conditions, for example, hypertension.

All three pathways: the ERK1/2 pathway, the PI3K/Akt pathway, and the ROCK1/ROCK2 pathway are crucial in precipitating deleterious events such as hypertrophy and hyper-contractility in VSMCs, which further lead to cardiovascular complications manifested in CVD such as hypertension and arrhythmia.^{99,119,144}

Our results indicate that oxytocin may prevent these changes in the dynamics of the vasculature by reducing the phosphorylation of ERK1/2, PI3K, and

Akt and by decreasing the activation of ROCK-1 and ROCK-2 in normotensive as well as hypertensive conditions (**Figure 5**).

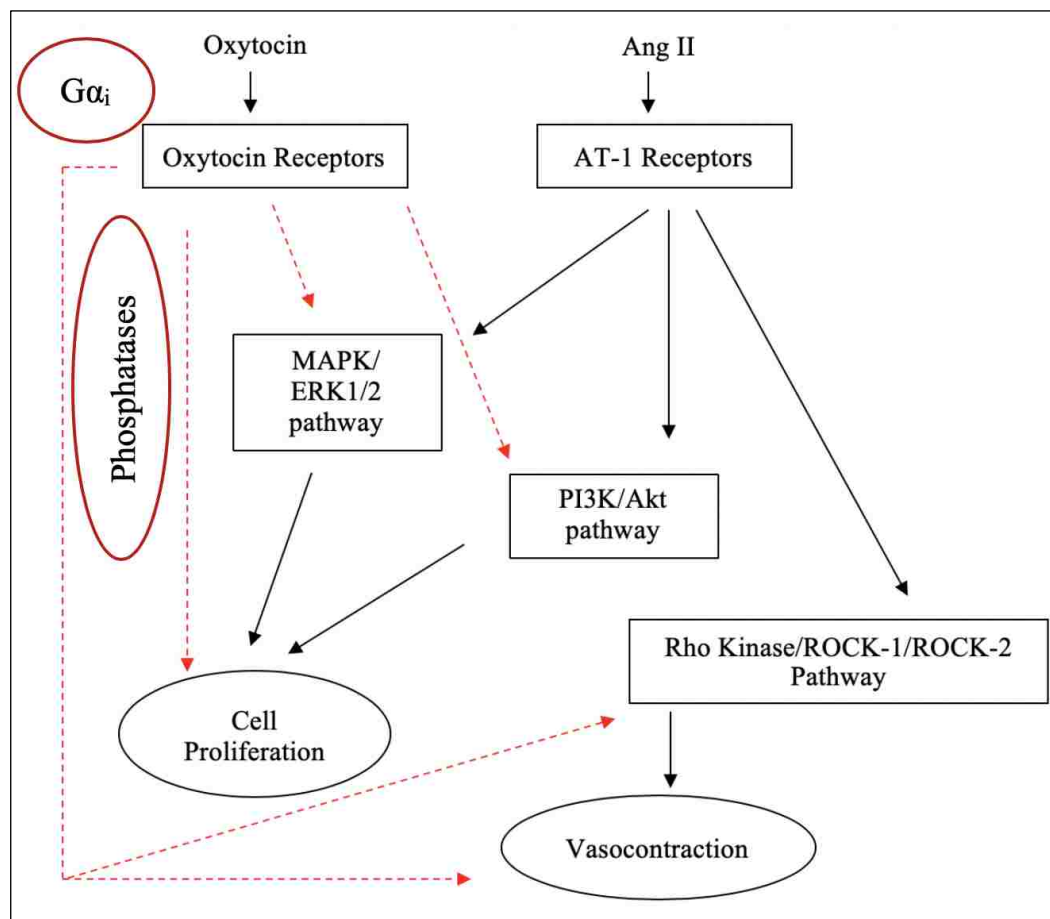


Figure 5. Inhibition of the ERK1/2 pathway, the PI3K/Akt pathway, and the Rho-kinase pathway by oxytocin in aortic VSMCs. Oxytocin mediates anti-proliferative effects by inhibiting phosphorylation of ERK1/2, PI3K, and Akt. Oxytocin deactivates Rho-kinase pathway involved in vasoconstriction of VSMCs by inhibiting the phosphorylation of ROCK-1 and ROCK-2. It may be possible that either via the recruitment of $G\alpha_i$ proteins or by stimulating phosphatases oxytocin decreases phosphorylation of ERK1/2, PI3K, Akt, ROCK-1, and ROCK-2. ERK indicates extracellular signal-regulated kinase; PI3K, phosphoinositide-3-kinase; ROCK, rho-associated coiled-coil containing protein kinase; Ang II, angiotensin II; AT-1, angiotensin type-1; $G\alpha_i$, G alpha inhibitory protein. Dotted red arrows indicate inhibition; black arrows, activation.

5.4. Oxytocin increased IL-6 in aortic VSMCs

One of the objectives of the current study was to investigate the effects of oxytocin on inflammation responses using aortic VSMCs. Hence, IL-6, a biomarker of inflammation, was measured.

In contrast to previous studies carried out on vascular endothelial cells,³⁴ the outcomes of the present study, conducted on aortic VSMCs, revealed that oxytocin significantly increased IL-6, but remarkably reduced Ang II-stimulated IL-6 levels.

It is essential to understand the role of IL-6 while interpreting these results. IL-6 has been known for its pleiotropic effects.¹⁷² It is involved in various physiological events including inflammation (production of CRP) and immunological responses (activation and differentiation of T-cells and B-cells).^{174,175,178,180,181}

Several studies have shown that an increase in IL-6 levels by any treatment(s) or peptide(s) under investigation can be detrimental, especially in conditions, for example rheumatoid arthritis and heart disease, wherein IL-6 mediated immunological and pathophysiological events contribute in worsening of the symptoms.^{293,294}

However, the effects of IL-6 can be both harmful and protective.¹⁹² It is the duration of the pathogenic stimuli or pathophysiological conditions that determines which one of the two effects IL-6 will manifest. For example, IL-6 elicits cardioprotective effects on cardiomyocytes when exposed to acute pathophysiological condition(s).¹⁹²

Conversely, IL-6 signaling can prove detrimental if the exposure to pathophysiological condition(s) is chronic. In such a scenario, IL-6 may result in pathological changes, for instance, reduced cardiac contractility, pathological cardiac hypertrophy, and ultimately leading to decreased cardiac function.^{197,198}

In this context, based on this study findings, oxytocin-induced IL-6 secretion in VSMCs could be vascular protective, as oxytocin-induced IL-6 secretion might boost the immunological defense required to counter the pathogenic stimulus for a shorter period. The fact that oxytocin's effect on IL-6 was consistent in VSMCs isolated from hypertensive rats suggests oxytocin could similarly bolster immune defense against an acute exposure to a pathogen in diseased condition. However, further *in vivo* studies are required to explore the beneficial effect of oxytocin-induced IL-6 secretion against an acute exposure to pathogenic stimuli.

Another aspect of the present study was to determine the impact of oxytocin on Ang II-stimulated IL-6 secretion. The study results demonstrated that oxytocin reduced Ang II-stimulated IL-6 secretion not only in aortic VSMCs from normotensive rats but also in aortic VSMCs from hypertensive rats, indicating that oxytocin could show protective effects against Ang II-induced inflammation (increase in IL-6), which more likely happens in a chronic basis under cardiovascular disease conditions such as atherosclerosis and rheumatoid arthritis.

Based on the study findings that the singular treatment of oxytocin increased IL-6 secretion, it can be suggested that oxytocin might provide beneficial effects in conditions where a short-term inflammatory response is required to arrest the host

damage, for example, in cardiomyocytes where short-term IL-6 signaling provided cardioprotective effects.¹⁹² Furthermore, oxytocin's antagonistic action against Ang II-induced IL-6 secretion could provide protection against chronic inflammation such as in coronary heart disease and atherosclerosis.^{293,294}

5.5. Oxytocin increased ROS in aortic VSMCs

The current study was carried out to determine the effect of oxytocin on oxidative stress. Oxidative stress is often at the center of many pathophysiological conditions in the CVS.²⁹⁵ It is caused by an imbalance between oxidants and antioxidants.²⁹⁶ The oxidants are ROS such as hydrogen peroxide (H₂O₂) and superoxide (O₂⁻). On the other hand, antioxidants include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GRx).²⁹⁷

In cardiovascular cells such as VSMCs, endothelial cells, and cardiomyocytes, ROS is primarily produced by NADPH oxidase.²¹⁷ Others include mitochondrial transport chain and uncoupling of eNOS.^{225,227} The vasoconstrictor peptides such as Ang II and ET -1 increase oxidative stress via increased production of ROS, contributing to the pathogenesis of CVD including hypertension.^{219,221}

The results of the ROS measurement in the present study showed that oxytocin increased ROS production in aortic VSMCs. These results are in contrast with the effect of oxytocin on ROS production in vascular endothelial cells as reported in previous studies.³⁴ Also, oxytocin's pre-treatment with Ang II further increased ROS production, suggesting an additive effect. These effects of oxytocin were consistent in SHR VSMCs. The basal levels of ROS were higher in Wistar VSMCs, and consequently, oxytocin-induced ROS production was higher in Wistar VSMCs than SHR VSMCs.

These results suggest that oxytocin increases oxidative stress and further potentiate Ang II-induced oxidative stress in cultured aortic VSMCs. However, it

is essential to mention that the current study employed the in vitro experimental models, the effect of oxytocin was measured on isolated aortic VSMCs, of which the findings showed oxytocin increased ROS production. It cannot be assumed that oxytocin would show similar effects in vivo, where VSMCs are not separate and co-exist with endothelial cells.

It is worth noting endothelial cells, VSMCs, and their interaction plays a crucial role in maintaining vascular homeostasis. However, events such as increased ROS production causing oxidative stress and reduced availability of NO can lead to endothelial dysfunction. This can adversely impact the functions of VSMCs. VSMCs in such conditions are prone to excessive proliferation and hyper-contraction owing to lack of vasorelaxants (NO) and can lead to the development of CVD, for example, atherosclerosis.²⁹⁸

As stated earlier, oxytocin has been shown to induce protective effects in vascular endothelial cells via reduction of ROS production. It might be that in vivo, where both endothelial cells and VSMCs co-exist, oxytocin-mediated opposite effects on ROS production in VSMCs and endothelial cells might negate each other. This might not disrupt the overall redox equilibrium and may not adversely impact oxidative stress in blood vessels. This could be useful in conditions associated with increased oxidative stress such as hypertension, atherosclerosis, and rheumatoid arthritis.

However, the present study did not investigate the interaction between endothelial cells and VSMCs with regards to oxytocin's effect on ROS production, and this could be a subject of future investigation where oxytocin's effect on ROS

production in VSMCs and endothelial cells can be determined simultaneously by employing co-cultures of VSMCs and endothelial cells and in vivo studies.

5.6. Summary of the dissertation research

In the last decade and a half, the status of oxytocin as a contractor of the pregnant uterus, and an initiator of milk-ejection reflex have transcended to a hormone eliciting protective effects in the CVS, in general, and on cardiovascular cells including cardiomyocytes and vascular endothelial cells, in particular. However, oxytocin's impact on VSMCs, which form an integral layer of blood vessels and play a crucial role in physiological and pathophysiological conditions associated with CVS, have not been well-established. This study was carried out to investigate oxytocin's effect on proliferation, oxidative stress, inflammation, and on vasocontraction pathway in aortic VSMCs isolated from both normotensive and hypertensive rats.

In addition, this study also examined the interaction of oxytocin with Ang II, a vital hormone of the RAAS in the CVS. Ang II-induced proliferation, oxidative stress, inflammation, and vasocontraction of VSMCs have been associated with the development and worsening of various vascular diseases, including hypertension and atherosclerosis. This study investigated the effect of oxytocin's pre-treatment on Ang II-induced proliferation, oxidative stress, inflammation, and Ang II-stimulated vasocontraction pathway in VSMCs in aortic VSMCs isolated from both normotensive and hypertensive rats.

The present study employed primary aortic VSMCs isolated from normotensive Wistar rats and hypertensive SHR. For investigating the effect of oxytocin, and its interaction with Ang II, on the proliferation of VSMCs, this study utilized various methods: Hemocytometer method for cell counting; MTT assay for

cell viability assessment; ³H-Thymidine incorporation assay for cell growth measurement; and flow cytometry to determine apoptosis and necrosis.

To gain a better understanding of the impact of oxytocin on VSMCs proliferation, the two key proliferation signal transduction pathways were investigated: the ERK1/2 pathway and the PI3K/Akt pathway. Western blot technique was utilized to study the effect of oxytocin on these two pathways. For studying the impact of oxytocin, and its interaction with Ang II, on the vasoconstriction pathway in VSMCs, the ROCK pathway was investigated. The western blot technique was utilized to study oxytocin's effect on the ROCK pathway.

To investigate the effect of oxytocin, and its interaction with Ang II, on inflammation and oxidative stress, the ELISA-based IL-6 assay, and the fluorescent spectroscopy-based ROS assay were carried out, respectively.

The outcomes of proliferation studies indicated that oxytocin reduces VSMCs proliferation by inhibiting the phosphorylation of ERK1/2, PI3K p110 α , and Akt. Besides, oxytocin also reduced Ang II-induced proliferation by reducing Ang II-mediated phosphorylation of ERK1/2, PI3K p110 α , and Akt not only in VSMCs isolated from normotensive rats but also in hypertensive rats. This may have clinical relevance in conditions associated with higher levels of Ang II and Ang II-mediated hyper-signaling of proliferation pathways in the CVS, such as in hypertension and atherosclerosis. Nonetheless, further in vivo studies are required to demonstrate the protective effects of oxytocin against deleterious effects of Ang II in these pathophysiological conditions.

The study findings further revealed that oxytocin and its pre-treatment with Ang II led to dysregulation of the ROCK pathway via inhibition of the activation of ROCK1 and ROCK2. These findings suggest a possible cardioprotective role of oxytocin in conditions where there is an excessive vasoconstriction of blood vessels linked with Ang II-mediated hyper-signaling of ROCK pathway, for example, hypertension. However, this study was limited in its scope with regards to oxytocin's effect on vasoconstriction as the study did not directly measure vasoconstriction of VSMCs and rather investigated the vasoconstriction pathway, and this could be further investigated in the future.

Furthermore, the results of an ELISA-based IL-6 assay indicated that oxytocin's treatment increased IL-6 secretion in aortic VSMCs. This may be beneficial in conditions where a short-term increase in IL-6 release bolsters immunological response against a pathogenic stimulus for a shorter duration. Also, our findings demonstrated that oxytocin reduced the Ang II-mediated IL-6 secretion in aortic VSMCs isolated from both Wistar rats and SHRs. This could be favorable in conditions associated with Ang II-induced prolonged inflammation such as rheumatoid arthritis. However, further studies investigating oxytocin's effect on inflammation and its interaction with Ang II on the inflammatory response, especially in vascular conditions associated with aggravated inflammation such as atherosclerosis, are required.

The ROS assay findings showed that oxytocin increased ROS production and its pre-treatment with Ang II further increased ROS production in aortic VSMCs isolated from both Wistar rats and SHRs. This suggests that oxytocin might

increase oxidative stress in cultured VSMCs. However, it cannot be assumed that in vivo, oxytocin would exhibit similar effects. In vivo, VSMCs co-exist with vascular endothelial cells, wherein oxytocin has been previously shown to reduce ROS production. It could very well be that the opposite effects of oxytocin on ROS production in VSMCs and endothelial cells cancel out each other and might not disturb the overall redox equilibrium. Henceforth, oxytocin might not contribute to a change in oxidative stress in blood vessels, which could be significant in pathophysiological conditions associated with increased oxidative stress in CVS. Nevertheless, further studies would be required where oxytocin's effect on ROS can be assessed simultaneously in both VSMCs and endothelial cells, such as in vitro studies employing co-culturing of VSMCs and endothelial cells and in vivo studies.

5.7. Conclusion

VSMCs undergo various pathophysiological changes in hypertension and other CVD, which render them as a potential target for newer approaches for their therapeutic management. The present *in vitro* study investigated the effects of oxytocin on cell proliferation, inflammation, oxidative stress, and also on the pathways involved in cell proliferation and vasoconstriction in primary aortic VSMCs, isolated from normotensive (Wistar rats) and hypertensive rats (SHRs).

This study demonstrated that oxytocin inhibits proliferation and increases inflammation (IL-6) and oxidative stress (ROS) in aortic VSMCs obtained from Wistar rats and SHRs. Based on the study outcomes, it can be suggested that the anti-proliferative effect of oxytocin is mediated by inhibition of ERK1/2 and PI3K/Akt phosphorylation. Also, oxytocin deactivated Rho-kinase signaling pathway, a critical pathway involved in the contraction of VSMCs, by inhibiting the phosphorylation of ROCK-1 and ROCK-2.

The increased proliferation in VSMCs and hypercontraction mediated by peptides such as Ang II, worsen hypertension and precipitate other CVD and their progression. Oxytocin not only decreased VSMCs proliferation but also reduced Ang II-induced VSMCs proliferation and Ang II-stimulated vasoconstriction pathways.

Overall, this study indicates that oxytocin might have therapeutic potential in the management of CVD including hypertension. However, future *in vivo* studies are required to examine the cardiovascular protective effect of oxytocin in hypertension.

5.8. Future recommendation

The present study utilized all the existing techniques available to assess the effect of oxytocin and its pretreatment with Ang II on cell proliferation in VSMCs. However, the study was limited in its scope with regards to the direct measurement of vasocontraction in aortic VSMCs.

The current study indirectly assessed vasocontraction by investigating the vasocontraction pathway — ROCK pathway. In the future, this aspect can be addressed. The effect of oxytocin on vasocontraction can be studied by determining the intracellular calcium levels in aortic VSMCs. This can be achieved by employing techniques such as fluorescent-based calcium measurement assay and more directly employing organ bath studies using vascular smooth muscle tissue.

Furthermore, to understand the mechanism of how oxytocin decreases the phosphorylation of the ERK1/2, PI3K p110 α , and Akt, G α_i inhibitors may be employed. Since oxytocin receptors are GPCRs and have been previously shown to recruit different G-proteins in different cells and in different physiological conditions, it is a likely that oxytocin recruits G α_i and thus inhibits ERK1/2 and PI3K/Akt-mediated downstream signaling pathways involved in the proliferation of VSMCs.

Another such possibility could be that oxytocin stimulates phosphatases and consequently inhibits the phosphorylation of ERK1/2, PI3K p110 α , and Akt. This can be explored in future research endeavors.

Also, the present study employed in vitro experimental models. All the study experiments were performed on primary aortic VSMCs isolated from Wistar

rats and SHRs. This study has generated sufficient scientific evidence to warrant the use of in vivo experimental models in the future. And, based on the results of a future in vivo study, the next step would be to conduct a pilot clinical trial as oxytocin is a peptide that has been approved by the food and drug administration (FDA), and has been subjected to various clinical trials.

As demonstrated by the study outcomes, this research highlighted the significant vascular protective effects of oxytocin and its antagonistic actions on Ang II effects in VSMCs isolated from both normotensive and hypertensive rats. These findings suggest that oxytocin may have therapeutic implication in conditions where hyper-activation of Ang II leads to hyper-signaling of Ang II-mediated signaling pathways resulting in the development and progression of various CVD, including hypertension. However, future in vivo studies and clinical studies would be required to explore the protective effect of oxytocin in CVD.

Bibliography

1. Dale HH. On some physiological actions of ergot. *J Physiol*. 1906;34(3):163-206.
2. McCarthy MM, Altemus M. Central nervous system actions of oxytocin and modulation of behavior in humans. *Mol Med Today*. 1997;3(6):269-275.
3. Gimpl G, Fahrenholz F. The oxytocin receptor system: structure, function, and regulation. *Physiol Rev*. 2001;81(2):629-683.
4. Ivell R, Richter D. Structure and comparison of the oxytocin and vasopressin genes from rat. *Proc Natl Acad Sci U S A*. 1984;81(7):2006-2010.
5. Fanelli F, Barbier P, Zanchetta D, de Benedetti PG, Chini B. Activation mechanism of human oxytocin receptor: a combined study of experimental and computer-simulated mutagenesis. *Mol Pharmacol*. 1999;56:214-225.
6. Hoare S, Copland JA, Strakova Z, et al. The proximal portion of the COOH terminus of the oxytocin receptor is required for coupling to Gq, but Not Gi: Independent mechanisms for elevating intracellular calcium concentrations from intracellular stores. *J Biol Chem*. 1999;274(40):28682-28689.
7. Gorewit RC, Wachs EA, Sagi R, Merrill WG. Current concepts on the role of oxytocin in milk ejection. *J Dairy Sci*. 1983;66(10):2236-2250.
8. Strathearn L, Iyengar U, Fonagy P, Kim S. Maternal oxytocin response during mother–infant interaction: Associations with adult temperament. *Horm Behav*. 2012;61(3):429-435.
9. Borrow AP, Cameron NM. The role of oxytocin in mating and pregnancy. *Horm Behav*. 2012;61(3):266-276.
10. Song XR, Zhao XH, Bai XH, Lü YH, Zhang HJ, Wang YX LR. Application of

- oxytocin antagonists in thaw embryo transfer. *Chinese J Obstet Gynecol*. 2013;48(9):667-670.
11. Lee MR, Wehring HJ, McMahon RP, et al. Relationship of plasma oxytocin levels to baseline symptoms and symptom changes during three weeks of daily oxytocin administration in people with schizophrenia. *Schizophr Res*. 2015;172(1-3):165-168.
 12. Van Ijzendoorn M, Bakermans-Kranenburg MJ. The role of oxytocin in parenting and in augmentative pharmacopsychotherapy. *Psychoneuroendocrinology*. 2015;61(453):2.
 13. Alfirevic Z, Kelly AJ, Dowswell T. Intravenous oxytocin alone for cervical ripening and induction of labour. *Cochrane Database Syst Rev*. 2009;(4):1-247.
 14. Chilimigras JL, Berman DR, Romero VC, et al. Methods of induction of labour: a systematic review. *BMC Pregnancy Childbirth*. 2011;11(84):1-19.
 15. Ferguson JN, Aldag JM, Insel TR, Young LJ. Oxytocin in the medial amygdala is essential for social recognition in the mouse. *J Neurosci*. 2001;21(20):8278-8285.
 16. Winslow JT, Insel TR. The social deficits of the oxytocin knockout mouse. *Neuropeptides*. 2002;36(2-3):221-229.
 17. Ferguson JN, Young LJ, Hearn EF, et al. Social amnesia in mice lacking the oxytocin gene. *Nat Genet*. 2000;25(3):284-288.
 18. Palgi S, Klein E, Shamay-tsoory S. The role of oxytocin in empathy in PTSD. *Psychol Trauma*. 2017;9(1):1-7.
 19. Milewski M, Goodey A, Lee D, et al. Rapid absorption of dry-powder intranasal oxytocin. *Pharm Res*. 2016;33(8):1936-1944.

20. Leng G, Ludwig M. Intranasal oxytocin: Myths and delusions. *Biol Psychiatry*. 2016;79(3):243-250.
21. Bethlehem RAI, van Honk J, Auyeung B, Baron-Cohen S. Oxytocin, brain physiology, and functional connectivity: A review of intranasal oxytocin fMRI studies. *Psychoneuroendocrinology*. 2013;38(7):962-974.
22. Gutkowska J, Jankowski M. Oxytocin revisited: Its role in cardiovascular regulation. *J Neuroendocr*. 2012;24(4):599-608.
23. Gutkowska J, Jankowski M, Antunes-Rodrigues J. The role of oxytocin in cardiovascular regulation. *Braz J Med Biol Res*. 2014;47(3):206-214.
24. Gutkowska J, Jankowski M, Mukaddam-Daher S, McCann SM. Oxytocin is a cardiovascular hormone. *Braz J Med Biol Res*. 2000;33(6):625-633.
25. Jankowski M, Wang D, Hajjar F, Mukaddam-Daher S, McCann SM, Gutkowska J. Oxytocin and its receptors are synthesized in the rat vasculature. *Proc Natl Acad Sci U S A*. 2000;97(11):6207-6211.
26. Cicutti NJ, Smyth CE, Rosaeg OP, Wilkinson M. Oxytocin receptor binding in rat and human heart. *Can J Cardiol*. 1999;15(11):1267-1273.
27. Mukaddam-Daher S, Yin Y-L, Roy J, Gutkowska J, Cardinal R. Negative inotropic and chronotropic effects of oxytocin. *Hypertension*. 2001;38(2):292-296.
28. Favaretto AL V, Ballejo GO, Albuquerque-Arajo WIC, Gutkowska J, Antunes-Rodrigues J, McCann SM. Oxytocin releases atrial natriuretic peptide from rat atria in vitro that exerts negative inotropic and chronotropic action. *Peptides*. 1997;18(9):1377-1381.
29. Gutkowska J, Jankowski M, Lambert C, Mukaddam-Daher S, Zingg HH, McCann

- SM. Oxytocin releases atrial natriuretic peptide by combining with oxytocin receptors in the heart. *Proc Natl Acad Sci U S A*. 1997;94(21):11704-11709.
30. Houshmand F, Faghihi M, Zahediasl S. Role of atrial natriuretic peptide in oxytocin induced cardioprotection. *Hear Lung Circ*. 2015;24(1):86-93.
 31. Goetz K. Physiology and pathophysiology of atrial peptides. *Am J Physiol*. 1988;254(1):E1-E15.
 32. Danalache BA, Paquin J, Donghao W, Grygorczyk R, Moore JC, Mummery et al CL. Nitric oxide signaling in oxytocin-mediated cardiomyogenesis. *Stem Cells*. 2007;25(3):679-688.
 33. Uchida S, Fuke S, Tsukahara T. Upregulations of gata4 and oxytocin receptor are important in cardiomyocyte differentiation processes of P19CL6 cells. *J Cell Biochem*. 2007;100(3):629-641.
 34. Szeto A, Nation DA, Mendez AJ, Dominguez-Bendala J, Brooks LG, Schneiderman et al N. Oxytocin attenuates NADPH-dependent superoxide activity and IL-6 secretion in macrophages and vascular cells. *Am J Physiol Endocrinol Metab*. 2008;295(6):1495-1501.
 35. Risk M. Diabetes and Cardiovascular Disease. *Circulation*. 2013;36:1-19.
 36. Heeschen C, Lehmann R, Honold J, et al. Profoundly reduced neovascularization capacity of bone marrow mononuclear cells derived from patients with chronic ischemic heart disease. *Circulation*. 2004;109(13):1615-1622.
 37. Fadini GP, Sartore S, Schiavon M, et al. Diabetes impairs progenitor cell mobilisation after hindlimb ischaemia-reperfusion injury in rats. *Diabetologia*. 2006;49(12):3075-3084.

38. Kim YS, Kwon JS, Hong MH, et al. Restoration of angiogenic capacity of diabetes-insulted mesenchymal stem cells by oxytocin. *BMC Cell Biol.* 2013;14(1):38.
39. Cattaneo M, Chini B, Vicentini L. Oxytocin stimulates migration and invasion in human endothelial cells. *Br J Pharmacol.* 2008;153(4):728-736.
40. Cassoni P, Marrocco T, Bussolati B, Allia E, Munaron L, Sapino et al A. Oxytocin induces proliferation and migration in immortalized human dermal microvascular endothelial cells and human breast tumor-derived endothelial cells. *Mol Cancer Res.* 2006;4(6):351-360.
41. Cattaneo MG, Lucci G, Vicentini LM. Oxytocin stimulates in vitro angiogenesis via a Pyk-2/Src-dependent mechanism. *Exp Cell Res.* 2009;315(18):3210-3219.
42. Rzuclidlo EM, Martin KA, Powell RJ. Regulation of vascular smooth muscle cell differentiation. *J Vasc Surg.* 2007;45(6):25-32.
43. Hamblin M, Chang L, Fan Y, Zhang J, Chen YE. PPARs and the cardiovascular system. *Antioxid Redox Signal.* 2009;11(6):1415-1452.
44. Insull W. The pathology of atherosclerosis: Plaque development and plaque responses to medical treatment. *Am J Med.* 2009;122(1):3-14.
45. Ahmed MA, Elosaily GM. Role of oxytocin in deceleration of early atherosclerotic inflammatory processes in adult male rats. *Int J Clin Exp Med.* 2011;4(3):169-178.
46. Kobayashi H, Yasuda S, Bao N, et al. Postinfarct treatment with oxytocin improves cardiac function and remodeling via activating cell-survival signals and angiogenesis. *J Cardiovasc Pharmacol.* 2009;54(6):510-519.
47. Al-Amaran FF, Shahkolahi M. Oxytocin ameliorates the immediate myocardial injury in heart transplant through down regulation of the neutrophil dependent

- myocardial apoptosis. *Hear Views*. 2014;15(2):37-45.
48. Bulut EC, Abueid L, Ercan F, Süleymanoğlu S, Ağırbaşı M, Yeğen BÇ. Treatment with oestrogen-receptor agonists or oxytocin in conjunction with exercise protects against myocardial infarction in ovariectomized rats. *Exp Physiol*. 2016;101(5):612-627.
 49. Dostal DE, Baker KM. The cardiac renin-angiotensin system conceptual, or a regulator of cardiac function? *Circ Res*. 1999;85(7):643-650.
 50. Baker KM, Booz GW, Dostal DE. Cardiac actions of angiotensin II: Role of an intracardiac renin-angiotensin system. *Ann Rev Physiol*. 1992;54(39):227-241.
 51. Mehta PK, Griendling KK. Angiotensin II cell signaling: Physiological and pathological effects in the cardiovascular system. *Am J Physiol Cell Physiol*. 2007;292(1):82-97.
 52. Chiu WC, Juang JM, Chang SN, et al. Angiotensin II regulates the LARG/RhoA/MYPT1 axis in rat vascular smooth muscle in vitro. *Acta Pharmacol Sin*. 2012;33(12):1502-1510.
 53. Ferrario CM. Role of angiotensin II in cardiovascular disease — Therapeutic implications of more than a century of research. *JRAAS*. 2006;7(1):3-14.
 54. Olson ER, Shamhart PE, Naugle JE, Meszaros JG. Angiotensin II-induced extracellular signal-regulated kinase 1/2 activation is mediated by protein kinase C δ and intracellular calcium in adult rat cardiac fibroblasts. *Hypertension*. 2008;51(3):704-711.
 55. Touyz RM, Deng L-Y, He G, Wu X-H, Schiffrin EL. Angiotensin II stimulates DNA and protein synthesis in vascular smooth muscle cells from human arteries: role of

- extracellular signal-regulated kinases. *J Hypertens.* 1999;17(7):907-916.
56. Mugabe BE, Yaghini FA, Song CY, Buharalioglu CK, Waters CM, Malik KU. Angiotensin II-induced migration of vascular smooth muscle cells is mediated by p38 mitogen-activated protein kinase-activated c-Src through spleen tyrosine kinase and epidermal growth factor receptor transactivation. *J Pharmacol Exp Ther.* 2010;332(1):116-124.
 57. Yue H, Li W, Desnoyer R, Karnik SS. Role of nuclear unphosphorylated STAT3 in angiotensin II type 1 receptor-induced cardiac hypertrophy. *Cardiovasc Res.* 2010;85(1):90-99.
 58. Chen D, Liu J, Rui B, et al. GSTpi protects against angiotensin II-induced proliferation and migration of vascular smooth muscle cells by preventing signal transducer and activator of transcription 3 activation. *Biochim Biophys Acta Mol Cell Res.* 2014;1843(2):454-463.
 59. Touyz RM. Reactive oxygen species and angiotensin II signaling in vascular cells - Implications in cardiovascular disease. *Braz J Med Biol Res.* 2004;37(8):1263-1273.
 60. Jian Cheng Z, Vapaatalo H, Mervaala E, Professor A. Angiotensin II and vascular inflammation. *Med Sci Monit.* 2005;11(6):194-205.
 61. Sironi L, Calvio AM, Arnaboldi L, et al. Effect of valsartan on angiotensin II – induced plasminogen muscle cells. *Hypertension.* 2001;37(3):961-966.
 62. Stefansson S, Lawrence D a. The serpin PAI-1 inhibits cell migration by blocking integrin alpha V beta 3 binding to vitronectin. *Nature.* 1996;383(6599):441-443.
 63. Heymans S, Luttun a, Nuyens D, et al. Inhibition of plasminogen activators or matrix metalloproteinases prevents cardiac rupture but impairs therapeutic

- angiogenesis and causes cardiac failure. *Nat Med.* 1999;5(10):1135-1142.
64. Ridker PM, Gaboury CL, Conlin PR, Seely EW, Williams GH, Vaughan DE. Stimulation of plasminogen activator inhibitor in vivo by infusion of angiotensin II. Evidence of a potential interaction between the renin- angiotensin system and fibrinolytic function. *Circulation.* 1993;87(6):1969-1973.
 65. Nakamura S, Nakamura I, Ma L, Vaughan DE, Fogo AB. Plasminogen activator inhibitor-1 expression is regulated by the angiotensin type 1 receptor in vivo. *Kidney Int.* 2000;58(1):251-259.
 66. van Leeuwen RT, Kol A, Andreotti F, Kluft C, Maseri A, Sperti G. Angiotensin II increases plasminogen activator inhibitor type 1 and tissue-type plasminogen activator messenger RNA in cultured rat aortic smooth muscle cells. *Circulation.* 1994;90(1):362-368.
 67. Simon DI, Simon NM. Plasminogen activator inhibitor-1: A novel therapeutic target for hypertension? *Circulation.* 2013;128(21):2286-2288.
 68. Afonso LC, Edelson GW, Sowers JR. Metabolic abnormalities in hypertension. *Curr Opin Nephrol Hypertens.* 1997;6(3):219-223.
 69. Sweitzer NK. What is an angiotensin converting enzyme inhibitor? *Circulation.* 2003;108(3):e16-e18.
 70. Macdougall IC. The role of ACE inhibitors and angiotensin II receptor blockers in the response to epoetin. *Nephrol Dial Transplant.* 1999;14(8):1836-1841.
 71. Michel JB, Li Z, Lacolley P. Smooth muscle cells and vascular diseases. *Cardiovasc Res.* 2012;95(2):135-137.
 72. Touyz RM, Alves-Lopes R, Rios FJ, et al. Vascular smooth muscle contraction in

- hypertension. *Cardiovasc Res.* 2018;114(4):529-539.
73. Timpl R. Macromolecular organization of basement membranes. *Curr Opin Cell Biol.* 1996;8(5):618-624.
74. Hedin U, Roy J, Tran PK. Control of smooth muscle cell proliferation in vascular disease. *Curr Opin Lipidol.* 2004;15(5):559-565.
75. Zhang MJ, Zhou Y, Chen L, et al. An overview of potential molecular mechanisms involved in VSMC phenotypic modulation. *Histochem Cell Biol.* 2016;145(2):119-130.
76. Fukata Y, Kaibuchi K, Amano M, Kaibuchi K. Rho-Rho-kinase pathway in smooth muscle contraction and cytoskeletal reorganization of non-muscle cells. *Trends Pharmacol Sci.* 2001;22(1):32-39.
77. Thyberg J. Phenotypic modulation of smooth muscle cells during formation of neointimal thickenings following vascular injury. *Histol Histopathol.* 1998;13(3):871-891.
78. Touyz RM, Briones AM. Reactive oxygen species and vascular biology: Implications in human hypertension. *Hypertens Res.* 2011;34(1):5-14.
79. Brown DI, Griendling KK, Mášová-chrastinová L, et al. Nox proteins in signal transduction. 2010;47(12):1707-1714.
80. Rivard A, Andres V. Vascular smooth muscle cell proliferation in the pathogenesis of atherosclerotic cardiovascular diseases. *Histol Histopathol.* 2000;15(2):557-571.
81. Xu S, Fu J, Chen J, Xiao P, Lan T, Le et al K. Development of an optimized protocol for primary culture of smooth muscle cells from rat thoracic aortas. *Cytotechnology.* 2009;61(1):65-72.

82. Desk R, Williams L, Health K. Signal Transduction of Mechanical Stresses in the Vascular Wall. *Hypertension*. 1998;32:338-345.
83. Seger R, Krebs EG. The MAPK signaling cascade. *FASEB*. 1995;9(9):726-735.
84. Rose BA, Force T, Wang Y. Mitogen-activated protein kinase signaling in the heart: Angels versus demons in a heart-breaking tale. *Physiol Rev*. 2013;90(4):1-63.
85. Reusch HP, Chan G, Ives HE, Nemenoff RA. Activation of JNK / SAPK and ERK by mechanical strain in vascular smooth muscle cells depends on extracellular matrix composition. *Arter Thromb Vasc Biol*. 1997;244(237):239-244.
86. Bogoyevitch MA, Ngoei KRW, Zhao TT, Yeap YYC, Ng DCH. c-Jun N-terminal kinase (JNK) signaling: recent advances and challenges. *Biochim Biophys Acta*. 2010;1804(3):463-475.
87. Dhanasekaran DN, Reddy EP. JNK signaling in apoptosis. *Oncogene*. 2008;27(48):6245-6251.
88. Johnson GL. The c-Jun kinase/stress-activated pathway: Regulation, function and role in human disease. *Biochim Biophys Acta*. 2007;1773(8):1341-1348.
89. Ohanian J, Cunliffe P, Ceppi E, Alder A, Heerkens E, Ohanian V. Activation of p38 mitogen-activated protein kinases by endothelin and noradrenaline in small arteries, regulation by calcium influx and tyrosine kinases, and their role in contraction. *Arterioscler Thromb Vasc Biol*. 2001;21(12):1921-1927.
90. Feoktistov I, Goldstein AE, Biaggioni I. Role of p38 mitogen-activated protein kinase and extracellular signal-regulated protein kinase kinase in adenosine A2B receptor-mediated interleukin-8 production in human mast cells. *Mol Pharmacol*. 1999;55(4):726-734.

91. Plataniias LC. The p38 mitogen-activated protein kinase pathway and its role in interferon signaling. *Pharmacol Ther.* 2003;98(2):129-142.
92. Sharma G, He J, Bazan HEP. p38 and ERK1/2 coordinate cellular migration and proliferation in epithelial wound healing: Evidence of cross-talk activation between MAP kinase cascades. *J Bio Chem.* 2003;278(24):21989-21997.
93. Roskoski R. ERK1/2 MAP kinases: Structure, function, and regulation. *Pharmacol Res.* 2012;66(2):105-143.
94. Zhu L, Gao P, Jin X, Fang N, Liu Z, Wang H. Differential ERK1/2 signaling and hypertrophic response to endothelin-1 in cardiomyocytes from SHR and Wistar-Kyoto rats: A potential target for combination therapy of hypertension. *Curr Vasc Pharmacol.* 2015;13(4):467-474.
95. Watson MH, Venance SL, Pang SC, Mak AS. Smooth muscle cell proliferation. Expression and kinase activities of p34cdc2 and mitogen-activated protein kinase homologues. *Circ Res.* 1993;73(1):109-117.
96. Hedges JC, Oxhorn BC, Carty M, et al. Phosphorylation of caldesmon by ERK MAP kinases in smooth muscle. *Am J Physiol Cell Physiol.* 2000;278(4):718-726.
97. For E, Switching AC. Role of caldesmon in the calcium regulation of smooth muscle thin filaments. Evidence for a cooperative switching mechanism. *J Bio Chem.* 2008;283(1):47-56.
98. Roberts RE. The extracellular signal-regulated kinase (ERK) pathway: A potential therapeutic target in hypertension. *J Exp Pharmacol.* 2012;4:77-83.
99. Muslin AJ. MAPK signalling in cardiovascular health and disease: Molecular mechanisms and therapeutic targets. *Clin Sci.* 2008;115(7):203-218.

100. Touyz RM, Deschepper C, Park JB, et al. Inhibition of mitogen-activated protein/extracellular signal-regulated kinase improves endothelial function and attenuates Ang II-induced contractility of mesenteric resistance arteries from spontaneously hypertensive rats. *J Hypertens*. 2002;20(6):1127-1134.
101. Babu G. Phosphorylation of Elk-1 by MEK / ERK pathway is necessary for c-fos gene activation during cardiac myocyte hypertrophy. *J Mol Cell Cardiol*. 2000;32(8):1447-1457.
102. Gonzalez-reyes A, Menaouar A, Yip D, Danalache B. Molecular mechanisms underlying oxytocin-induced cardiomyocyte protection from simulated ischemia–reperfusion. *Mol Cell Endocrinol*. 2015;412:170-181.
103. Rimoldi V, Reversi A, Taverna E, et al. Oxytocin receptor elicits different EGFR/MAPK activation patterns depending on its localization in caveolin-1 enriched domains. *Oncogene*. 2003;22(38):6054-6060.
104. Allen JA, Yu JZ, Dave RH, Bhatnagar A, Roth BL, Rasenick MM. Caveolin-1 and lipid microdomains regulate Gs trafficking and attenuate Gs/adenylyl cyclase signaling. *Mol Pharmacol*. 2009;76(5):1082-1093.
105. Morello F, Perino A, Hirsch E. Phosphoinositide 3-kinase signalling in the vascular system. *Cardiovasc Res*. 2009;82(2):261-271.
106. Li F, Li L, Pan W, et al. PI3K/Akt signaling transduction pathway is involved in rat vascular smooth muscle cell proliferation induced by apelin-13. *Acta Biochim Biophys Sin*. 2010;42(6):396-402.
107. Carnevale D, Lembo G. PI3K γ in hypertension: A novel therapeutic target controlling vascular myogenic tone and target organ damage. *Cardiovasc Res*.

- 2012;95(4):403-408.
108. Perrotta M, Lembo G, Carnevale D. The multifaceted roles of PI3K γ in hypertension, vascular biology, and inflammation. *Int J Mol Sci.* 2016;17(11):1-9.
 109. Ghigo A, Laffargue M, Li M, Hirsch E. PI3K and calcium signaling in cardiovascular disease. *Circ Res.* 2017;121(3):282-292.
 110. Aoyagi T, Matsui T. Phosphoinositide-3 kinase signaling in cardiac hypertrophy and heart failure. *Curr Pharm Des.* 2011;17(18):1818-1824.
 111. Cantley LC. The phosphoinositide 3-kinase pathway. *Science.* 2002;296(5573):1655-1657.
 112. Jean S, Kiger AA. Classes of phosphoinositide 3-kinases at a glance. *J Cell Sci.* 2014;127(5):923-928.
 113. Wymann MP, Pirola L. Structure and function of phosphoinositide 3-kinases. *Biochim Biophys Acta.* 1998;1436(1-2):127-150.
 114. Lupieri A, Smirnova N, Malet N, Gayral S, Laffargue M. PI3K signaling in arterial diseases: Non redundant functions of the PI3K isoforms. *Adv Bio Reg.* 2015;59:4-18.
 115. Oudit GY, Penninger JM. Cardiac regulation by phosphoinositide 3-kinases and PTEN. *Cardiovasc Res.* 2009;82(2):250-260.
 116. Hanada M, Feng J, Hemmings BA. Structure, regulation and function of PKB/AKT - A major therapeutic target. *Biochim Biophys Acta.* 2004;1697(1-2):3-16.
 117. Coffey P, Jin J, Woodgett J. Protein kinase B (c-Akt): A multifunctional mediator of phosphatidylinositol 3-kinase activation. *Biochem J.* 1998;335(1):1-13.
 118. Manning BD, Toker A. AKT/PKB signaling: Navigating the network. *Cell.*

- 2017;169(3):381-405.
119. Oudit GY, Sun H, Kerfant BG, Crackower MA, Penninger JM, Backx PH. The role of phosphoinositide-3 kinase and PTEN in cardiovascular physiology and disease. *J Mol Cell Cardiol.* 2004;37(2):449-471.
 120. Le Blanc C, Mironneau C, Barbot C, et al. Regulation of vascular L-type calcium channels by phosphatidylinositol 3,4,5-trisphosphate. *Cir Res.* 2004;95(3):300-307.
 121. Hirsch E, Patrucco E, Vecchione C, et al. Protection from angiotensin II-mediated vasculotoxic and hypertensive response in mice lacking PI3K γ . *J Exp Med.* 2005;201(8):1217-1228.
 122. Nayler WG. Calcium channels and their involvement in cardiovascular disease. *Biochem Pharmacol.* 1992;43(1):39-46.
 123. Fisher M, Grotta J. New uses for calcium channel blockers: Therapeutic implications. *Drugs.* 1993;46(6):961-962.
 124. Garat C, Crossno J, Sullivan T, Reusch J, Klemm D. Inhibition of phosphatidylinositol 3-kinase/Akt signaling attenuates hypoxia-induced pulmonary artery remodeling and suppresses CREB depletion in arterial smooth muscle cells. *J Cardiovasc Pharmacol.* 2013;62(6):539-548.
 125. Carnevale D, Vecchione C, Mascio G, et al. PI3K γ inhibition reduces blood pressure by a vasorelaxant Akt / L-type calcium channel mechanism. *Cardiovasc Res.* 2012;93:200-209.
 126. Bernardo BC, Weeks KL, Pretorius L, McMullen JR. Molecular distinction between physiological and pathological cardiac hypertrophy : Experimental findings and therapeutic strategies. *Pharmacol Ther.* 2010;128(1):191-227.

127. Kemi OJ, Ellingsen Ø. Physiological cardiac hypertrophy. In: Mooren FC, ed. *Encyclopedia of Exercise Medicine in Health and Disease*. Berlin, Heidelberg: Springer Berlin Heidelberg; 2012:171-175.
128. Heineke J, Molkentin JD. Regulation of cardiac hypertrophy by intracellular signalling pathways. *Nat Rev Mol Cell Biol*. 2006;7(8):589-600.
129. McMullen J, Jennings G. Differences between pathological and physiological cardiac hypertrophy: Novel therapeutic strategies to treat heart failure. *Clin Exp Pharmacol Physiol*. 2007;34(4):255-262.
130. McMullen JR, Shioi T, Huang W, et al. The insulin-like growth factor 1 receptor induces physiological heart growth via the phosphoinositide 3-kinase (p110 α) pathway. *J Biol Chem*. 2004;279(6):4782-4793.
131. Debusch B, Treskov I, Lupu TS, et al. Akt1 is required for physiological cardiac growth. *Circulation*. 2006;113(17):2097-2104.
132. McMullen JR, Shioi T, Zhang L, et al. Phosphoinositide 3-kinase (p110 α) plays a critical role for the induction of physiological, but not pathological, cardiac hypertrophy. *Proc Natl Acad Sci U S A*. 2003;100(21):12355-12360.
133. Oudit GY, Crackower MA, Eriksson U, et al. Phosphoinositide 3-kinase gamma-deficient mice are protected from isoproterenol-induced heart failure. *Circulation*. 2003;108(17):2147-2152.
134. Matsui T, Amano M, Yamamoto T, et al. Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP binding protein Rho. *EMBO J*. 1996;15(9):2208-2216.
135. Kimura K, Eguchi S. Angiotensin II type-1 receptor regulates RhoA and Rho-

- kinase/ROCK activation via multiple mechanisms. Focus on — angiotensin II induces RhoA activation through SHP2-dependent dephosphorylation of the RhoGAP p190A in vascular smooth muscle cells. *Am J Physiol Cell Physiol.* 2009;297(5):C1059-C1061.
136. Leung T, Chen X, Manser E, Lim L. The p160 RhoA-binding kinase ROK α is a member of a kinase family and is involved in the reorganization of the cytoskeleton. *Mol Cell Biol.* 1996;19:5513-5327.
137. Maekawa M, Ishizaki T, Boku S, et al. Signaling from Rho to the actin cytoskeleton through protein kinases ROCK and LIM-kinase. *Science.* 1999;285(5429):895-898.
138. Etienne-manneville S. Rho GTPases in cell biology. *Nature.* 2002;420:629-636.
139. Ridley AJ. Rho GTPases and cell migration. *J Cell Sci.* 2001;114:2713-2722.
140. Mukai Y, Shimokawa H, Matoba T, et al. Involvement of Rho-kinase in hypertensive vascular disease: A novel therapeutic target in hypertension. *FASEB J.* 2001;15(6):1062-1064.
141. Matsumoto Y, Uwatoku T, Oi K, et al. Long-term inhibition of Rho-kinase suppresses neointimal formation after stent implantation in porcine coronary arteries: Involvement of multiple mechanisms. *Arter Thromb Vasc Biol.* 2004;24(1):181-186.
142. Masumoto A, Hirooka Y, Shimokawa H, Hironaga K, Setoguchi S, Takeshita A. Possible involvement of Rho-kinase in the pathogenesis of hypertension in humans. *Hypertension.* 2001;38(6):1307-1310.
143. Maruhashi T, Noma K, Fujimura N, et al. Exogenous nitric oxide inhibits Rho-associated kinase activity in patients with angina pectoris: A randomized controlled

- trial. *Hypertens Res.* 2015;38(7):485-490.
144. Hartmann S, Ridley AJ, Lutz S. The function of Rho-associated kinases ROCK-1 and ROCK-2 in the pathogenesis of cardiovascular disease. *Front Pharmacol.* 2015;6(276):1-15.
 145. Matsui T, Maeda M, Doi Y, et al. Rho-kinase phosphorylates COOH-terminal threonines of ezrin/radixin/moesin (ERM) proteins and regulates their head-to-tail association. *J Cell Biol.* 1998;140(3):647-657.
 146. Julian L, Olson MF. Rho-associated coiled-coil containing kinases (ROCK): structure, regulation, and functions. *Small GTPases.* 2014;5:e29846.
 147. Jacobs M, Hayakawa K, Swenson L, et al. The structure of dimeric ROCK I reveals the mechanism for ligand selectivity. *J Biol Chem.* 2006;281(1):260-268.
 148. Yamaguchi H, Kasa M, Amano M, Kaibuchi K, Hakoshima T. Molecular mechanism for the regulation of rho-kinase by dimerization and its inhibition by fasudil. *Structure.* 2006;14(3):589-600.
 149. Rahman A, Davis B, Cecilia L, et al. The small GTPase Rac1 is required for smooth muscle contraction. *J Physiol.* 2014;592(5):915-926.
 150. Seko T, Ito M, Kureishi Y, et al. Activation of RhoA and inhibition of myosin phosphatase as important components in hypertension in vascular smooth muscle. *Circ Res.* 2003;92(4):411-418.
 151. Behuliak M, Bencze M, Van I, Kuneš J, Zicha J. Basal and activated calcium sensitization mediated by RhoA/Rho kinase pathway in rats with genetic and salt hypertension. *Biomed Res Int.* 2017;2017:1-13.
 152. Relation T, Vessel B, Stiffness W, Huveneers S, Daemen MJAP, Hordijk PL.

- Between Rho(k) and a hard place: The relation between vessel wall stiffness, endothelial contractility, and cardiovascular disease. *Circ Res.* 2015;116(5):895-908.
153. Crestani S, Webb RC, Silva-santos JE. High-salt intake augments the activity of the RhoA/ROCK pathway and reduces intracellular calcium in arteries from rats. *Am J Hypertens.* 2017;30(4):389-399.
154. Komers R, Oyama TT, Anderson S. Effects of systemic inhibition of Rho kinase on blood pressure and renal haemodynamics in. *Br J Pharmacol.* 2011;162(1):163-174.
155. Scalbert E, Bril A, Lockhart B, et al. RhoA guanine exchange factor expression profile in arteries: Evidence for a Rho kinase-dependent negative feedback in angiotensin II-dependent hypertension. *Am J Physiol Cell Physiol.* 2012;302(9):1394-1404.
156. Zhou Q, Wei SS, Wang H, et al. Crucial role of ROCK2-mediated phosphorylation and upregulation of FHOD3 in the pathogenesis of angiotensin II-induced cardiac hypertrophy. *Hypertension.* 2017;69(6):1070-1083.
157. Carbone ML, Brégeon J, Devos N, et al. Angiotensin II activates the RhoA exchange factor Arhgef1 in humans. *Hypertension.* 2015;65(6):1273-1278.
158. Chang J, Xie M, Shah VR, et al. Activation of Rho-associated coiled-coil protein kinase 1 (ROCK-1) by caspase-3 cleavage plays an essential role in cardiac myocyte apoptosis. *Proc Natl Acad Sci U S A.* 2006;103(39):14495-14500.
159. Yang X, Li Q, Lin X, et al. Mechanism of fibrotic cardiomyopathy in mice expressing truncated Rho-associated coiled-coil protein kinase 1. *FASEB J.* 2012;26(5):2105–2116.

160. Davies S, Reddy H, Caivano M, Cohen P. Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J*. 2000;351:95-105.
161. Shi J, Wei L. Rho kinases in cardiovascular physiology and pathophysiology: The effect of fasudil. *J Cardiovasc Pharmacol*. 2013;62(4):341-354.
162. Masumoto A, Mohri M, Shimokawa H, Urakami L, Usui M, Takeshita A. Suppression of coronary artery spasm by the Rho-kinase inhibitor fasudil in patients with vasospastic angina. *Circulation*. 2002;105(13):1545-1547.
163. Fukumoto Y, Matoba T, Ito A, et al. Acute vasodilator effects of a Rho-kinase inhibitor, fasudil, in patients with severe pulmonary hypertension. *Heart*. 2005;91(3):391-392.
164. Shimokawa H, Hiramori K, Iinuma H, et al. Anti-anginal effect of fasudil, a Rho-kinase inhibitor, in patients with stable effort angina: A multicenter study. *J Cardiovasc Pharmacol*. 2002;40(5):751-761.
165. Zhang P, Chen H, Meng L, et al. Acute effects of Rho-kinase inhibitor fasudil on pulmonary arterial hypertension in patients with congenital heart defects. *Circ J*. 2015;79(6):1342-1348.
166. Chang R, Liu B, Wu J, et al. The level of ROCK1 and ROCK2 in patients with pulmonary hypertension in plateau area. *Sci Rep*. 2018;8(1):1-8.
167. Ishikura K, Yamada N, Ito M, et al. Beneficial acute effects of rho-kinase inhibitor in patients with pulmonary arterial hypertension. *Circ Res*. 2006;70(2):174-178.
168. Kaibuchi K, Matsumoto Y, Abe K, et al. Long-term treatment with a Rho-kinase inhibitor improves monocrotaline-induced fatal pulmonary hypertension in rats. *Circ Res*. 2003;94(3):385-393.

169. Dong M, Liao JK, Fang F, et al. Increased Rho kinase activity in congestive heart failure. *Eur J Hear Fail.* 2012;14(9):965-973.
170. Ocaranza MP, Gabrielli L, Mora I, et al. Markedly increased Rho-kinase activity in circulating leukocytes in patients with chronic heart failure. *Am Hear J.* 2011;161(5):931-937.
171. Ito K, Masumoto A, Inokuchi K, et al. Rho-Kinase inhibitor improves increased vascular resistance and impaired vasodilation of the forearm in patients with heart failure. *Circulation.* 2005;111(21):2741-2747.
172. Morales-Montor J. The role of the pleiotropic cytokine interleukin – 6 (IL-6) during disease. *Mod Asp Immunobiol.* 2005;16:21-26.
173. Anda TK, Akahashi TT. Interleukin-6 and cardiovascular diseases. *Jpn Hear J.* 2004;45(2):183-193.
174. Hirano T, Yasukawa K, Harada H, et al. Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. *Nature.* 1986;324(6092):73-76.
175. Lotz M, Jirik F, Kabouridis P, et al. B cell stimulating factor 2/interleukin 6 is a co-stimulant for human thymocytes and T lymphocytes. *J Exp Med.* 1988;167(3):1253-1258.
176. Gauldie J, Richards C, Harnish D, Lansdorp P, Baumann H. Interferon beta 2/B-cell stimulatory factor type 2 shares identity with monocyte-derived hepatocyte-stimulating factor and regulates the major acute phase protein response in liver cells. *Proc Natl Acad Sci U S A.* 1987;84(20):7251-7255.
177. Luger TA, Krutmann J, Kirnbauer R, et al. IFN-beta 2/IL-6 augments the activity of

- human natural killer cells. *J Immunol*. 1989;143(4):1206-1209.
178. Kaplanski G, Marin V, Montero-Julian F, Mantovani A, Farnarier C. IL-6: A regulator of the transition from neutrophil to monocyte recruitment during inflammation. *Trends Immunol*. 2003;24(1):25-29.
179. Streetz KL, Luedde T, Manns MP, Trautwein C. Interleukin 6 and liver regeneration. *Gut*. 2000;47(2):309-312.
180. Del-Giudice M, Gangestad S. Rethinking IL-6 and CRP: Why they are more than inflammatory biomarkers, and why it matters. *Brain Behav Immun*. 2018;70:61-75.
181. Verma S, Li S-H, Badiwala M V, et al. Endothelin antagonism and interleukin-6 inhibition attenuate the proatherogenic effects of C-reactive protein. *Circulation*. 2002;105(16):1890-1896.
182. Somers W, Stahl M, Seehra JS. A crystal structure of interleukin 6: Implications for a novel mode of receptor dimerization and signaling. *EMBO J*. 1997;16(5):989-997.
183. Mihara M, Hashizume M, Yoshida H, Suzuki M, Shiina M. IL-6/IL-6 receptor system and its role in physiological and pathological conditions. *Clin Sci*. 2012;122(4):143-159.
184. Kotake S, Sato K, Kim KJ, et al. Interleukin-6 and soluble interleukin-6 receptors in the synovial fluids from rheumatoid arthritis patients are responsible for osteoclast-like cell formation. *J Bone Min Res*. 1996;11(1):88-95.
185. Robak T, Gladalska A, Stepien H, Robak E. Serum levels of interleukin-6 type cytokines and soluble interleukin-6 receptor in patients with rheumatoid arthritis. *Mediat Inflamm*. 1998;7(5):347-353.
186. Nishimoto N, Sasai M, Shima Y, et al. Improvement in Castleman's disease by

- humanized anti-interleukin-6 receptor antibody therapy. *Blood*. 2000;95(1):56-61.
187. Nishimoto N, Yoshizaki K, Miyasaka N, et al. Treatment of rheumatoid arthritis with humanized anti-interleukin-6 receptor antibody: A multicenter, double-blind, placebo-controlled trial. *Arthritis Rheum*. 2004;50(6):1761-1769.
188. Bacchiega BC, Bacchiega AB, Usnayo MJG, Bedirian R, Singh G, Pinheiro G da RC. Interleukin 6 inhibition and coronary artery disease in a high-risk population: A prospective community-based clinical study. *J Am Heart Assoc*. 2017;6(3):1-9.
189. Mesri M, Altieri DC. Endothelial cell activation by leukocyte microparticles. *J Immunol*. 1998;161(8):4382-4387.
190. Loppnow H, Libby P. Proliferating or interleukin-1-activated human vascular smooth muscle cells secrete copious interleukin-6. *J Clin Invest*. 1990;85(3):731-738.
191. Gwechenberger M, Mendoza LH, Youker KA, et al. Cardiac myocytes produce interleukin-6 in culture and in viable border zone of reperfused infarctions. *Circulation*. 1999;99(4):546-551.
192. Fontes JA, Rose NR, Hopkins J. The varying faces of IL-6: From cardiac protection to cardiac failure. *Cytokine*. 2015;74(1):62-68.
193. Wollert KC, Taga T, Saito M, et al. Cardiotrophin-1 activates a distinct form of cardiac muscle cell hypertrophy: Assembly of sarcomeric units in series VIA gp130/leukemia inhibitory factor receptor-dependent pathways. *J Biol Chem*. 1996;271(16):9535-9545.
194. Terrell AM, Crisostomo PR, Wairiuko GM, Wang M, Morrell ED, Meldrum DR. Jak/STAT/SOCS signaling circuits and associated cytokine-mediated inflammation

- and hypertrophy in the heart. *Shock*. 2006;26(3):226-234.
195. Wollert KC, Drexler H. The role of interleukin-6 in the failing heart. *Heart Fail Rev*. 2001;6(2):95-103.
 196. Yamauchi-Takahara K, Kishimoto T. Cytokines and their receptors in cardiovascular diseases — role of gp130 signalling pathway in cardiac myocyte growth and maintenance. *Int J Exp Pathol*. 2000;81(1):1-16.
 197. Yang S, Ma Y, Messina JL, et al. Mechanism of cardiac depression after trauma-hemorrhage: Increased cardiomyocyte IL-6 and effect of sex steroids on IL-6 regulation and cardiac function. *Am J Physiol Heart Circ Physiol*. 2004;287(5):2183-2191.
 198. Prabhu SD. Cytokine-induced modulation of cardiac function. *Circ Res*. 2004;95(12):1140-1153.
 199. Yu X, Kennedy RH, Liu SJ. JAK2/STAT3, not ERK1/2, mediates interleukin-6-induced activation of inducible nitric-oxide synthase and decrease in contractility of adult ventricular myocytes. *J Biol Chem*. 2003;278(18):16304-16309.
 200. van der Wal AC, Becker AE, van der Loos CM, Das PK. Site of intimal rupture or erosion of thrombosed coronary atherosclerotic plaques is characterized by an inflammatory process irrespective of the dominant plaque morphology. *Circulation*. 1994;89(1):36-44.
 201. Rajavashisth TB, Xu XP, Jovinge S, et al. Membrane type 1 matrix metalloproteinase expression in human atherosclerotic plaques: evidence for activation by proinflammatory mediators. *Circulation*. 1999;99(24):3103-3109.
 202. Biasucci LM, Liuzzo G, Fantuzzi G, et al. Increasing levels of interleukin (IL)-1Ra

- and IL-6 during the first 2 days of hospitalization in unstable angina are associated with increased risk of in-hospital coronary events. *Circulation*. 1999;99(16):2079-2084.
203. Tsutamoto T, Hisanaga T, Wada A, et al. Interleukin-6 spillover in the peripheral circulation increases with the severity of heart failure, and the high plasma level of interleukin-6 is an important prognostic predictor in patients with congestive heart failure. *J Am Coll Cardiol*. 1998;31(2):391-398.
204. Birks EJ, Yacoub MH. The role of nitric oxide and cytokines in heart failure. *Coron Artery Dis*. 1997;8(6):389-402.
205. Lommi J, Pulkki K, Koskinen P, et al. Haemodynamic, neuroendocrine and metabolic correlates of circulating cytokine concentrations in congestive heart failure. *Eur Hear J*. 1997;18(10):1620-1625.
206. Kubota T, Miyagishima M, Alvarez RJ, et al. Expression of proinflammatory cytokines in the failing human heart: comparison of recent-onset and end-stage congestive heart failure. *J Hear Lung Transpl*. 2000;19(9):819-824.
207. Raymond RJ, Dehmer GJ, Theoharides TC, Deliargyris EN. Elevated interleukin-6 levels in patients with asymptomatic left ventricular systolic dysfunction. *Am Hear J*. 2001;141(3):435-438.
208. Kranzhöfer R, Schmidt J, Pfeiffer CAH, Hagl S, Libby P, Kübler W. Angiotensin induces inflammatory activation of human vascular smooth muscle cells. *Arter Thromb Vasc Biol*. 1999;19(7):1623-1629.
209. Wright CJ, Dennery PA. Manipulation of gene expression by oxygen: A primer from bedside to bench. *Pediatr Res*. 2009;66(1):3-10.

210. Redza-Dutordoir M, Averill-Bates DA. Activation of apoptosis signalling pathways by reactive oxygen species. *Biochim Biophys Acta*. 2016;1863(12):2977-2992.
211. Ryter SW, Kim HP, Hoetzel A, et al. Mechanisms of cell death in oxidative stress. *Antioxid Redox Signal*. 2007;9(1):49-89.
212. Mittal M, Siddiqui MR, Tran K, Reddy SP, Malik AB. Reactive oxygen species in inflammation and tissue injury. *Antioxid Redox Signal*. 2014;20(7):1126-1167.
213. Paravicini TM, Touyz RM. NADPH oxidases, reactive oxygen species, and hypertension: Clinical implications and therapeutic possibilities. *Diabetes Care*. 2008;31(2):170-180.
214. Hafstad AD, Nabeebaccus AA, Shah AM. Novel aspects of ROS signalling in heart failure. *Basic Res Cardiol*. 2013;108(4):359.
215. Kaneto H, Katakami N, Matsuhisa M, Matsuoka T. Role of reactive oxygen species in the progression of type 2 diabetes and atherosclerosis. *Mediat Inflamm*. 2010;2010(2):1-11.
216. Panth N, Paudel KR, Parajuli K. Reactive oxygen species: A key hallmark of cardiovascular disease. *Adv Med*. 2016;2016:1-12.
217. Van Heerebeek L, Meischl C, Stoker W, Meijer CJLM, Niessen HWM, Roos D. NADPH oxidase(s): New source(s) of reactive oxygen species in the vascular system? *J Clin Pathol*. 2002;55(8):561-568.
218. Montezano AC, Burger D, Ceravolo GS, Yusuf H, Montero M, Touyz RM. Novel Nox homologues in the vasculature: Focusing on Nox4 and Nox5. *Clin Sci*. 2011;120(4):131-141.
219. Rajagopalan S, Kurz S, Munzel T, et al. Angiotensin II-mediated hypertension in

- the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. *J Clin Invest.* 1996;97(8):1916-1923.
220. Garrido AM, Griendling KK. NADPH oxidases and Angiotensin II receptor signaling. *Mol Cell Endocrinol.* 2009;302(2):148-158.
221. Loomis ED, Sullivan JC, Osmond DA, Pollock DM, Pollock JS. Endothelin mediates superoxide production and vasoconstriction through activation of NADPH oxidase and uncoupled nitric-oxide synthase in the rat aorta. *J Pharmacol Exp Ther.* 2005;315(3):1058-1064.
222. Tsai M-H, Jiang MJ. Reactive oxygen species are involved in regulating alpha1-adrenoceptor-activated vascular smooth muscle contraction. *J Biomed Sci.* 2010;17(67):1-10.
223. Ushio-Fukai M. Vascular signaling through G protein-coupled receptors: New concepts. *Curr Opin Nephrol Hypertens.* 2009;18(2):153-159.
224. Bendall JK, Douglas G, McNeill E, Channon KM, Crabtree MJ. Tetrahydrobiopterin in cardiovascular health and disease. *Antioxid Redox Signal.* 2014;20(18):3040-3077.
225. Roe ND, Ren J. Nitric oxide synthase uncoupling: A therapeutic target in cardiovascular diseases. *Vasc Pharmacol.* 2012;57(5-6):168-172.
226. Elks CM, Mariappan N, Haque M, Guggilam A, Majid DSA, Francis J. Chronic NF-kappa B blockade reduces cytosolic and mitochondrial oxidative stress and attenuates renal injury and hypertension in SHR. *Am J Physiol Ren Physiol.* 2009;296(2):298-305.

227. Siasos G, Tsigkou V, Kosmopoulos M, et al. Mitochondria and cardiovascular diseases—from pathophysiology to treatment. *Ann Transl Med.* 2018;6(12):1-22.
228. Schulz E, Gori T, Munzel T. Oxidative stress and endothelial dysfunction in hypertension. *Hypertens Res.* 2011;34(6):665-673.
229. Montezano AC, Touyz RM. Reactive oxygen species and endothelial function — role of nitric oxide synthase uncoupling and Nox family nicotinamide adenine dinucleotide phosphate oxidases. *Basic Clin Pharmacol Toxicol.* 2012;110(1):87-94.
230. Brandes RP. Triggering mitochondrial radical release: A new function for NADPH oxidases. *Hypertension.* 2005;45(5):847-848.
231. Andrukhiv A, Costa AD, West IC, Garlid KD. Opening mitoKATP increases superoxide generation from complex I of the electron transport chain. *Am J Physiol Hear Circ Physiol.* 2006;291(5):2067-2074.
232. Chan SHH, Wu KLH, Chang AYW, Tai M-H, Chan JYH. Oxidative impairment of mitochondrial electron transport chain complexes in rostral ventrolateral medulla contributes to neurogenic hypertension. *Hypertension.* 2009;53(2):217-227.
233. Wang Y, Branicky R, Noë A, Hekimi S. Superoxide dismutases: Dual roles in controlling ROS damage and regulating ROS signaling. *J Cell Biol.* 2018;217(6):1915-1928.
234. Lee MY, Griendling KK. Redox signaling, vascular function, and hypertension. *Antioxid Redox Signal.* 2008;10(6):1045-1059.
235. Torres M, Forman HJ. Redox signaling and the MAP kinase pathways. *Biofactors.* 2003;17(1-4):287-296.

236. Son Y, Cheong Y-K, Kim N-H, Chung H-T, Kang DG, Pae H-O. Mitogen-activated protein kinases and reactive oxygen species: How can ROS activate MAPK Pathways? *J Signal Transduct.* 2011;2011:1-6.
237. Finkel T. Signal transduction by reactive oxygen species. *J Cell Biol.* 2011;194(1):7-15.
238. Irani K. Oxidant signaling in vascular cell growth, death, and survival : A review of the roles of reactive oxygen species in smooth muscle and endothelial cell mitogenic and apoptotic signaling. *Circ Res.* 2000;87(3):179-183.
239. Pinto Ym, Paul M, Ganten D. Lessons from rat models of hypertension - from Goldblatt to Genetic Engineering [Review]. *Cardiovasc Res.* 1998;39(1):77-88.
240. Lin HIUYU, Lee YEET, Chan YINWAH, Tse G. Animal models for the study of primary and secondary hypertension in humans (Review). *Biomed Rep.* 2016;5(6):653-659.
241. Doggrell S a, Brown L. Rat models of hypertension, cardiac hypertrophy and failure. *Cardiovasc Res.* 1998;39:89-105.
242. Battle T, Arnal JF, Challah M, Michel JB. Selective isolation of rat aortic wall layers and their cell types in culture — application to converting enzyme activity measurement. *Tissue Cell.* 1994;26(6):943-955.
243. Cadena-Herrera D, Esparza-De Lara JE, Ramírez-Ibañez ND, et al. Validation of three viable-cell counting methods: Manual, semi-automated, and automated. *Biotechnol Rep.* 2015;7:9-16.
244. Yue H, Tanaka K, Furukawa T, Karnik SS, Li W. Thymidine phosphorylase inhibits vascular smooth muscle cell proliferation via upregulation of STAT3. *Biochim*

- Biophys Acta*. 2012;1823(8):1316-1323.
245. Riss TL, Moravec RA, Niles AL, et al. Cell viability assays. In: Sittampalam G, Coussens N, Brimacombe K, eds. *Assay Guidance Manual*. Bethesda, MD: Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2013:357-387.
246. Madhavan H. Simple Laboratory methods to measure cell proliferation using DNA synthesis property. *J Stem Cells Regen Med*. 2007;3(1):12-14.
247. Noiseux N, Borie M, Desnoyers A, Menaouar A, Stevens LM, Mansour et al S. Preconditioning of stem cells by oxytocin to improve their therapeutic potential. *Endocrinology*. 2012;153(11):5361-5372.
248. Selliah N, Eck S, Green C, et al. Flow Cytometry Method Validation Protocols. *Curr Protoc Cytom*. 2019;87(1):e53.
249. Brown M, Wittwer C. Flow cytometry: Principles and clinical applications in hematology. *Clin Chem*. 2000;46(8 II):1221-1229.
250. Wlodkowic D, Skommer J, Darzynkiewicz Z. Flow cytometry-based apoptosis detection. *Methods Mol Biol*. 2009;559:1-14.
251. Kanduc D, Mittelman A, Serpico R, et al. Cell death: Apoptosis versus necrosis. *Int J Oncol*. 2002;21(1):165-170.
252. Krysko D V, Vanden Berghe T, D'Herde K, Vandenabeele P. Apoptosis and necrosis: Detection, discrimination and phagocytosis. *Methods*. 2008;44(3):205-221.
253. Jaimes M, Inokuma M, McIntyre C, Mittar D. Detection of apoptosis using the BD Annexin V FITC assay on the BD FACSVerserTM system. *BD Biosci*. 2011;(8):1-12.

254. Beckman Coulter. *BD LSRFORTESSA X-20 - Overview BD Biosciences*. CA,USA.
255. Shah K, Maghsoudlou P. Enzyme-linked immunosorbent assay (ELISA): The basics. *Br J Hosp Med*. 2016;77(7):98-101.
256. Kong Y, Gao Y, Lan D, et al. Trans-repression of NFkappaB pathway mediated by PPARgamma improves vascular endothelium insulin resistance. *J Cell Mol Med*. 2019;23(1):216-226.
257. Eruslanov E, Kusmartsev S. Identification of ROS using oxidized DCFDA and flow-cytometry. *Methods Mol Biol*. 2010;594:57-72.
258. Popowich DA, Vavra AK, Walsh CP, et al. Regulation of reactive oxygen species by p53: Implications for nitric oxide-mediated apoptosis. *Am J Physiol Hear Circ Physiol*. 2010;298(6):2192-2200.
259. Mahmood T, Yang P-C. Western blot: Technique, theory, and trouble shooting. *N Am J Med Sci*. 2012;4(9):429-434.
260. West S. *Western Blotting Handbook and Troubleshooting Guide*. 2004th ed. Pierce Biotechnology Inc.; 2004.
261. Li X, Tong G, Zhang Y, Liu S, Jin Q, Chen et al H. Neferine inhibits angiotensin II-stimulated proliferation in vascular smooth muscle cells through heme oxygenase-1. *Acta Pharmacol Sin*. 2010;31(6):679-686.
262. Castejon AM, Zollner E, Tristano AG, Cubeddu LX. Upregulation of angiotensin II-AT1 receptors during statin withdrawal in vascular smooth muscle cells. *J Cardiovasc Dis Diagn*. 2007;50(6):708-711.
263. Silverio A, Cavallo P, Rosa R De, Galasso G. Big health data and cardiovascular diseases: A challenge for research, an opportunity for clinical care. *Front Med*.

- 2019;6(2):1-10.
264. Benjamin EJ, Muntner P, Bittencourt MS. Heart disease and stroke statistics — 2019 update: A report from the American Heart Association. *Circulation*. 2019;139(10):e56–e528.
265. Lozano R, Naghavi M, Foreman K, et al. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: A systematic analysis for the Global Burden of Disease Study 2010. *Lancet*. 2012;380(9859):2095-2128.
266. Bansilal S, Castellano JM, Fuster V. Global burden of CVD: Focus on secondary prevention of cardiovascular disease. *Int J Cardiol*. 2015;201:1-7.
267. Arnett DK, Blumenthal RS, Albert MA, et al. 2019 ACC/AHA guideline on the primary prevention of cardiovascular disease. *J Am Coll Cardiol*. 2019;3(26029):1-101.
268. Gorewit RC, Wachs EA, Sagi R, Merrill WG. Current concepts on the role of oxytocin in milk ejection. *J Dairy Sci*. 1983;66(10):2236-2250.
269. Strathearn L, Iyengar U, Fonagy P, Kim S. Maternal oxytocin response during mother-infant interaction: Associations with adult temperament. *Horm Behav*. 2012;61(3):429-435.
270. Hadrava V, Kruppa U, Russo RC, Lacourcière Y, Tremblay J, Hamet P. Vascular smooth muscle cell proliferation and its therapeutic modulation in hypertension. *Am Hear J*. 1991;122(4):1198-1203.
271. Lacolley P, Regnault V, Nicoletti A, Li Z, Michel JB. The vascular smooth muscle cell in arterial pathology: A cell that can take on multiple roles. *Cardiovasc Res*. 2012;95(2):194-204.

272. Clempus RE, Griendling KK. Reactive oxygen species signaling in vascular smooth muscle cells. *Cardiovasc Res.* 2006;71(2):216–225.
273. Dinh QN, Drummond GR, Sobey CG, Chrissobolis S. Roles of inflammation, oxidative stress, and vascular dysfunction in hypertension. *Biomed Res Int.* 2014;2014:1-11.
274. Higuchi S, Ohtsu H, Suzuki H, Shirai H, Frank GD, Eguchi S. Angiotensin II signal transduction through the AT 1 receptor: Novel insights into mechanisms and pathophysiology. *Clin Sci.* 2007;112(8):417-428.
275. Schmitz U, Berk BC. Angiotensin II signal transduction: Stimulation of multiple mitogen-activated protein kinase pathways. *Trends Endocrinol Metab.* 1997;8(7):261-266.
276. Saward L, Zahradka P. Angiotensin II activates phosphatidylinositol 3-kinase in vascular smooth muscle cells. *Circ Res.* 1997;81(2):249-257.
277. Dugourd C, Gervais M, Corvol P, Monnot C. Akt is a major downstream target of PI3-kinase involved in angiotensin II-induced proliferation. *Hypertension.* 2003;41(4):882-890.
278. Bregeon J, Loirand G, Pacaud P, Rolli-Derkinderen M. Angiotensin II induces RhoA activation through SHP2-dependent dephosphorylation of the RhoGAP p190A in vascular smooth muscle cells. *Am J Physiol Physiol.* 2009;297(5):C1062-C1070.
279. Liu L, Yao F, Lu G, et al. The Role of the Rho / ROCK Pathway in ang II and TGF- β 1-induced atrial remodeling. *PLoS One.* 2016;11(9):1-16.
280. Cheng Z, Vapaatalo H, Mervaala E. Angiotensin II and vascular inflammation. *Med*

- Sci Monit.* 2005;11(6):194-205.
281. Lerman B, Harricharran T, Ogunwobi OO. Oxytocin and cancer: An emerging link. *World J Clin Oncol.* 2018;9(5):74-82.
282. Whittington K, Connors B, King K, Assinder S, Hogarth K, Nicholson H. The effect of oxytocin on cell proliferation in the human prostate is modulated by gonadal steroids: implications for benign prostatic hyperplasia and carcinoma of the prostate. *Prostate.* 2007;67(10):1132-1142.
283. Morita T, Shibata K, Kikkawa F, Kajiyama H, Ino K, Mizutani S. Oxytocin inhibits the progression of human ovarian carcinoma cells in vitro and in vivo. *Int J Cancer.* 2004;109(4):525-532.
284. Petersson M. Opposite effects of oxytocin on proliferation of osteosarcoma cell lines. *Regul Pept.* 2008;150(1-3):50-54.
285. Kurdi M, Booz GW. New take on the role of angiotensin II in cardiac hypertrophy and fibrosis. *Hypertension.* 2011;57(6):1034-1038.
286. Zafar R. An insight into pathogenesis of cardiovascular diseases. *J Cardiovasc Dis Diagn.* 2015;3(3):1-7.
287. Torres VA. Caveolin-1 controls cell proliferation and cell death by suppressing expression of the inhibitor of apoptosis protein survivin. *J Cell Sci.* 2006;119(9):1812-1823.
288. Devost D, Carrier ME, Zingg HH. Oxytocin-induced activation of eukaryotic elongation factor 2 in myometrial cells is mediated by protein kinase C. *Endocrinology.* 2008;149(1):131-138.
289. Kaul G, Pattan G, Rafeequi T. Eukaryotic elongation factor-2 (eEF2): Its regulation

- and peptide chain elongation. *Cell Biochem Funct.* 2011;29(3):227-234.
290. Zhang W, Elimban V, Nijjar MS. Role of mitogen-activated protein kinase in cardiac hypertrophy and heart failure. *Exp Clin Cardiol.* 2003;8(4):173-183.
291. Wang Y. Mitogen-activated protein kinases in heart development and diseases. *Circulation.* 2007;116:1413-1423.
292. Klein BY, Tamir H, Welch MG. PI3K/Akt responses to oxytocin stimulation in Caco2BBB gut cells. *J Cell Biochem.* 2011;112(11):3216-3226.
293. Madhok R, Crilly A, Watson J, Capell HA. Serum interleukin 6 levels in rheumatoid arthritis: Correlations with clinical and laboratory indices of disease activity. *Ann Rheum Dis.* 1993;52(3):232-234.
294. Wainstein M V., Mossmann M, Araujo GN, et al. Elevated serum interleukin-6 is predictive of coronary artery disease in intermediate risk overweight patients referred for coronary angiography. *Diabetol Metab Syndr.* 2017;9(1):1-7.
295. Dhalla NS, Temsah RM, Netticadan T. Role of oxidative stress in cardiovascular diseases. *J Hypertens.* 2000;18(6):655-673.
296. Kisaoglu A, Borekci B, Yapca OE, Bilen H, Suleyman H. Tissue damage and oxidant/antioxidant balance. *Eurasian J Med.* 2013;45(1):47-49.
297. Pham-Huy LA, He H, Pham-Huy C. Free radicals, antioxidants in disease and health. *Int J Biomed Sci.* 2008;4(2):89-96.
298. Li M, Qian M, Kyler K, Xu J. Endothelial–vascular smooth muscle cells interactions in atherosclerosis. *Front Cardiovasc Med.* 2018;5(10):1-8.

