إقرار

أنا الموقع أدناه مقدم الرسالة التي تحمل العنوان:

Histopathological and Histochemical Study of Tramadol on Rabbit's Liver and Kidney

دراسة نسيجية مرضية و نسيجية كيميائية لتأثير الترامادول على كبد وكلية الأرانب

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Date: التاريخ: 2015/7/25

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Histopathological and Histochemical Study of Tramadol on Rabbit's Liver and Kidney

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Submitted in Partial Fulfillment for the Degree
Master of Biological Sciences - Zoology

2015

بسم الله الرحمن الرحيم

الجامعة الإسلامية – غزة
The Islamic University - Gaza
مكتب نائب الرئيس للبحث العلمي والدراسات العليا
هاتف داخلي: 1150

ج س غ/35/
الرقم Ref 2015/05/19
التاريخ Date .....

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دراسة نسيجية مرضية ونسيجية كيميائية لتأثير الترامادول على كبد وكلية الأرانب

Histopathological and Histochemical Study of Tramadol on Rabbit's Liver and Kidney

وبعد المناقشة التي تمت اليوم الثلاثاء 01 شعبان 1436هـ، الموافق 2015/05/19م الساعة الثانية عشرة ظهراً، اجتمعت لجنة الحكم على الأطروحة والمكونة من:

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وبعد المداولة أوصت اللجنة بمنح الباحث درجة الماجستير في **كلية** *العلوم*/ **قسم العلوم الحياتية – علم الحيوان.**

واللجنة إذ تمنحه هذه الدرجة فإنها توصيه بتقوى الله ولزوم طاعته وأن يسخر علمه في خدمة دينه ووطنه.

والله ولي التوفيق،،،

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Dedication

To my lovely family ...

Acknowledgment

My deepest and profound acknowledgments are to my supervisors **Prof. Dr. Ismail Abdelaziz**, Professor of Comparative Anatomy, Faculty of Science and **Dr. Abdelmonem Lubbad**, Associate Professor of Pathology, Faculty of Medicine, Islamic University of Gaza. For their continuous support, generous helps, and fruitful constructive suggestions. I could not have imagined having a better supervisors and mentors for my master thesis.

My deep respect and appreciation to my family member; their tender care and patience have not ceased even in my worst moments.

I would like to thank the staff of biology and biotechnology department at the Islamic university especially **Dr. Mohamed Abou Auda** and **Mr. Ashraf Alshafae**, for their assistance, numerous suggestion and time. To all the colleagues for their help.

Abstract

Background: Tramadol is used worldwide and is listed in many medical guidelines to treat both acute and chronic pain. It is a synthetic opiate analgesic with serotonin and noradrenaline reuptake inhibitor properties. There is growing evidence of abuse of tramadol in some African and West Asian countries. Abuse of tramadol is reported by Egypt, Gaza, Jordan, and Saudi Arabia. Tramadol has some side effects and adverse reactions and may cause psychological and physical addiction similar to that of other opiates.

Aim: The present study was designed a follow up of the histopathological and histochemical response which might be induced in the liver and kidney as a consequence of tramadol application.

Materials and Methods: Tramadol was injected to six groups of adult rabbits weighted (1000±200 g). The 1^{st}, 2^{nd} , 3^{rd}, 4^{th}, 5^{th} and 6^{th} groups were daily injected with tramadol (40mg/kg. body weight) for 10, 20, 30, 40 and 50 days respectively. The 6^{th} group treated with tramadol for 30 days and left for 10 days for recovery. Control group injected with distilled water. Formalin-fixed liver and kidney were processed by the standard paraffin wax technique, sectioned in (4–5µm) thicknesses, stained with Harris's alum heamatoxylin and eosin for histopathological investigation. For the histochemical study, sections were stained with periodic acid-Schiff's method to demonstrate total carbohydrates and with mercury bromophenol blue method to demonstrate total proteins.

Results: Several alterations were observed. The changes in the liver histological structure include congestion in the central vein, diffuse of Kupffer cells, karyolysis and complete pyknosis of many cells were noticed after treatment with tramadol for 30, 40 and 50 days and congestion in sinusoids after treatment for 10 days. Moderate inflammatory cells infiltration, piecemeal necrosis and fibroblastic cells proliferation was observed in groups treated for 30 day. On the other hand, vascular degeneration in the epithelial cells lining the renal tubules at the cortical zone with pyknotic and karyolysed nuclei were noticed in kidney of rabbits treated with tramadol for 30, 40 and 50 days. In addition, swelling in the lining epithelium of the renal tubules and the presence of inflammatory cellular infiltration, expansion of glomerulus, glomerular tuft atrophy, focal tubular necrosis and mild mononuclear cell infiltration were noticed. More excessive necrosis of tubular epithelial cells, dilated tubules and expansion of glomerulus were observed after 40 and 50 days of treatment. In recovery group the rabbits treated with tramadol for 30 dose and left for 10 days without administration of tramadol, some of the previous observations were disappeared. The liver of rabbits treated with tramadol for 10 days preserved the normal contents of glycogen similar to that of control animals, while Specimens treated for 30 days showed mild glycogen depletion involving many hepatic cells. Rabbits treated for 50 days showed marked glycogen depletion in comparison to the rabbits hepatocytes of the control. Examination of kidney sections of the rabbits treated with tramadol for 10 days revealed a normal PAS reaction, while kidneys of rabbits treated for 30

and 50 days of tramadol showed marked diminution in PAS positive material in the renal corpuscles and tubules. The animals received tramadol for 10 days did not manifest obvious changes in the protein contents of their hepatocytes. Hepatocytes of rabbits treated with tramadol for 30 and 50 days demonstrated a severe reduction of protein contents in comparison to the hepatocytes of the control rabbits. Examination of kidney sections of the rabbits injected with tramadol for 30 and 40 days manifested obvious changes in the protein contents of their kidney cells. The glomeruli and renal tubules have lost the most protein contents and became slightly less stainable than the control animal. The protein contents as well as the general appearance of the hepatocytes and renal tissues were approximately restored after administration of tramadol and leave rabbits for a recovery period.

Conclusion: Tramadol can causes histological and histochemical changes in kidney and liver, especially when high or repeated low dose of it are used.

Key words: Tramadol, Histopathology, Histochemistry, Liver, Kidney.

دراسة نسيجية مرضية ونسيجية كيميائية لتأثير الترامادول على كبد وكلية الأرانب

المستخلص

المقدمة: يستخدم عقار الترامادول في جميع أنحاء العالم، وهو مدرج ضمن العديد من الارشادات الطبية لعلاج الآلام الحادة والمزمنة على حد سواء. وهو مسكن أفيوني اصطناعي يعمل على تثبيط امتصاص السيروتونين والنورادرينالين. هناك أدلة متزايدة على تعاطي الترامادول في بعض البلدان الأفريقية وغرب آسيا، حيث تفيد التقارير بأن هناك إساءة لاستخدامه في مصر وغزة والأردن والمملكة العربية السعودية. للترامادول بعض الآثار الجانبية وردود الفعل السلبية ومن الممكن أن يسبب الإدمان النفسي والجسدي مماثلاً للمواد الأفيونية المخدرة الأخرى.

الهدف: صمم هذا البحث لدراسة الاستجابة النسيجية المرضية والنسيجية الكيميائية التي يمكن أن يسببها عقار الترامادول في الكبد و الكلى و توضيح أي آثار ضارة محتملة أخرى.

المواد و الطرق: حقن الترامادول في ٦ مجموعات من الأرانب البالغة وزنها (١٠٠٠ ± ٢٠٠ غرام) ، حيث أعطيت جرعة بمقدار ٤٠ ملجرام/كجم من وزن الجسم لمدة ١٠ ، ٢٠، ٣٠، ٤٠، ٥٠ يومًا على التوالي. وأعطيت مجموعة سادسة ترامادول لمدة ٣٠ يوما ومن ثم تركت لفترة ١٠ أيام للاستشفاء، واستخدمت مجموعة من الأرانب كمجموعة ضابطة. بغرض الفحص النسيجي، أخذت عينات الكبد والكلى من الأرانب وثبتت في محلول الفورمالين وتم معالجتها للحصول على مقاطع شمعية ثم قطعت بسمك ٤-٥ ميكرون. لدراسة التأثيرات النسيجية المرضية تم صباغة الشرائح بصبغة الهيمتوكسلين و الايوسين ، أما للدراسة النسيجية الكيميائية فصبغت الشرائح بصبغة شف للحمض البيريودي لتوضيح الكربوهيدرات ، وصبغة أزرق البروموفينول الزئبقي لتوضيح البروتينات.

النتائج: ظهرت العديد من التغيرات ، شملت تغيرات في البنية النسيجية للكبد واحتقان في الوريد المركزي بعد اعطاء الترامادول لمدة ٣٠ ، ٤٠ و ٥٠ يوم كذلك تشتت لخلايا كوبفر وانتشارها بين الخلايا المتنكرزة. ولوحظ أيضًا انتشار للخلايا الالتهابية و الخلايا الليفية بشكل معتدل في المجموعات المعالجة لمدة ٣٠ يوم و انحلالاً للأنوية و تكثف كامل لها في عدد من الخلايا. كذلك تحللاً للأوعية الدموية في الخلايا الطلائية المبطنة للأنابيب الكلوية في منطقة القشرة مع تغلظ للأنوية وتحلل بعضها في كلى الأرانب أعطيت ترامادول لمدة ٣٠ و ٤٠ و ٥٠ يوم. و كانت التغيرات الشديدة في الانابيب الملتوية القريبة حيث ظهرت فجوات سيتوبلازمية في الخلايا الطلائية المكونة لها ، وكان هناك تنكرز بدرجات عالية للخلايا الطلائية للأنابيب الكلوية وتوسع فيها بعد ٥٠ يوما من العلاج بالترامادول . وقد اختفت بعض الملامح المرضية السابقة في مجموعة الاستشفاء. ظهرت المحتويات الطبيعية من الجليكوجين في كبد الأرانب التي عولجت بالترامادول لمدة ١٠ أيام ، حيث كان مشابهًا للعينة الضابطة . بينما العينات التي عولجت بالترامادول لمدة ٣٠ يوم أظهرت استنزاف للجليكوجين بشكل معتدل وانطوى ذلك على العديد من الخلايا الكبدية. وأظهرت الأرانب المعالجة لمدة ٥٠ جرعة استنزاف بشكل أكبر للجليكوجين مقارنة مع خلايا كبد حيوانات المجموعة الضابطة. وأظهر فحص أنسجة الكلى من الأرانب التي أعطيت ترامادول لمدة ١٠ أيام استجابة طبيعية لتفاعل شيف للحمض البيريودي PAS في الكبيبات، ومحفظة بومان، كذلك في الأغشية القاعدية من الأنابيب الكلوية والحدود الفرشائية للأنابيب الملتوية القريبة. وبينت الملاحظة المجهرية لكلى الأرانب التي عولجت لمدة ٣٠ و ٥٠ يوما بالترامادول انتقاصاً ملحوظ في المواد المستجيبة لصبغة PAS في كبيبات الكلى والأنابيب الكلوية. ولم تبدِ الحيوانات التي تلقت ترامادول لمدة ١٠ أيام أي تغييرات واضحة في محتويات البروتين في الخلايا الكبدية. بينما أظهرت خلايا الكبد من الأرانب أعطيت ترامادول لمدة ٣٠ و ٥٠ يوما انخفاض حاد في المحتوى البروتيني بالمقارنة مع خلايا الكبد من أرانب المجموعة الضابطة. أما المقاطع النسيجية لكلى الأرانب التي حقنت بالترامادول لمدة ٣٠ و ٤٠ يومًا فأبدت تغييرات واضحة في محتواها البروتيني. وتجلت تلك التغيرات في الكبيبات والأنابيب الكلوية حيث فقدت معظم محتواها من البروتين وأصبح أقل بشكل واضح مقارنةً مع حيوانات المجموعة الضابطة. وبعد ترك الأرانب لفترة الاستشفاء لوحظ تحسنا واستعادة للمحتوى البروتيني فضلا عن المظهر العام للأنسجة.

الخلاصة: يمكن أن يسبب الترامادول تغيرات نسيجية وهستوكيميائية في الكبد والكلى خاصة عند اعطائه بجرعات عالية أو جرعات قليلة بشكل مستمر.

الكلمات الرئيسية: الترامادول، أمراض الأنسجة، الكيمياء النسيجية، الكبد، الكلى.

CONTENTS

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LIST OF ABBRIVIATIONS

BF: Bile duct proliferation

CV: Central vein

C: Congestion of sinusoids

CY: Cytoplasmic vacuolation

D: Dilation of sinusoids

DT: Distal convoluted tubules

EGC: Expansion of the glomerular chamber

HD: Hydropic degeneration

F: Fatty change

FTN: Focal tubular necrosis

GTA: Glomerular tuft atrophy

HA: Hepatic artery

HP: Hepatic parenchyma

IF: lymphoplasmacytic peri-portal infiltrates

KY: Karyolitic nuclei

KC: Kupffer cells

LB: lobule boundary

LP: Limiting plate

MCI: Mononuclear cell infiltration

N: Necrotic cells

PV: Portal vein

PY: Pyknotic nuclei

PT: Proximal convoluted tubules

R: Ruptured limiting plate

Chapter 1

INTRODUCTION

1.1 Overview

Opioids are the most powerful painkillers. Their use is appropriate for the treatment of moderate-to-severe chronic pain (5–10 point pain intensity) with the goal to control this symptom and to improve functions and quality of life **(Huang and Mallet, 2013)**. Tramadol is a synthetic, centrally acting analgesic, available in Europe since 1977 and in the United States since 1995 for the treatment of pain syndromes previously amenable only to the opiate analogues **(Moore *et al.,* 1999)**.

Tramadol has been in clinical use for the relief of mild to moderate pain in human and veterinary medicine **(Pypendop and Ilkiw, 2008)**. Tramadol is also used perioperatively in veterinary anesthesia as it significantly reduces the requirements of volatile anesthetics and opioid agents **(Seddighi *et al.,* 2009)**. Tramadol, a synthetic racemic mixture of the 4-phenyl-piperidine analogue of codeine, has received widespread acceptance in human medicine since it was first introduced in 1977 in Germany **(Osterloh *et al.,* 1978 and Scott and Perry, 2000)**.

It has dual mode of action. Its analgesic efficacy is attributed to its partial affinity for the μ-opiate receptor and its inhibition of norepinephrine and serotonin reuptake **(Shadnia *et al.,* 2008)**. Tramadol is considered a safe drug devoid of many serious adverse effects of traditional opioids. However, recently, abuse and dependence of tramadol as well as toxicity and tramadol-related deaths have been increasingly reported **(Tjäderborn *et al.,* 2007)**.

Tramadol is rapidly absorbed orally; a peak concentration is detected 2-3 hours post oral dose. It has extensive tissue distribution. Thirty percent of the drug is excreted through the kidneys in an unchanged manner. Elimination half-life is 5–6 hours, while the remaining is metabolized in liver by N- and O-demethylation, followed by conjugation with glucuronic acid and sulphate. The active metabolite, o-desmethyl tramadol shows higher affinity for the μ-opioid receptors and has twice the analgesic potency of the parent drug **(Khandave *et al.,* 2010)**.

Tramadol causes respiratory depression, psychological and physical addiction similar to that of other opiates and the analgesic efficacy of tramadol can further be improved by combination with a non-opioid analgesic **(Lanier *et al.,* 2010)**. Repeated tramadol administration in such patients might lead to the accumulation of toxic metabolites in the body, increase the risk for pharmacokinetic interactions, and/or decrease the clearance of tramadol, thus increasing its potential for toxicity **(De Decker *et al.,* 2008 and Shadnia *et al.,***

2008). Nowadays addiction is an ever-increasing problem in the world and despite all efforts to prevent and control it, it continues to be a tremendous public health issue. Analgesics are among the most popular drugs which are being abused **(Rafati *et al.,* 2006)**.

Histopathological and biochemical changes due to chronic usage of morphine or tramadol in liver and kidney in rats had confirmed by **Atici et al. (2005)**. where, Serum ALT, AST, LDH, BUN and creatinin levels were significantly higher in morphine group compared to the control group. Serum LDH, BUN and creatinin levels were significantly increased in the morphine group compared to the tramadol group. Light microscopy revealed severe centrolobular congestion and focal necrosis in the liver of morphine and tramadol groups, but perivenular necrosis was present only in the morphine group. The main histopathologic finding was vacuolization in tubular cells in morphine and tramadol groups.

The present study was conducted to assess the histopathological and histochemical alterations of tramadol on liver and kidney tissues of domestic rabbits. The finding can then be extrapolated to human beings to assess the potential hazards in the human populations due to tramadol addiction.

1.2 Objectives

1.2.1 General objective

The general objective of the present study is to assess the toxic effects of tramadol on liver and kidney tissues of domestic rabbits.

1.2.2 Specific objectives

1. To investigate the histopathological effects of tramadol on the liver and kidney tissues through examination paraffin sections by microscope examination.
2. To test the effects of tramadol on the liver and kidney glycogen and total proteins by staining techniques.
3. To demonstrate the extent of reversibility and tissue repair in the target organs upon drug abstinence or withdrawal.

1.3 Significance

1. Tramadol is being extensively used among people in Gaza Strip with lake of protective measures. Hopefully, the results obtained from this investigation would be some value for the proper and safer therapeutic application of tramadol, that indispensable opiate.

2. Studies on tramadol toxicity on rabbit's tissue are limited in the literature and it's the first study in Palestine.
3. The result of the present study may be useful to a ware people particularly abuser people on the extent of tramadol toxicity.

Chapter 2

LITERATURE REVIEW

2.1 Definition of opioids

Opioid is the term used broadly to describe all compounds that work at the opioid receptors **(Trescot *et al.,* 2008)**. They are considered first-line treatment for hospitalized patients with moderate to severe pain **(Holden, 2008)**. They are currently the most effective pain-relieving pharmaceuticals. Opioids are the most potent and effective analgesics available and have become accepted as appropriate treatment for acute, cancer and non-cancer chronic pain **(Collet, 2001)**. However, they are also rewarding and their repeated use can lead to dependence and addiction. In fact, addiction to opioid analgesics is a growing socioeconomic and health problem with potentially serious consequences documented by a rise in deaths due to overdose **(Hall *et al.,* 2008 and Fields, 2011)**.

In the past two decades, the medical use of opioids has increased dramatically in the United States **(Okie, 2010)**. A parallel rise in opioid-related adverse effects such as emergency room visits and overdose mortality has also occurred **(Bohnert *et al.,* 2011)**. The commonly prescribed opioids in palliative care include morphine, tramadol, fentanyl, buprenorphine, methadone, oxycodone, diamorphine and codeine **(Klepstad *et al.,* 2011)** and these exhibit multi-system effects due to their interactions with receptors that are targeted by the endogenous opioid system.

Morphine (the archetypal opioid) consists of a benzene ring with a phenolic hydroxyl group at position 3 and an alcohol hydroxyl group at position 6 and at the nitrogen atom. Both hydroxyl groups can be converted to ethers or esters. For example, codeine is morphine that is O-methylated at position 3, while heroin is morphine O-acetylated at position 3 and 6 (diacetyl morphine). The tertiary form of the nitrogen appears to be crucial to the analgesia of morphine; making the nitrogen quaternary greatly decrease the analgesia, since it cannot pass into the central nervous system. Changes to the methyl group on the nitrogen will decrease analgesia as well, creating antagonists such as nalorphine. Morphine is optically active, and only the levorotatory isomer is an analgesic **(Andrea *et al.,* 2008)**.

2.2 The opioid system

The opioid system plays a major role in pain relief. Receptors in this system, which are called the opioid receptors belong to the G-protein-coupled receptor (GPCR) superfamily and they are mostly coupled to Gi/o type G-proteins **(Burford *et al.,* 2000)**. They exert their effects through the inhibition of different

types of neurotransmitters, such as noradrenaline, dopamine or acetylcholine **(Mansour *et al.,* 1995)**. Their endogenous ligands, namely the enkephalins, endorphins, dynorphins, nociceptin and endomorphins **(Holden *et al.,* 2005)** are called the endogenous opioids, and they are small peptide natured molecules functioning as neurotransmitters, neurohormones or neuromodulators **(Koneru *et al.,* 2009)**.

The opioid receptors are expressed widely in the central nervous system **(Mansour *et al.,* 1995)** as well as in the peripheral organs, such as the gastrointestinal tract **(Holzer, 2009)**. They have a substantial role in pain regulation, and opioid agonists, such as morphine and the more effective sufentanil **(Savoia *et al*., 2001)** were found to have a clear clinical efficacy for the attenuation of certain chronic and acute pain **(Pasternak and Pan, 2011)**.

2.3 Tramadol

Tramadol (2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)aminocyclohexanol), is widely used in the management of post-operative and chronic pains in animals including rabbits **(Saleem *et al.,* 2014)**. It is a centrally acting analgesic with efficacy and potency ranging between weak opioids and morphine **(Raffa *et al.,* 1992)**. It was appeared in the 1970s, but only approved by the Food and Drug Administration (FDA) in 1995 for the management, treatment and relief of moderate to severe pain conditions **(Bloor *et al.,* 2012; Eassa and El-Shazly, 2013 and El-Sayed *et al.,* 2013)**. The drug is structurally related to codeine and morphine, but it is 6000-times less potent than morphine and 10-times less potent than codeine **(Lavasani *et al.,* 2013 and Lee *et al.,* 2013)**.

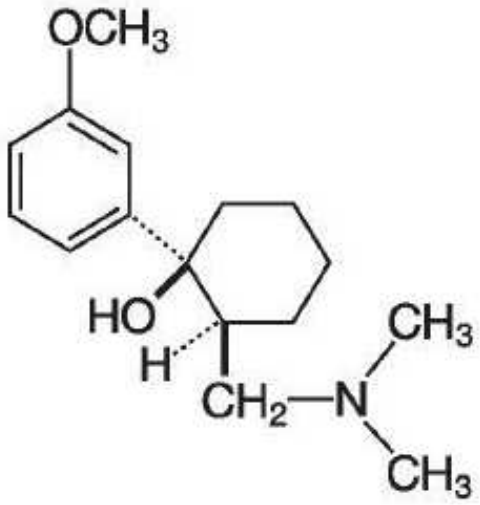

Figure 2.1: Chemical structure of tramadol **(Rouini *et al.,* 2008).**

2.3.1 Therapeutic uses

The analgesic actions of tramadol arise from agonist actions of the drug at the μ-opioid receptor and the blockade of serotonin and norepinephrine uptake **(Raffa *et al.,* 1993)**. An increased level of serotonin and norepinephrine blocks noxious stimuli via a direct effect on spinal receptors, and an inhibitory effect via descending noradrenergic and serotonergic systems to produce analgesia by non-opioid mechanisms **(Grond and Sablotzki, 2004)**. Thus, tramadol analgesia is multimodal and is partially blocked by naloxone and by antagonists of serotonin or norepinephrine neurotransmission **(Raffa *et al.,* 1992)**.

Studies have shown tramadol to be effective in both acute myocardial infarction and unstable angina **(Manji *et al.,* 1997)**. Tramadol has also been used in treatment of cancer pain, moderate to severe musculoskeletal pain; rheumatoid arthritis, restless legs syndrome, motor neuron disease, fibromyalgia, diabetic neuropathy and post herpetic neuralgia **(Raffa *et al.,* 1992 and Harati *et al.,* 2000)**. Moreover, tramadol may also be clinically applied for the management of premature ejaculation, the most common sexual disorder, affecting 20–30% of adult men **(Eassa and El-Shazly, 2013)**.

2.3.2 Mechanism of action

Tramadol has two distinct, synergistic mechanisms of action. It showed a weaker binding affinity for μ-opioid receptors than morphine, but had a 10 fold more affinity to μ-receptors than -K & δ- opioid receptors in rat in vitro studies **(Hennies *et al.*,1988)**. Mean median inhibition concentration (IC50) values for tramadol and morphine in displacing μ-receptor binding of radioactive ligands in rat brain membranes in vitro were 1.7x10-6 M and 4.6x10-9 M, respectively. Further, in animal and human models naloxone, a μ-receptor antagonist only partly reversed or blocked the tramadol induced antinociception even at higher doses **(Collart *et al.,* 1993)**, indicating that some of the antinociceptive effects of tramadol are not mediated by the opioid receptors alone.

Later, **Driessen *et al.* (1993)** investigated the non-opioid component of tramadol analgesia in two different studies using rat brain and demonstrated that tramadol inhibited the synaptosomal re-uptake of noradrenaline (NA) and 5-hydroxytryptamine (5-HT) by central neurones.

Both NA and 5-HT are monoamine neurotransmitters involved in the inhibition of nociception by descending inhibitory mechanisms **(Millan, 2002)**. Further evidence for monoamine reuptake inhibition by tramadol was provided by **Raffa *et al.* (1992)**. They found that both yohimbine (2-adrenoceptor blocker) and ritanserine (selective antagonist at 5-HT2 receptors) significantly reduced the antinociceptive action of intrathecally administered tramadol in the rat-tail flick test.

2.3.3 Metabolism of tramadol

The metabolism of tramadol has been investigated in a number of animal species (rats, mice, Syrian hamsters, guinea pigs, rabbits and dogs) as well as in humans **(Kukanich and Papich, 2004)**. Tramadol is rapidly and almost completely absorbed after oral administration. The mean peak plasma concentration occurs after two hours and its bioavailability is approximately 70% as a result of the first-pass metabolism in the liver. About 20% of the drug is bound to plasma proteins and the mean half-life is ca. 6 h **(Bloor *et al.,* 2012; Lavasani *et al.,* 2013 and Eassa and El-Shazly, 2013)**.

Tramadol is extensively metabolized in the liver via cytochrome isoenzymes P450 2D6, and P450 2B6 and P450 3A4, to O-desmethyltramadol (M1) and N-desmethyltramadol (M2) respectively, being the main phase-1 metabolites. These are further metabolized to three secondary metabolites, namely N, N-didesmethyltramadol,N,N,O-tridesmethyltramadol and N,O-desmethyltramadol. All metabolites are finally conjugated with glucuronic acid and sulfate before excretion in urine **(Ing Lorenzini *et al.,* 2012; Lavasani *et al.,* 2013 and El-Sayed *et al.,* 2013)**. **Wu *et al.* (2001**) reported that tramadol is converted in the liver to O-desmethyltramadol, which is an active substance and 2 to 4 times more potent than tramadol.

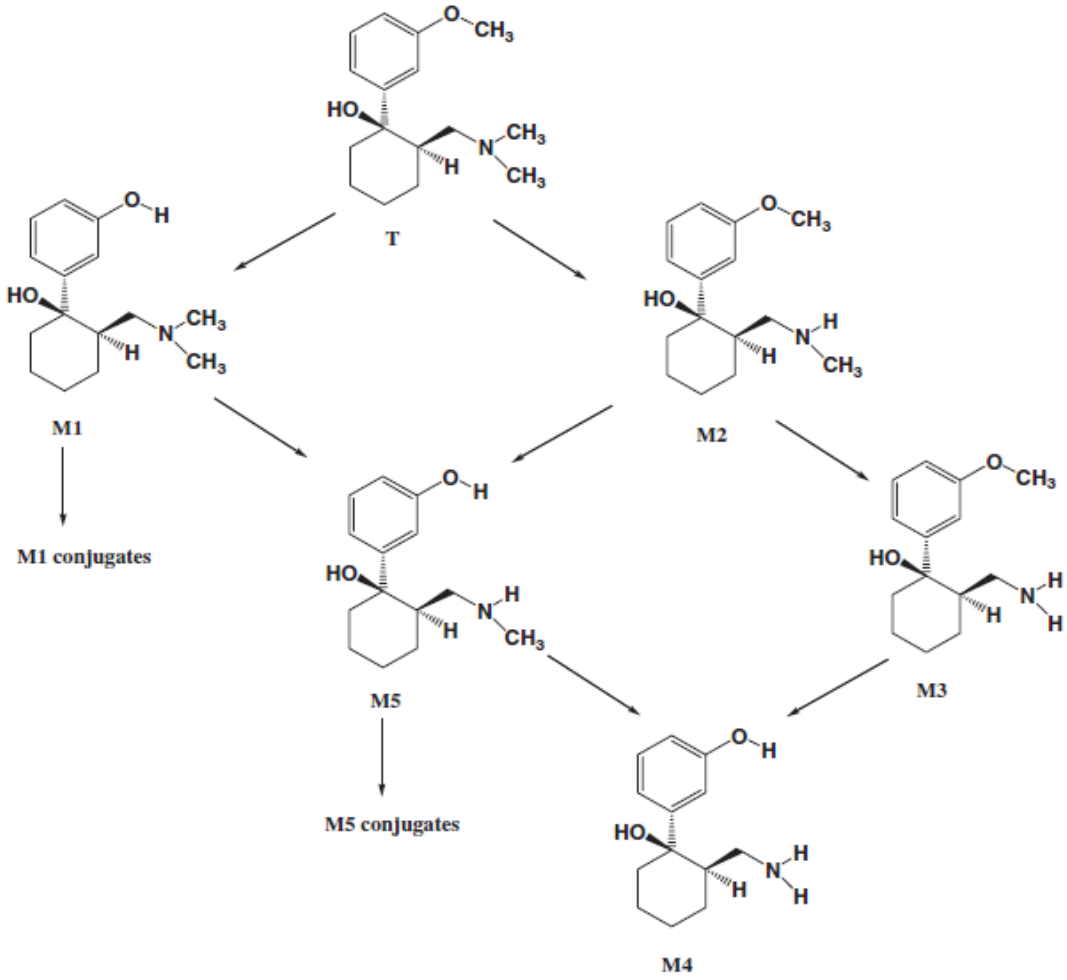

Figure 2.2: Metabolic pathways of tramadol **(Rouini et al., 2008)**.

Moreover, biotransformation results in inactive metabolites, which are excreted by kidneys **(Lee *et al.,* 1993 and Atici *et al.,* 2005)**. Likewise, if kidney or liver function is severely impaired, some dosage reduction (approximately by 50%) or extension of the dosage interval should be considered **(Klotz, 2003)**. A metabolite may have higher activity and/or greater toxicity than the original drug. Metabolites of the drugs that are excreted from kidneys may also cause cellular damage leading to kidney dysfunction **(Singhal *et al*., 1998)**.

2.3.4 Elimination of tramadol

The route of elimination almost totally involves the kidneys. Approximately 30% of the dose is excreted in the urine as unchanged drug, while 60% of the dose is excreted as metabolites. The remaining drug is eliminated in the feces, therefore biliary excretion is negligible **(Eassa and El-Shazly, 2013; El-Sayed *et al.,* 2013 and Lavasani *et al.,* 2013)**. **Klotz et al. (2003)** demonistrated that elimination of tramadol is primarily by the hepatic route (metabolism by CYP2D6 to an active metabolite and by CYP3A4 and CYP2B6) and partly by the renal route (up to 30% of dose). Elimination half-lives of the active agents range between 4.5 and 9.5 hours, and the total plasma clearance of tramadol is moderately high (600 ml/min).

2.3.5 Tramadol availability

Tramadol is available as drops, capsules and sustained-release formulations for oral use which can be administered either orally, intramuscularly, intravenously, or by patient-controlled analgesia **(Sunshine, 1994 and Desmeules *et al.,* 1996)**. Some studies indicated that tramadol dependence has been preclinically evaluated in different animal species **(Friderichs *et al.,* 1978 and Murano *et al.,* 1978)**. Some other studies showed that the intra-peritoneal administration of tramadol produces a dose-dependent antinociception **(Raffa *et al.,* 1992 and Miranda and Pinardi, 1998)** that remains unchanged after chronic administration.

2.3.6 Adverse side effects

Adverse side effects Opioids are usually prescribed for chronic pain but their use is frequently limited by their side effects. Whilst the long-term use of opiates in patients with chronic pain and also for cancer pain gets worse, evidence, based on a paucity of literature proving efficacy for long-term use, suggests that opiates fail to fulfill any of the key outcomes in terms of adequate pain relief, improved quality of life or improvements in functional capacity **(Farmer *et al.,* 2013)**.

Being an opioid, tramadol carries all possible risks known from other opiates **(Cicero *et al.,* 2005 and Adams *et al.,* 2006)**. Side effects include dizziness, headache, somnolence, nausea, constipation, sweating, pruritus, and central nervous system stimulation **(Kabel and van Puijenbroek, 2005)**. Tramadol causes respiratory depression, psychological and physical addiction similar to

that of other opiates and the analgesic efficacy of tramadol can further be improved by combination with a non-opioid analgesic **(Lanier *et al.,* 2010)**. Additionally, Tramadol use is associated with dependence, albeit it is generally considered safe at low dosage. Dopamine release in various regions of CNS is responsible for this phenomenon. Agonists of μ-opioid receptors, such as tramadol, stimulate the release of dopamine as well as inhibit GABA release, which in turn inhibits dopamine release **(Hassanian-Moghaddam *et al.,* 2013)**.

The effects of tramadol have been investigated in several animal species such as mice, rats, rabbits, dogs, and also in horses as well as in humans. Indeed, the drug is also used in veterinary medicine **(Cox *et al.,* 2010)**. **Casella *et al.* (2013)** found a significant increase of the maximum degree and slope of equine platelet aggregation after addition of tramadol in feeding condition. This could be explained by the fact that the inhibition of NE system blocks the release of prostacyclin and nitric oxide, both of which are known to be potent inhibitors of platelet. **Cox et al. (2010)** instead, reported short-term agitation, tremors, tachycardia and muscle twitching that occurred after rapid administration of tramadol in horses.

Bloor *et al.* (2012) overhauled the effects of tramadol during pregnancy, labor, delivery and lactation. Tramadol is classified as a category C drug by the Australian Therapeutic Goods Administration Evaluation Committee, i.e. drugs which have caused or may be suspected of causing harmful effects (that may be reversible) on the human fetus or neonate, without causing malformations. It would seem that the most cautious approach is to avoid its use around the time of conception and during the period of organogenesis, specifically in the first trimester. There is currently no clear evidence of fetal or neonatal harm, but increased fetal loss associated with typical maternal doses taken during early pregnancy has been reported. If chronic maternal tramadol use has occurred throughout pregnancy, there is a risk of a neonatal withdrawal syndrome. The drug offers limited analgesic effects during labor. During early lactation and breastfeeding, it appears unlikely to cause harm to healthy term infants. Unfortunately, there are no available data reporting this statement.

2.3.7 Social impacts of tramadol

Due to its opioid stimulant effects **(Duke *et al.,* 2011)**, the potential for tramadol abuse is an increasing public health concern. Clinically, tramadol is about 10-times less potent than morphine however; its unique pharmacological profile differentiates it from typical opioids. Also, Tramadol exhibits a lower liability for tolerance and dependence and has a low abuse potential **(Grond and Sablotzki, 2004)**.

Despite the fact that drug abuse is not a newly introduced issue to Egyptian society, the wide range of usage and illegal transactions are associated with tramadol abuse, making it the most easily accessible and readily provided drug at cheap cost **(Fawzi, 2010)**. It also seems that it is not only an Egyptian problem, but also a problem in neighboring countries. Their lower price and availability without prescription make them very popular. Thousands of young

men are developing dependence to a prescription painkiller used to alleviate the stress of living. They also use it to relieve psychosomatic symptoms such as headaches and abdominal pain, as well as depression and nervousness **(Progler, 2010)**. Students, laborers, and even professionals are buying large quantities of tramadol on the black market.

In Egypt, **Soueif *et al.* (1982)** reported several reasons for the different user categories. For secondary school students, the main reason was for entertainment in happy social occasions and socializing with friends. Workers and university students take drugs in situations of physical exhaustion and fatigue and to cope with psychosocial problems or difficult working conditions as well as at times of studying and examinations.

According to **(Soueif *et al.,* 1990)**, there is misconception that opiates are used to increase cognitive and sexual performances and to avoid physical exhaustion. Hence, it is not surprising to find that tramadol use has become recently very popular especially among youth and the middle-aged groups as a self-medication for premature ejaculatory function and for extended orgasm and to increase sexual pleasure **(Salem *et al.,* 2008)**. In contrast, there were some studies that showed negative impact of opiate use on cognitive functions **(Schindler *et al.,* 2004 and Fishbein *et al.,* 2007)**. Another study stated that opium users exhibit impaired sexual function (diminished libido and impaired sexual performance are common sequelae of chronic use) **(Van Ahlen *et al.,* 1995)**. Early clinical studies suggested that opiates may interfere with sex hormone secretion, although opiate addicts often equate the drug experience with sexual orgasm **(Mirin *et al.,* 1980)**.
Fawzi, (2010) reported that an increasingly alarming phenomenon of tramadol (Tramal, Amadol, Tramax, Contramal, Trama SR, Ultradol, Tramundin) abuse has been heavily demonstrated in the recent years. **Ezzat, (2014)** reported a catastrophic incidence of tramadol abuse after the 25th January Egyptian Revaluation; the report underlined the fact that the addiction rate in Cairo has jumped to comprise 7% of the capital's population during the study period. According to the Shura Council's Health Committee report, rising addiction rates were found to be partially attributable to the spread of erroneous concepts among young people. It is said that 30.6% of addicts believe that drugs increase physical abilities, whereas 36.6% associate drugs with getting over adversities and 34.8% do drugs to overcome depression.

2.3.8 Histopathological Aspects

Although considerable investigations have been carried out on Tramadol from certain biochemical and physiological points of view, yet a limited amount of publications seemed to be devoted to the possible histopathological and still fewer ones to the histochemical aspects of tramadol application. The liver and kidneys are responsible for the metabolism and excretion of tramadol **(Coughtrie *et al.,* 1989 and Milne *et al*., 1997)**. Morphine may cause hepatotoxicity and nephrotoxicity during its metabolism **(Van der Laan *et al*., 1995)**.

A significant increase in the levels of ALT, LDH and lipid peroxides was reported among chronic heroin users **(Panchenko *et al*., 1999)**. **Elyazji *et al.* (2013)** reported that ALT, AST, LDH, Blood urea nitrogen (BUN) and creatinine were significantly higher in tramadol treated groups compared to the control group. Furthermore, **Borzelleca *et al.* (1994)** reported increased levels of ALT, AST and LDH in rats after long-term usage of morphine like agent levo-alpha–acetylmethadol (LAAM) HCL. Centrolobular hypertrophy in the liver was also reported in the same study.

Experimental studies have also supported toxic effects of chronic use of opioids on liver and kidneys. **Nagmatsu *et al*. (1986)** demonstrated that addition of morphine to the isolated rat hepatocytes induced a marked decrease in the cells and resulted in cell death. Incubation of adult human hepatocytes with opioids, in therapeutic doses, for 24 h, is unlikely to produce irreversible damage to these cells in chemically defined culture conditions **(Gomez–Lechon *et al.,* 1988)**. In another experimental study, isolated rat hepatocytes exhibited a marked decrease in glutathione level when incubated with various concentrations of morphine and resulted in cell death **(William *et al.,* 1991)**.

Every drug has been associated with hepatotoxicity almost certainly due to the pivotal role of the liver in drug metabolism. Hepatic metabolism is first and foremost, a mechanism that converts drugs and other compounds into products that are more easily excreted and that usually have a lower pharmacologic activity than the portent compound **(Poppers,1980 and Tolman,1998)**. A metabolite may have higher activity and/or greater toxicity than the original drug. These metabolic products are able to induce free radicals and/or bind with glutathione (GSH), the natural scavenger of superoxide radicals. Both GSH conjugation and its subsequent depletion cause accumulation of free radicals as well as morphine metabolites induce directly and indirectly cellular toxicity **(Calignano *et al.,* 1992)** with enzymatic inactivation, DNA damage and/or lipid peroxidation **(Cavallo *et al.,* 2007)**.

Atici *et al.* (2005) reported that there is a renal tubular vacuolization, mononuclear cell infiltration, focal necrosis and haemorrhage as well as increase in BUN and creatinin levels in rats receiving morphine that can be considered as evidence of renal damage. Also, lipid peroxides were found significantly increased among chronic heroin users **(Panchenko *et al.,* 1999)**. Renal damage like focal cortico-medullary mineralization, focal regeneration in tubular epithelium, and mineral/ crystal deposition in intertubular region in kidneys has been shown after long-term use of LAAM **(Borzelleca *et al*., 1994)**. However, minimal histological changes confined to the tubular cells were observed in rats receiving long-term tramadol administration.

2.3.9 Histochemical Aspects

According to the best of our knowledge, there were a limited published histochemical studies about effects of tramadol on liver and kidney. But, several studies showed the abnormalities in the carbohydrate and protein

contents in the liver and kidney of experimental animals under several pathological agents.

Bekheet, (2010) showed that morphine caused a gradual decrease in the amount of carbohydrate contents in mice treated groups compared to control group and heterogenic distribution to PAS reaction was noticed in two neighboring areas, one containing an increased amount and the other showing a decreased amount. In contrast, In morphine treated groups, there was a general increase in protein (stainability) content proportional to the period of treatment and this is more pronounced in the basement membrane, nuclear structure and degenerated areas and moderate in the cytoplasm compared to the control group.

Chapter 3

MATERIALS AND METHODS

3.1 Experimental animals

A total of 47 healthy adult domestic rabbits weighing 1000±200 gm were obtained from local farms used in the present study. Animals were left for one week before experimentation to adapt to laboratory conditions. They were kept in metal cages. The animals were maintained under conditions of controlled temperature (22 ± 3) and humidity (30–70%) with 12 h light and dark cycle. A commercial balanced diet and water were provided ad libitum all over the experimental period.

Animals were divided randomly into seven groups (5-6 rabbits) and treated as follows:
Group I: Each rabbit was daily injected with tramadol (40mg/kg. body weight) for 10 days (according to **Atici *et al.,* 2005**).
Group II: Each rabbit was daily injected with tramadol (40mg/kg. body weight) for 20 days.
Group III: Each rabbit was daily injected with the same previous dose of tramadol for 30 days.
Group IV: Each rabbit was daily injected with the same previous dose of tramadol for 40 days.
Group V: Each rabbit was daily injected with the same previous dose of tramadol for 50 days.
Group VI: Each rabbit was daily injected with tramadol (40mg/kg. body weight) for 30 days left untreated for 10 days to recovery.
Group VII: Kept as a control, being injected intramuscularly with 1ml of distilled water.

3.2 Chemicals

The examined tramadol (Tramadol HCl ampoules 100mg) was obtained from Pharmacies PLC Betunia-Ramallah. All other chemicals were of analytical grade and were purchased from standard commercial suppliers.

3.3 Collection of samples

Following decapitation, the rabbits liver and kidney were dissected and the liver and kidney of each rabbit were freed from the surrounding connective tissues and organs, then excised. They were immediately immersed in saline solution (0.9% NaCl) for blood removal.

3.4 Histological and histochemical work up

For histological examination, the following steps were carried out daily from the beginning until the end of experimental duration.

3.4.1 Fixation

The following fixative reagent used for the routine heamatoxylin and eosin stain and histochemical stain was 10% buffered formalin **(Lillie, 1954)**.

3.4.2 Processing

The processing consisted of an initial two step fixation comprising tissue immersion in 10% buffered formalin for two hours each, followed by removal of fixative using distilled water for 30 minutes. Dehydration was then carried out by running the tissues through a graded series of alcohol (70%, 90%, and 100%). The tissues were initially exposed to 70% alcohol for 30 minutes followed by 90% alcohol for 1 hour and then two cycles of absolute alcohol, each for one hour. Dehydration was then followed by clearing the samples in several changes of xylene. It consisted of tissue immersion for an hour in a mixture comprising 50% alcohol and 50% xylene, followed by pure xylene for one and a half hour. Samples were then impregnated with molten paraffin wax, then embedded and blocked out. Paraffin sections (4–5um) were mounted and affixed to slides **(Underwood, 1985)**.

3.4.3 Items of staining

3.4.3.1 Routine histological stain

Sections were stained as a routine in Harris's alum heamatoxylin and eosin (Harris, 1900; Allen, 1992). Sections were de-paraffinized and hydrated to water, then stained with Harris's hematoxylin for 10 minutes. Sections were differentiated by dipping 3 - 4 times in 1% acid alcohol after a quick rinse in water, wash in running tap water for 20 minutes, counterstain with eosin from 15 seconds to 2 minutes depending on the age of the eosin, and the depth of the counterstain desired. At the end dehydration in 95% and absolute alcohols, two changes of two minutes each or until excess eosin is removed. Clearing in xylene, two changes of two minutes each and mount. The nuclei take a blue color while the cytoplasm take the deep pink color.

3.4.3.2 Carbohydrates demonstration

General carbohydrates were demonstrated following of **(Hotchkiss, 1948)** Periodic Acid-schiff (PAS) technique for 10% formalin's-fixed material. Sections were oxidized in 0.5% periodic acid for 5-10 minutes, washed for 5 minutes in

running tap water, rinsed in distilled water and stained with Schiff's reagent for 15 minutes, counterstain in Mayer’s hematoxylin for 1 minute. Sections were then rinsed in 3 changes of distilled water. The aldehydes-liberated by periodic acid-reacted with Schiff's reagent producing a certain component indicated by the development of magenta coloration and nuclei stained with a blue color.

3.4.3.3 Total proteins demonstration

Total proteins were visualized by using the Mercury Bromphenol Blue Method **(Mazia *et al.,* 1953)**. Paraffin sections, fixed in 10% formalin solution, were stained in 0.1% alcoholic bromphenol blue saturated with mercuric chloride. Proteins took a bluish stainability.

3.4.5 Examination

Mounted slides were examined and photographed under a light microscope (OPTIKA Microscopes, Italy) coupled with an image analyzer (OPTIKA Vision Pro, Microscopy Digital USB Camera).

Chapter 4

RESULTS

4.1 Histopathology of liver

A. Control group

Originally, the liver is invested by thin capsule of connective tissue, and the liver itself is distinguished into the connective tissue stroma and parenchymatous tissue or parenchyma. The capsular connective tissue is extended inwards with the liver substance making connective tissue septa or strands which divide the liver tissue roughly into individual lobules. Each hepatic lobule contained a thin walled central vein surrounded by hepatic cords radiating towards the periphery. The portal area includes a hepatic portal vein, a branch of the hepatic artery and a bile ductile (Figure 4.1.1). The portal area is delimited from the contiguous hepatic parenchyma by a limiting plate formed of linear band of hepatocytes.

The portal vein is the largest and widest one in this triad. The lumen might appear either empty or containing a few blood cells. The hepatic artery branch has a relatively narrow lumen and its lumen is usually devoid of blood cells. The hepatic bile ductule has a distinct wall of low cuboidal or rounded outline epithelial cells surrounded by a thin sheath of connective tissue. Besides, the portal triads contain a few mononuclear cells, mostly lymphocytes dispersed in its scanty connective tissue. The same figure, displays the bile ductile either having an oblong or rounded outline dependent on the plane of sectioning. It contains a comparatively wide cavity limited by a layer of cuboidal epithelium surrounded by a thin sheath of connective tissue.

The intralobular hepatic tissue (parenchyma) is made up of parenchymatous or hepatic cells organized in the form of radiation plates, each being one-cell thick. Thus, the hepatic cells obviously constitute a network structure around the central vein as elucidated in Figure 4.1.2. These cells alternate with narrow blood sinusoids that coverage toward the central vein in each lobule. The hepatic cells are cuboidal or polygonal in shape, relatively large in size and exhibit distinct limiting membranes. The size of these cells depend largely on their location in the lobule, the peripheral ones being comparatively larger in size than those lying in the pericentral areas.

Each hepatic cell has a centrally rounded or oval vesicular nucleus and a prominent nucleolus with one or two prominent nuclei, in addition to a number of chromatin particles. Occasionally some liver cells are binucleated. The cytoplasm is homogeneous, and finely granulated. The hepatic sinusoids are limited by a discontinuous layer of flattened endothelial cells, intervened by

certain large phagocytic kupffer cells with a large oval nucleus, which could also be detected elsewhere in the liver tissue or blood stream.

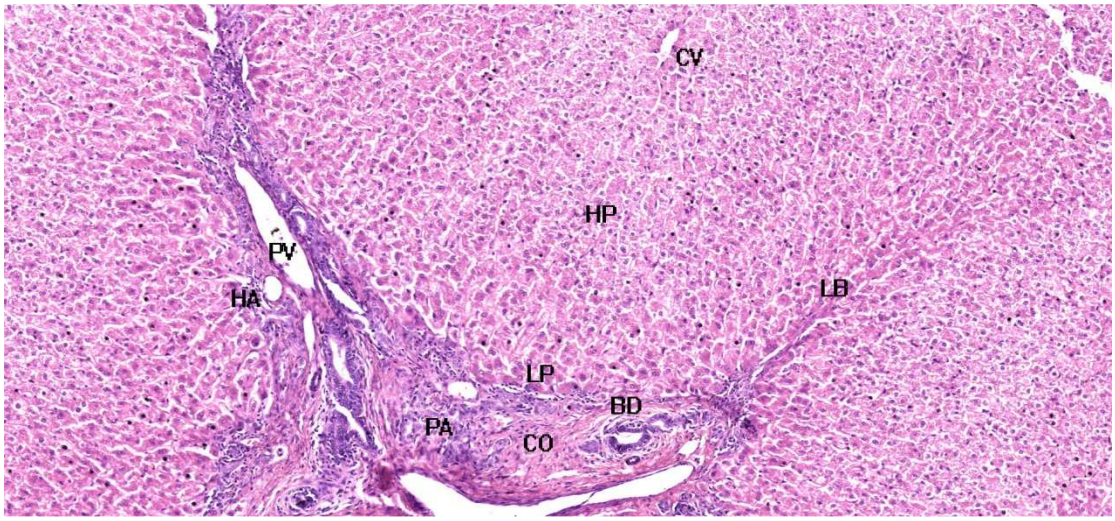

Figure 4.1.1: Hepatic tissue of the control rabbits group showing the general disposition the liver tissue. Central vein (CV), hepatic parenchyma (HP), lobule boundary (LB), the limiting plate (LP), portal area (PA) is clearly illustrated encountering a typical portal triad consisting of a tributary of hepatic portal vein (PV), hepatic artery (HA), the connective tissue (CO) and small bile ductile (BD). Sections stained with H&E, (X100).

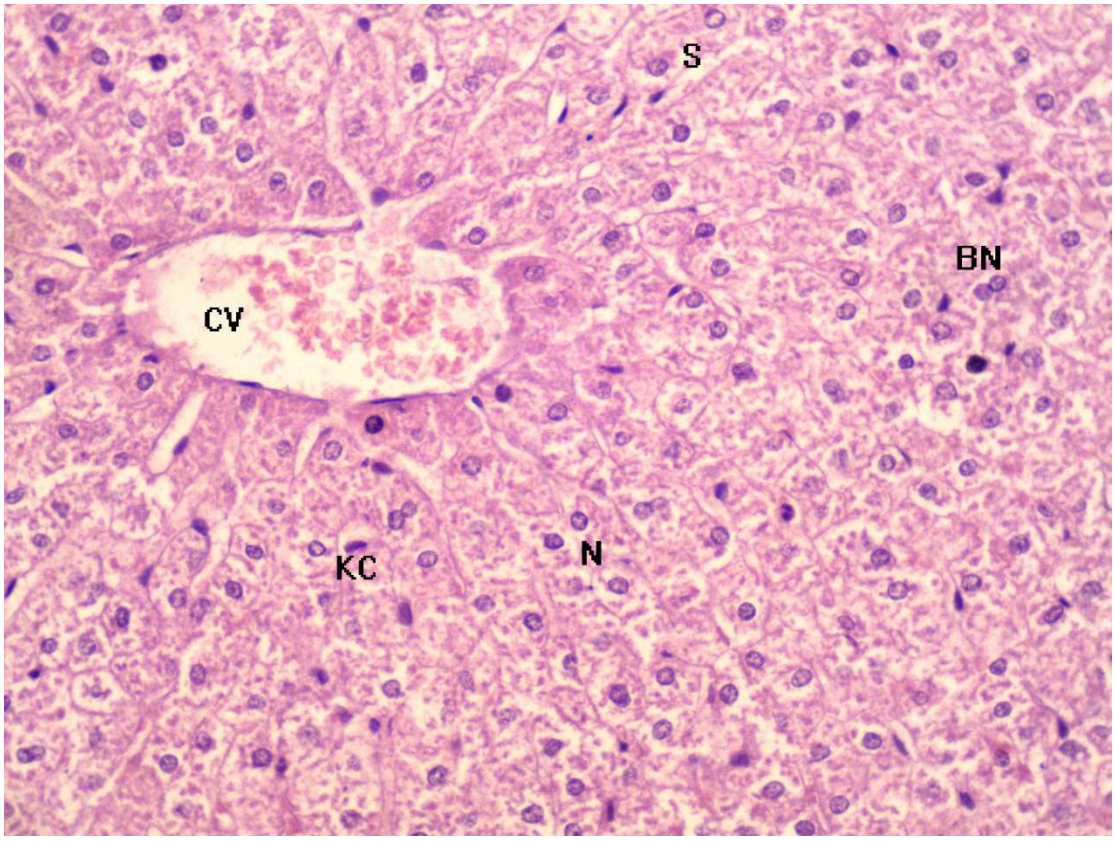

Figure 4.1.2: Hepatic tissue of the control rabbits group showing normal hepatic architecture, hepatocytes (H), with their normal nuclei (N), kupffer cells (KC) some cells are binucleated (BN), sinusoids (S) and central vein (CV). Sections stained with H&E, (X400).

B. Treated groups

It is worthy to point out that the histopathological, as well as the histochemical alterations, produced in the present study by the five applied therapeutic periods of tramadol (40mg/kg. body weight) were found to be relatively very much similar. Nevertheless, the most concrete results are those obtained in rabbits subjected to group 3,4 and 5 which treated for 30,40 and 50 days respectively. Therefore, to avoid repletion, it was preferred to restrict those observations to the group 3 and 4 besides those subjected to group 5.

The lobular architecture in the liver of tramadol-treated rabbits was preserved in all treated rabbits. In comparison with the control group (Figure 4.1.2), the following histological alterations were detected under the light microscope in the liver of tramadol-treated rabbits. The liver of rabbits treated for short period, 10 days showed some blood cells among the hepatic cells and congestion of sinusoids (Figure 4.1.3).

Diffuse kupffer cells between the sinusoids and degenerative hepatocytes were showed in rabbits liver treated with tramadol for 30, 40 and 50 days. On the other hand the inflammatory cells infiltration and fibroblastic cells proliferation (moderate) observed in groups treated for 30 days.

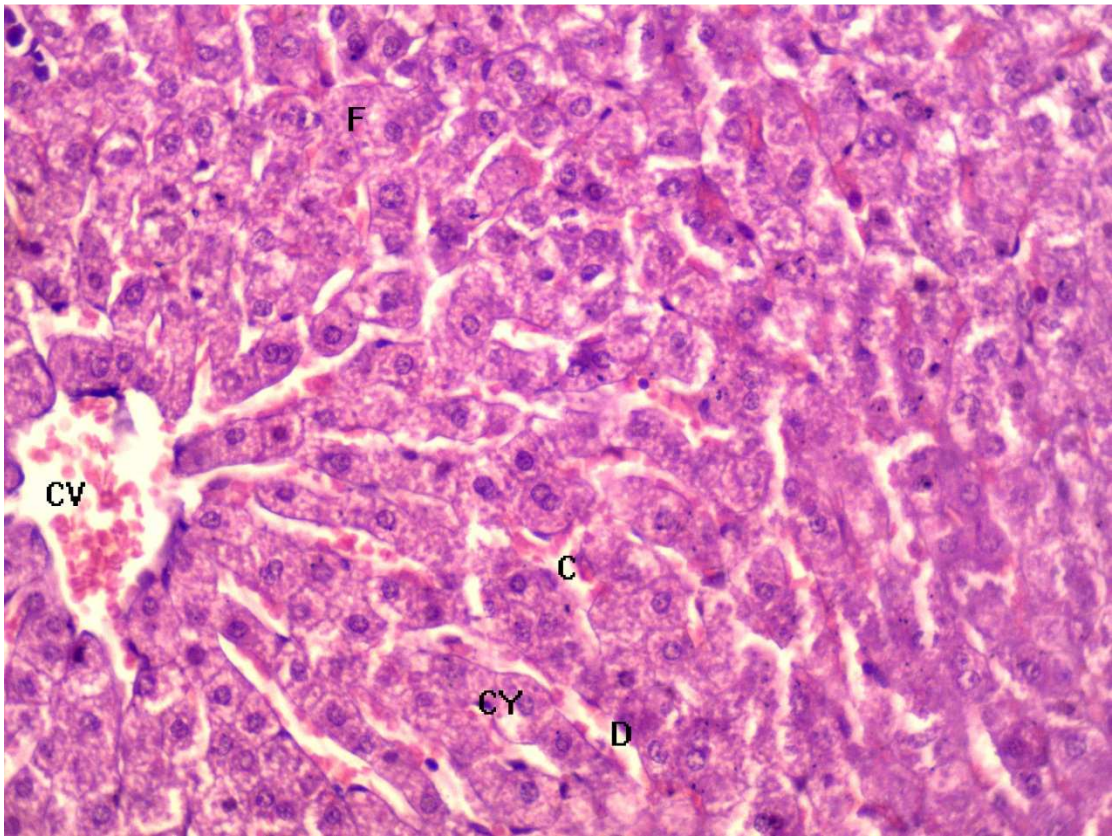

Figure 4.1.3: Hepatic tissue of tramadol-treated rabbits received (40mg/kg. body weight) for 10 days showing mild congestion of sinusoids (C), dilation of sinusoids (D), mild fatty changes (F) and intra-cytoplasmic vacuolation (CY). Sections stained with H&E, X400.

Also, these groups that treated for 30 days and more revealed severe liver damage including fatty changes, steatosis, degeneration of hepatocytes and sporadic spotty well-defined foci of parenchymal necrosis were noticed in some hepatocytes (Figures 4.1.4, 4.1.5 and 4.1.6). Furthermore, mild degree of hydropic changes and cytoplasm swelling of the hepatocytes were seen and increased in severity with increasing the dose and duration of tramadol treatment and were associated with necrosis.

Other sections in the liver of rabbits treated with tramadol for 30, 40 and 50 days showed ruptured limiting plate into the liver parenchyma (Figure 4.1.4 and Figure 4.1.5), piecemeal necrosis accompanied with inflammatory cell infiltrations and portal inflammatory infiltration is apparent around the portal

structure in liver tissue of groups treated with tramadol especially in liver tissue of rabbits treated for 40 days (Figure 4.1.5). This change appeared early in groups treated with tramadol for 30 days and more for other groups more of exposure.

In general, cytoplasmic vacuolation and pyknotic nuclei of some hepatocytes were seen especially in the necrotic ones, karyolysis, proliferation of kupffer cells and bile ductless also prominent in the liver tissue. Occasional binucleation was also observed in tramadol-treated rabbits treated for 10 days and more (Figures 4.1.5, 4.1.6 and 4.1.7).

Regarding recovery symptoms, when a patch of the above (40mg/kg. body weight) tramadol treated rabbits were left for 10 days post-drug withdrawal, then their liver was subjected to microscopic examination, it became apparent that only partial recovery seemed to occur. In such specimens, some parenchymal cells showed clear indications of improvement while others were still necrotic, the tissue is less fibrotic, less pyknotic nuclei and the bile duct proliferated (Figure 4.1.7).

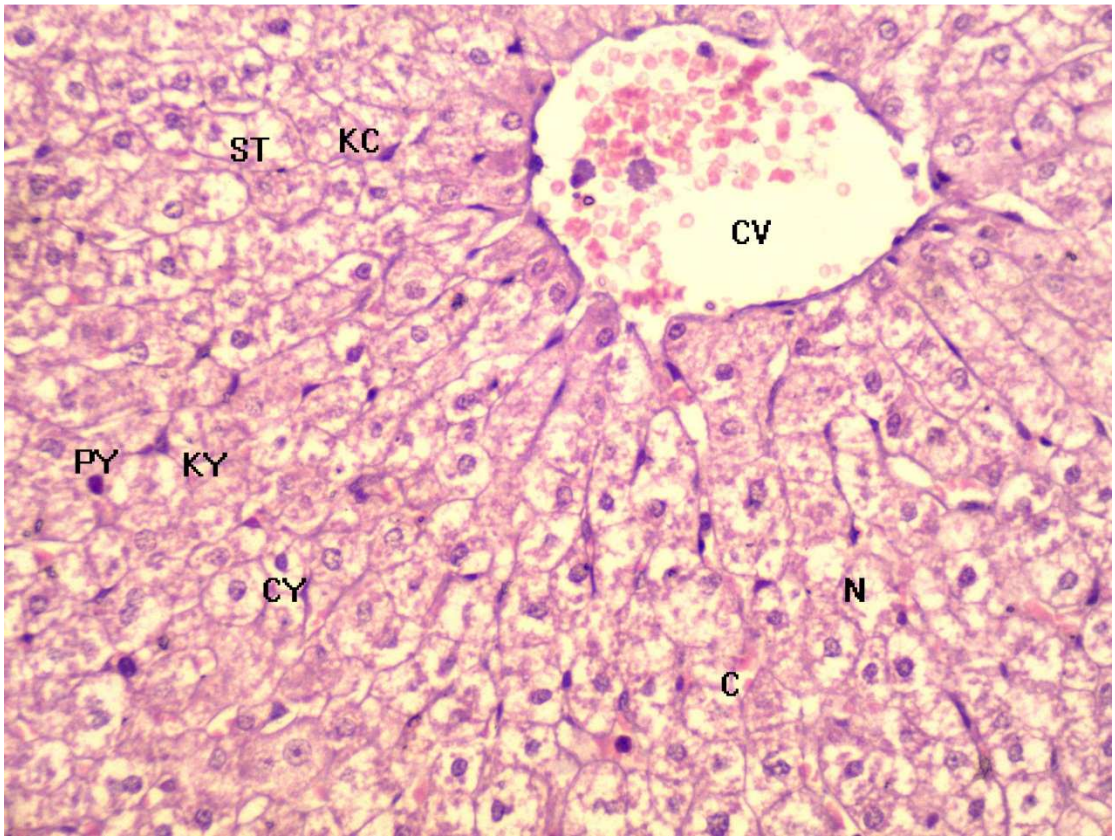

Figure 4.1.4: Hepatic tissue of tramadol-treated rabbits received (40mg/kg. body weight) for 30 days showing mild congestion of sinusoids (C), marked steatosis (ST), necrotic cells(N), cytoplasmic vacuolation (CY), pyknotic nuclei (PY), karyolysis (KY), swelling of Kupffer cells (KC) and moderate fatty changes. Sections stained with H&E, (X400).

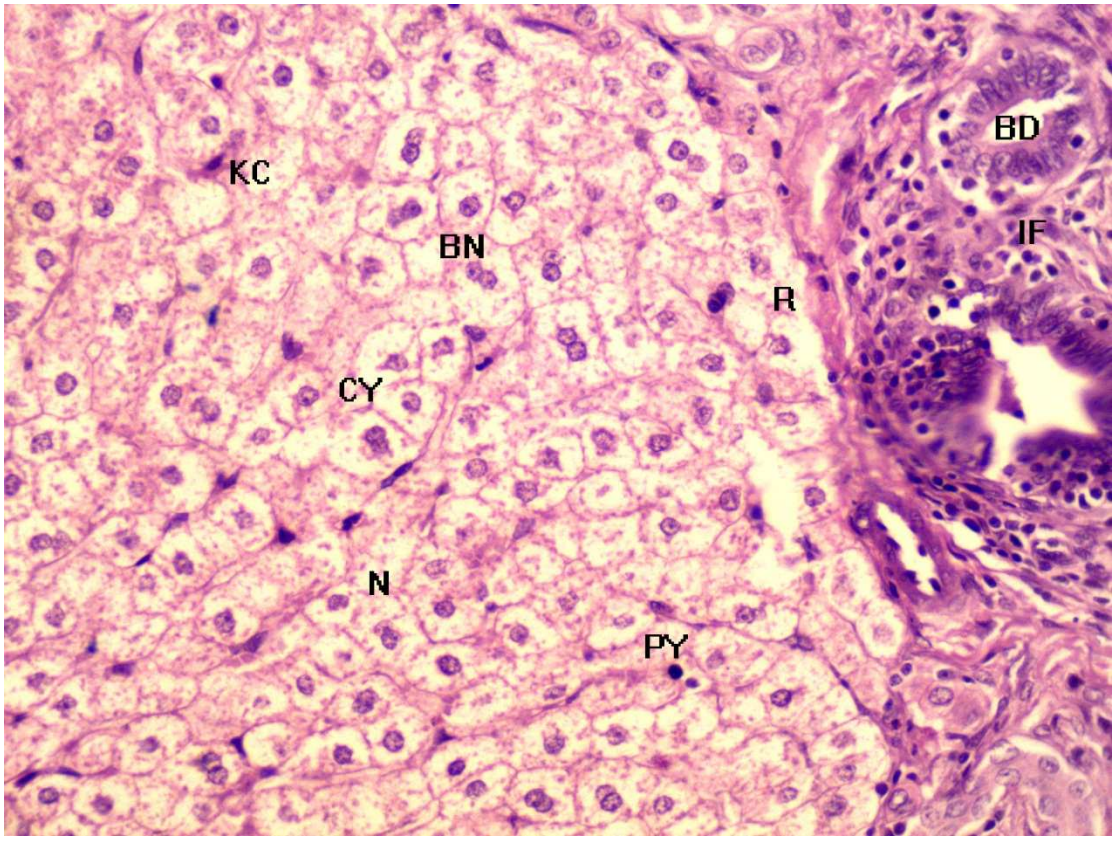

Figure 4.1.5: Hepatic tissue of tramadol-treated rabbits received (40mg/kg. body weight) for 40 days showing mild lymphoplasmacytic peri-portal infiltrates (IF), ruptured limiting plate into the liver parenchyma (R), Kupffer cell swelling (KC), some cells are binucleated (BN), piecemeal necrosis accompanied with inflammatory cell infiltrations (N) and fatty changes accompanied with cytoplasmic vacuolation (CY). Sections stained with H&E, (X400).

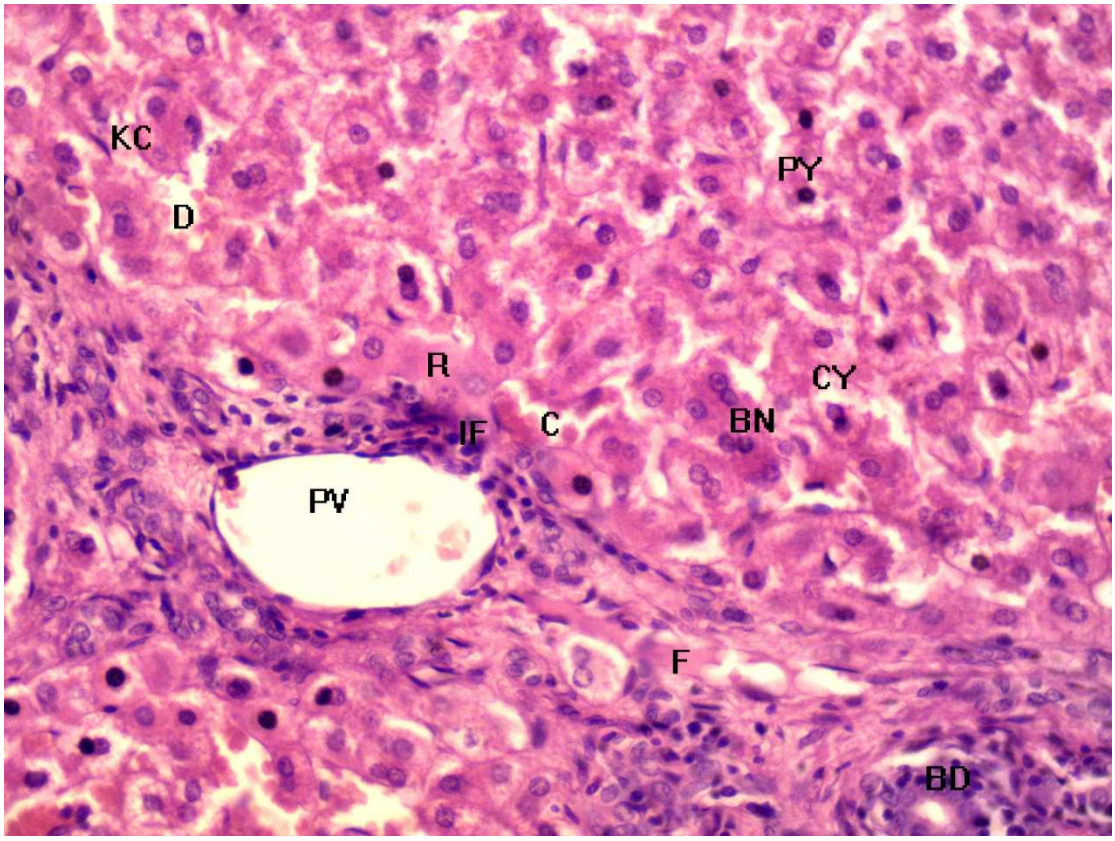

Figure 4.1.6: Hepatic tissue of tramadol-treated rabbits received (40mg/kg. body weight) for 50 days showing cytoplasmic vacuolation (CY), mononuclear inflammatory infiltrate (IF), periportal fibrosis (F), dilated sinusoids (D), ruptured limiting plate (R), congestion of sinusoids (C), pyknotic nuclei (PY), binucleation (BN), Kupffer cells swelling (KC) and the cells are completely necrotic. Sections stained with H&E, (X400).

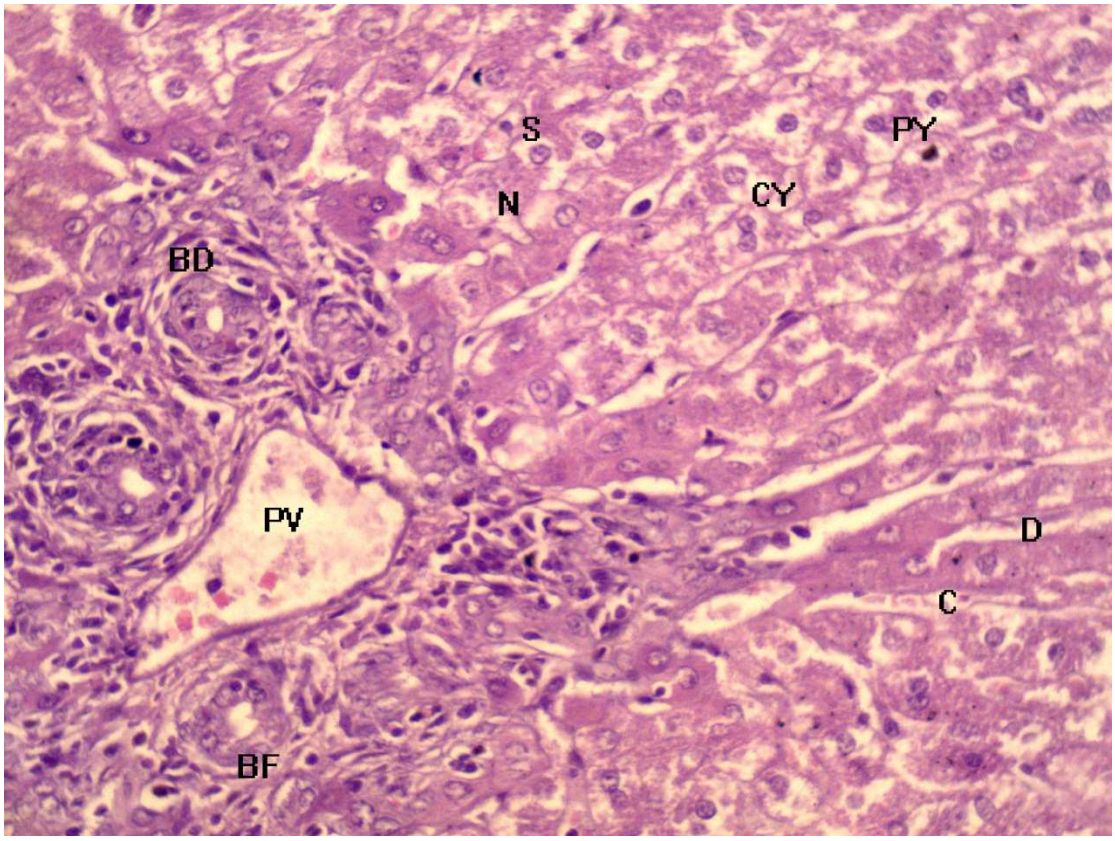

Figure 4.1.7: Hepatic tissue of tramadol-treated rabbits received (40mg/kg. body weight) for 30 days and left for a recovery period (10 days) showing partial improvement in the liver parenchyma whereas the tissue is less fibrotic, less pyknotic nuclei (PY) and less necrotic cells (N), but there is an existence some of the past histopathological changes, moderate steatosis (ST), karyolitic nuclei (KY), cytoplasmic vacuolation (CY), bile duct proliferation (BF), dilation of sinusoids (D) and congestion of sinusoids (C). Sections stained with H&E, (X400).

4.2 Histopathology of kidney

A. Control group

The kidney of control rabbit is a bean-shaped covered by a firm connective tissue-capsule composed of collagenous fibrous and few elastic fibers. The substance of the kidney is differentiated into two regions; an outer cortex and an inner medulla. The latter is formed of conical pyramids, the apices of which are directed lowered the renal pelvis. Each medullary pyramid with the corresponding part of the cortex represents a renal lobe.

The renal lobes consist of uriniferous tubules and stromal tissue. The latter is formed of a network of connective tissue. The uriniferous tubules are composed of two principal portions; an active part or the nephron, which is the structural and functional unit of kidney, and an excretory part of collecting tubule. The nephron is formed of the renal or Malpighian corpuscle, proximal convoluted tubule, descending and ascending limbs of Henle's loop and distal convoluted tubule. The cortex, in between the medullary rays, consists of Malpighian corpuscles and both proximal and distal convoluted tubules while the medulla consists mainly of the descending and ascending limbs of Henle's loops.

However, the collecting tubules are located in both cortical and medullary regions. The Malpighian corpuscle consists of a tuft of blood capillaries, the glomerulus and Bowman's capsule. The latter is a double walled cup formed of two layers of simple squamous epithelium, an outer parietal layer and an inner visceral one separated by a capsular space. The parietal layer is continuous with the wall of the proximal convoluted tubule. It is composed of simple squamous epithelium resting on a thin basement membrane and surrounded by a thin layer of connective tissue. The glomerulus consists of number of capillary loops of an afferent and efferent arteriole separated by reticular connective tissue fibers.

Kidney specimens obtained from the control group showed normal histological structures of the glomeruli and the renal tubules in the cortical and medullary portions (Figure 4.2.1).

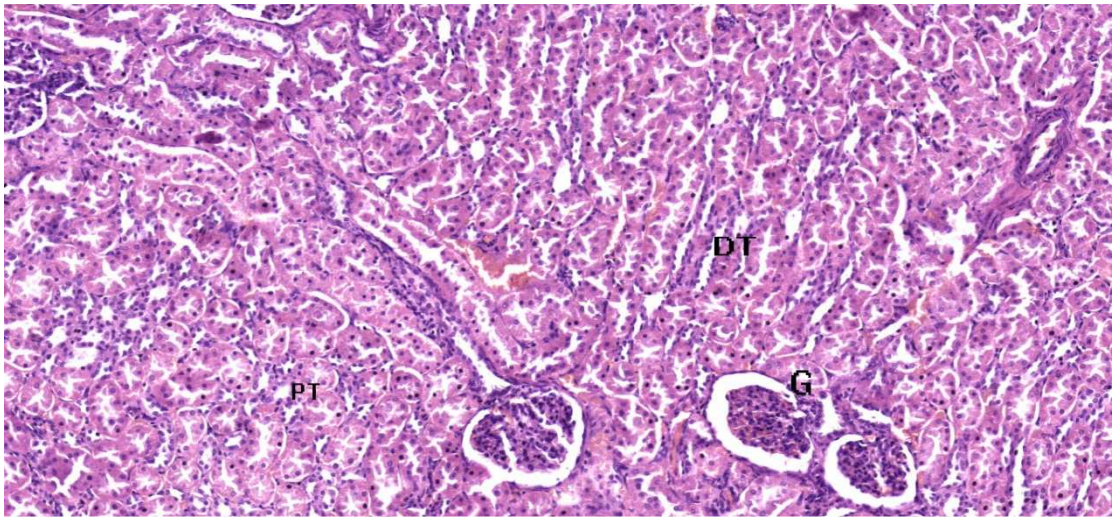

Figure 4.2.1: Photomicrograph of Kidney section of rabbits from control group showing the normal histological structure of the glomerulus (G) surrounded by proximal convoluted tubules (PT) and distal convoluted tubules (DT) in the cortical portion. Sections stained with H&E, (X100).

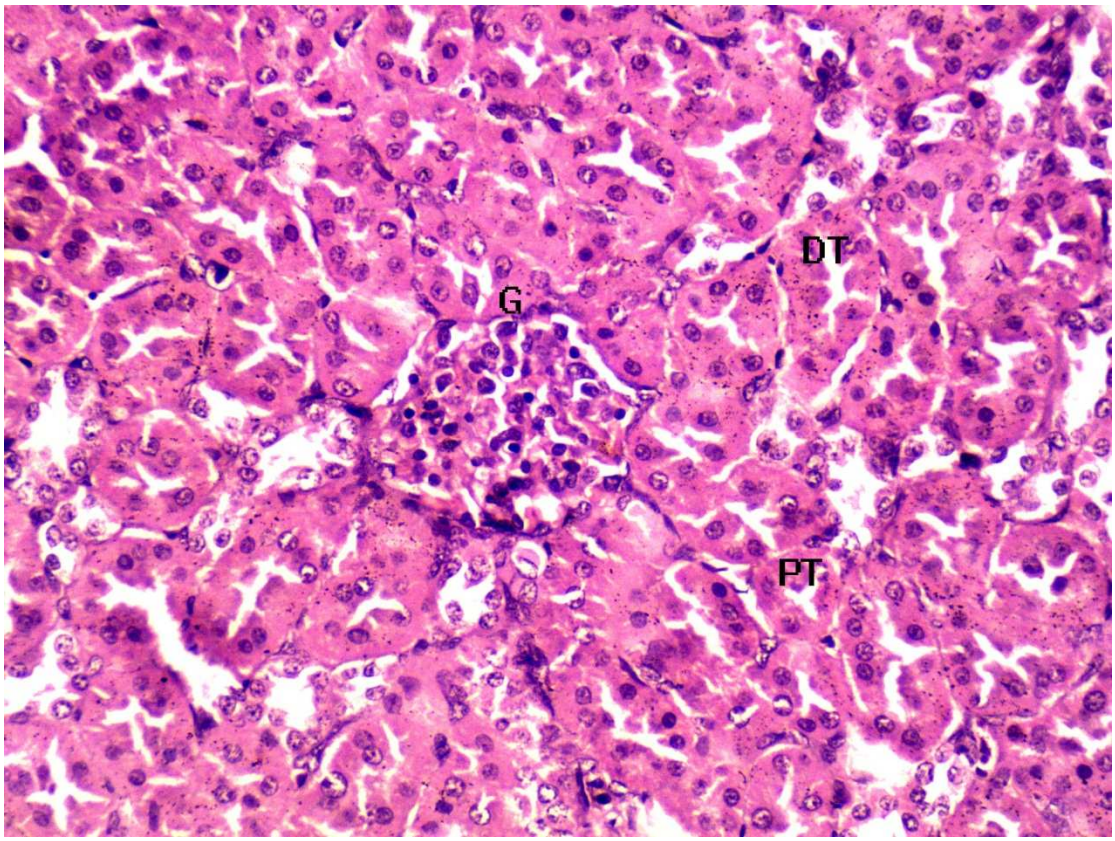

Figure 4.2.2: Photomicrograph of rabbit's kidney section from the control group showing the histological structure of the normal renal structure, glomerulus (G), distal tubule (DT) and proximal tubule (PT). Sections stained with H&E, (X400).

B. Treated groups

Specimens taken from group 1 and 2 which treated for 10 and 20 days exhibited the normal structure of kidney and there is no significance alterations. Examination of specimens taken from group 3 and 4 which treated for 30 and 40 days showed variable pathological changes in glomeruli and some parts of the urinary tubules. Such changes illustrated an existence of swelling in the lining epithelium of the glomerulus. Also, they were represented by renal injury with tubular cell swelling and dilation in the interlobular cortical blood vessels were noticed (Figure 4.2.3). In addition, swelling in the lining epithelium of the renal tubules and the presence of inflammatory cellular infiltration were noticed (Figure 4.2.4 and Figure 4.2.5).

Moreover, Figures 4.2.3 and 4.2.4 showed degeneration in the epithelial cells lining the renal tubules at the cortical zone with pyknotic and karyolysed nuclei. The most severe changes were observed in the proximal convoluted tubules which consisted of marked hydropic degeneration and cytoplasmic vacuolization of tubular epithelial cells. The alterations in cortical region include expansion of glomerulus, glomerular tuft atrophy, focal tubular necrosis and mild mononuclear cell infiltration (Figure 4.2.4). More excessive necrosis of tubular epithelial cells, dilated tubules and expansion of glomerulus were observed after 50 days of tramadol treatment (Figure 4.2.5)

In recovery group the rabbits treated with tramadol for 30 dose and left for 10 days without administration of tramadol, some of the previous observations were disappeared such as the expansion of glomerular chamber is reduced, the cells are less hydropic degeneration and no inflammatory infiltration were observed (Figure 4.2.6).

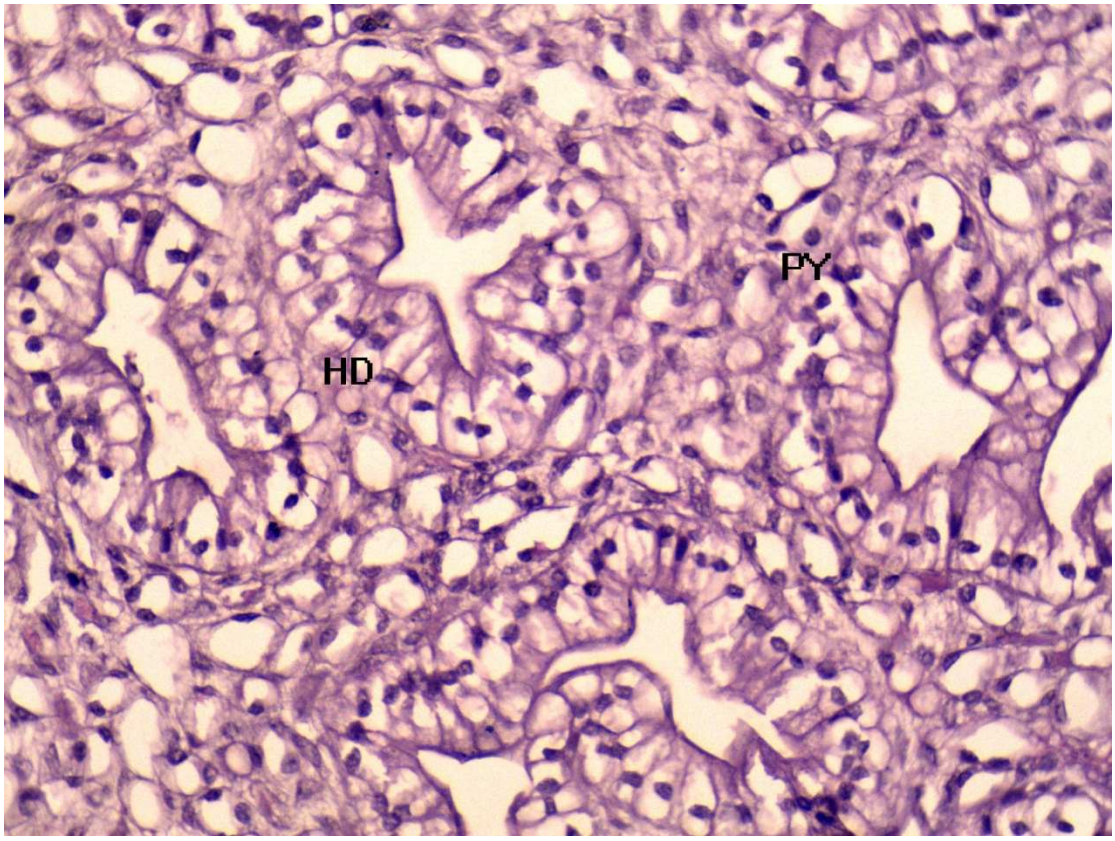

Figure 4.2.3: Photomicrograph of rabbits kidney in the medullary portion after treated with tramadol for 30 days showing marked hydropic degeneration (HD) accompanied with mild mononuclear cell infiltration and pyknotic nuclei (PY). Sections stained with H&E, (X400).

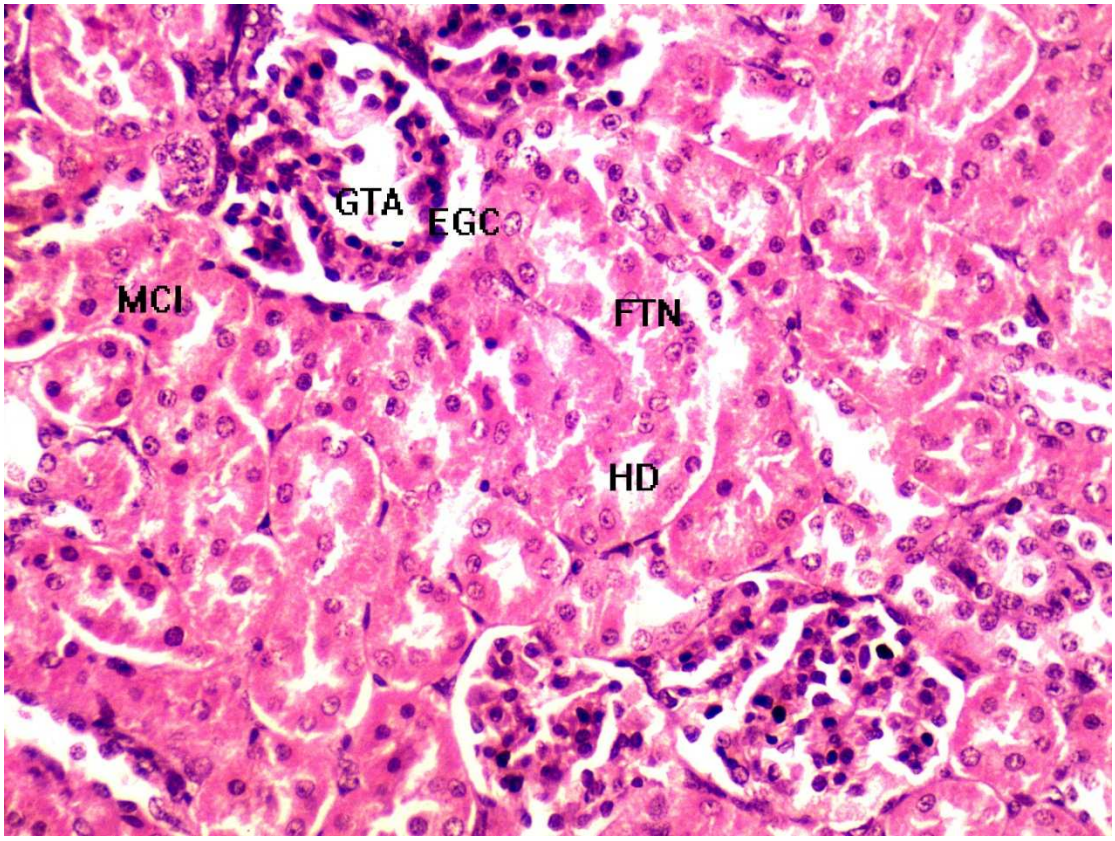

Figure 4.2.4: Photomicrograph of rabbits kidney section after treated with tramadol for 40 days showing expansion of the glomerular chamber (EGC), glomerular tuft atrophy(GTA), focal tubular necrosis (FTN), hydropic degeneration (HD) and mild mononuclear cell infiltration (MCI). Sections stained with H&E, (X400).

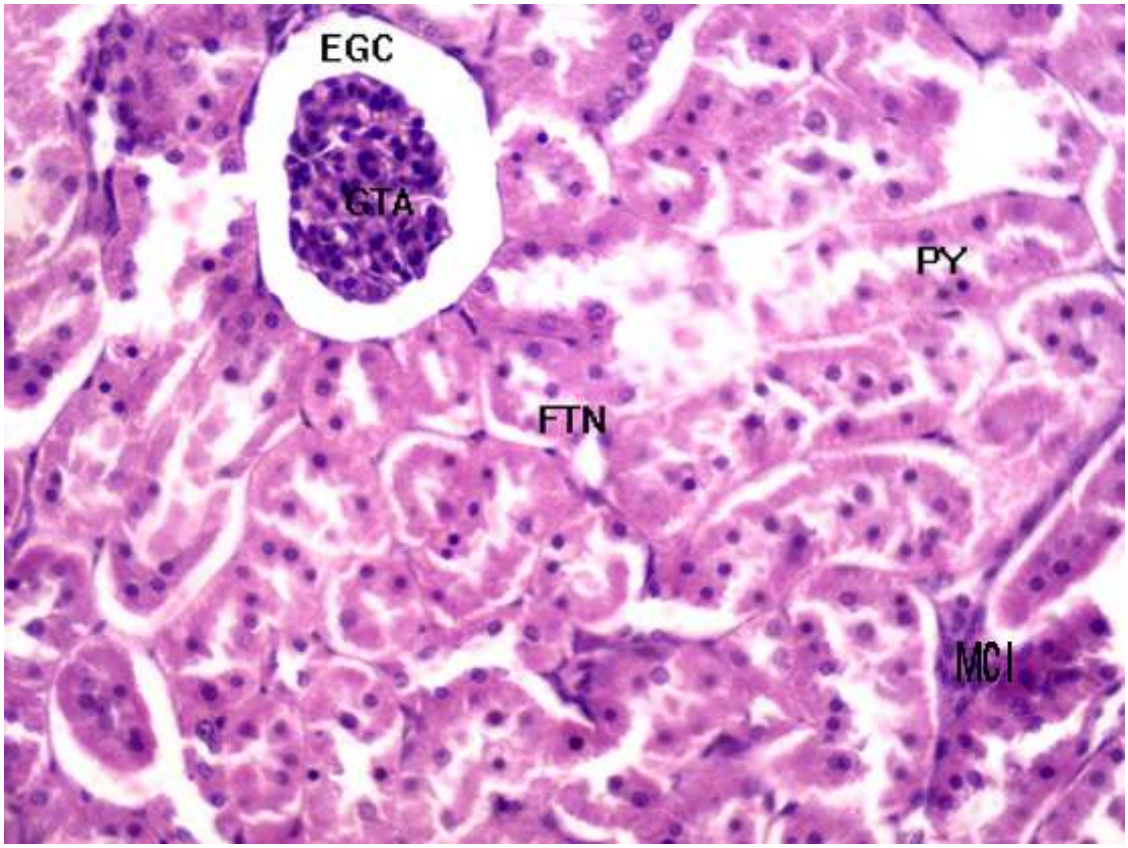

Figure 4.2.5: Photomicrograph of rabbits kidney section after treated with tramadol for 50 days showing expansion of the glomerular chamber (EGC), glomerular tuft atrophy (GTA), focal tubular necrosis (FTN), and mild mononuclear cell infiltration (MCI). Sections stained with H&E, (X400).

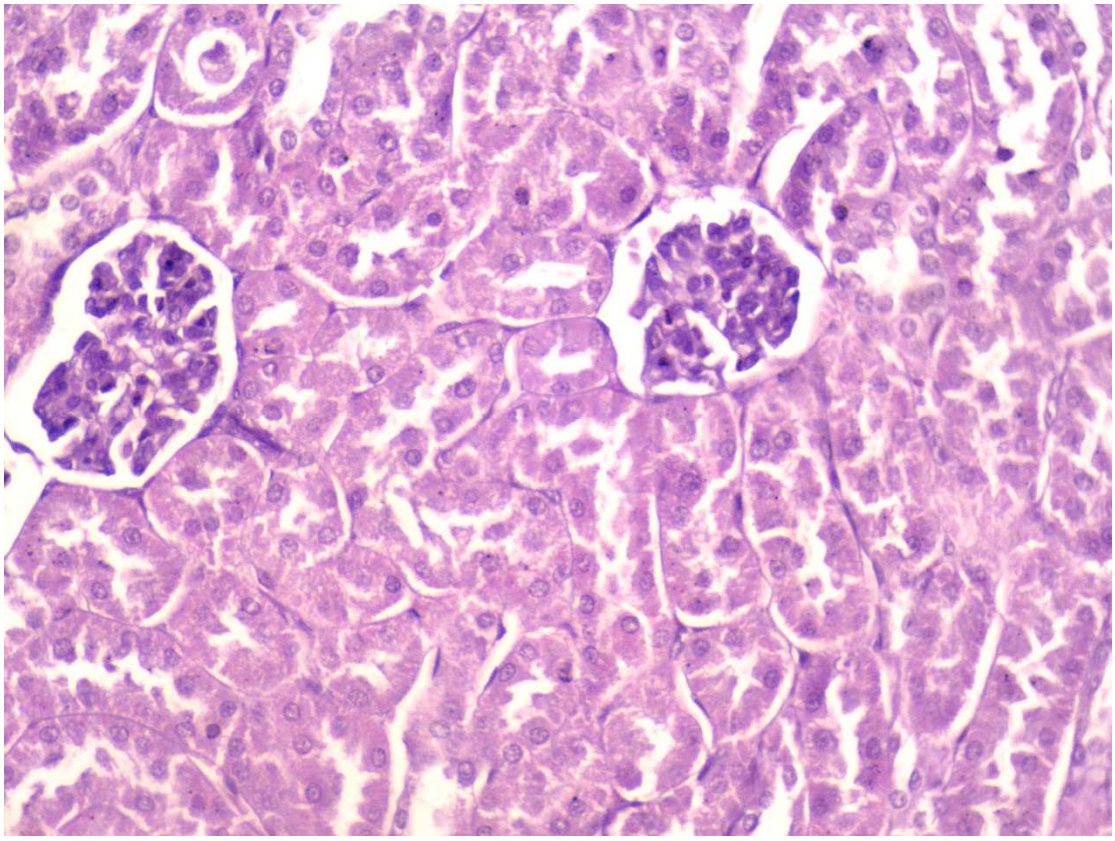

Figure 4.2.6: Photomicrograph of rabbits kidney section after treated with tramadol for 30 days and left for a recovery period showing partial improvement in the tubules of kidney and renal corpuscles, no inflammatory infiltrate, expansion of glomerulus is reduced and existence some of the past alterations as hydropic degeneration. Sections stained with H&E, (X400).

4.3 Histochemistry of liver polysaccharides

A. control group

Examination of liver of control rabbits stained with PAS showed normal content of glycogen particles which appeared as deeply red purple colored PAS positive inclusions densely located inside the cytoplasm. Most of the PAS positive products were displaced laterally towards one side of the cell (glycogen migration/flight phenomenon) caused by the effects of the fixative on the tissue. None of the nuclei of all liver cells acquired any positive stainability in that case which indicates the non-existence of any PAS-positive materials (Figure 4.3.1).

B. Treated groups

The liver of rabbits treated with tramadol (40mg/kg. body weight) intramuscularly for 10 days showed weakly stained with PAS positive materials compared with the control animals (Figure 4.3.2). However, rabbits treated with tramadol for 30 days showed a marked decrease in glycogen content of the hepatic cells especially in those located in the peripheral lobular areas (Figure 4.3.3).

On the 50^{th} day after tramadol administration, glycogen diminution became more obvious and most of the hepatic cells appeared to have lost a considerable proportion of their polysaccharide (glycogen) inclusions in comparison to the rabbits hepatocytes of the control group. However, in such cases, loss of glycogen was more striking in the peripheral lobular cells than those occupying the pericentral areas. Nevertheless, that depletion was not uniform in different cells of the same material. In some cells, glycogen contents appeared to be less affected and still displayed a noticeable positive PAS-reaction (Figure 4.3.4)

Regarding recovery of polysaccharide (glycogen) contents in rabbits which had received a 30 dose of tramadol, it was found that only partial recovery was achieved ten days after tramadol withdrawal. In such cases, only some of the hepatic cells had almost restored their normal staining affinity for PAS technique while others appeared still poor in their glycogen contents (Figure 4.3.5).

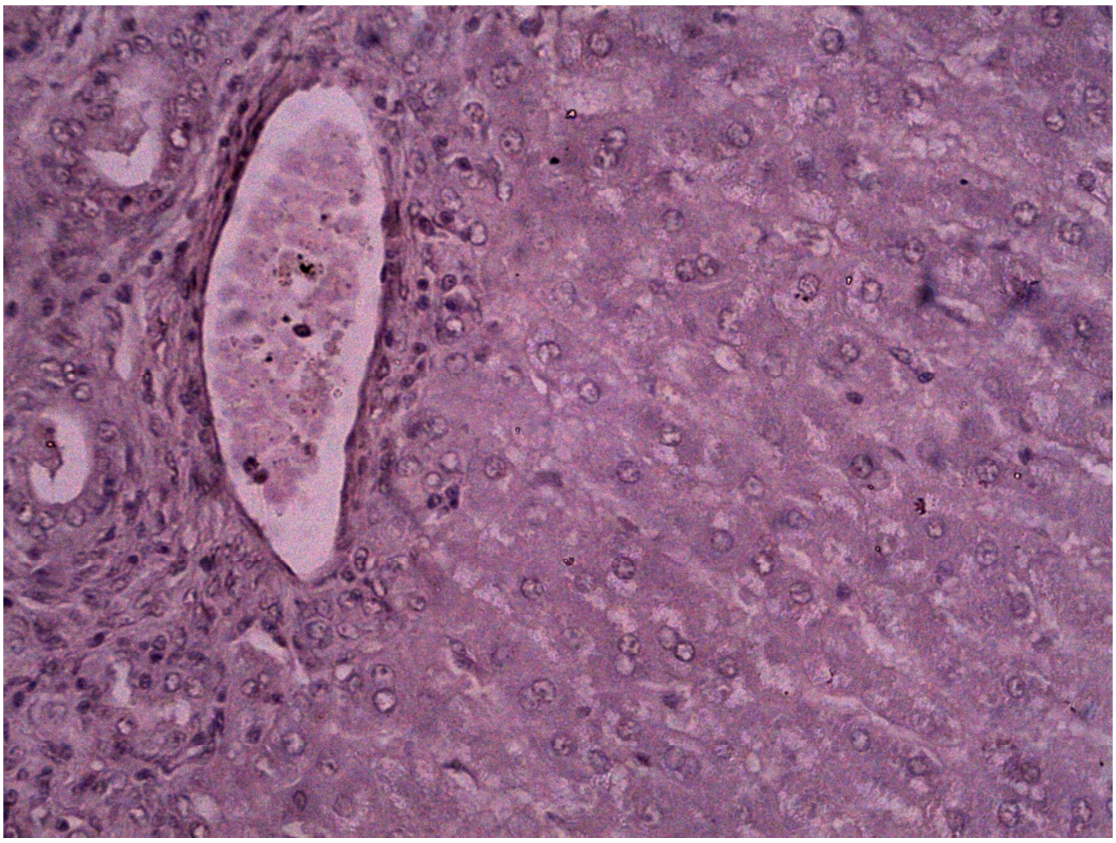

Figure 4.3.1: A photomicrograph of a liver section of rabbits in the control group showing the normal content of glycogen as deeply purple colored PAS-positive inclusions densely located in the cytoplasm. (PAS X400).

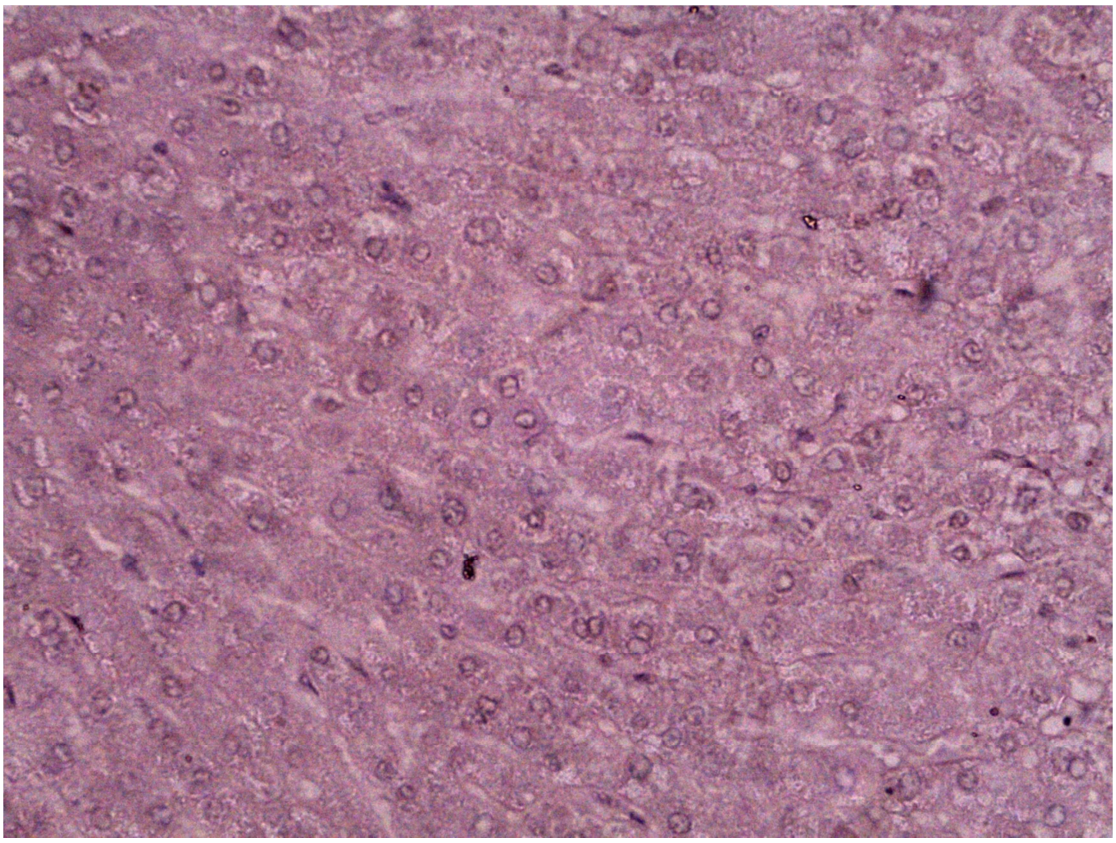

Figure 4.3.2: A photomicrograph of a liver section of a rabbit given (40mg/kg. body weight) of tramadol intramuscularly for 10 days (low dose) showing approximately the normal appearance of glycogen in the hepatocytes. (PAS X400).

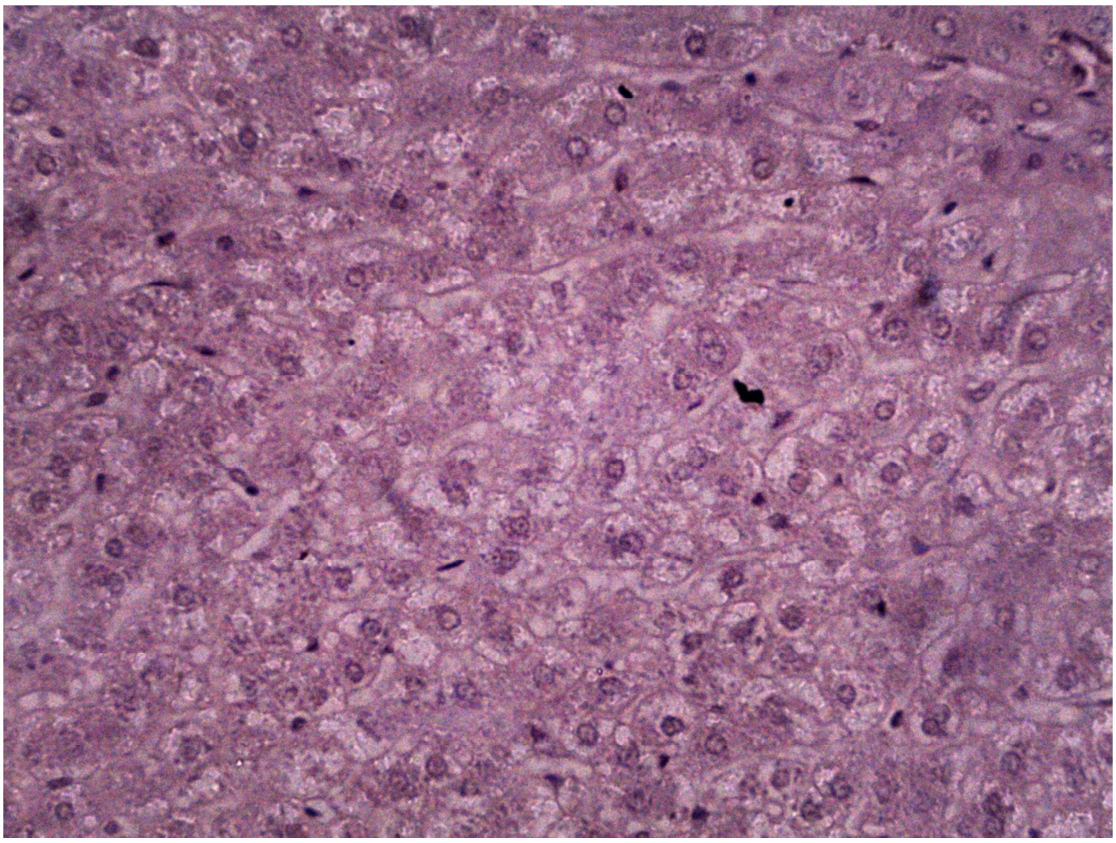

Figure 4.3.3: A photomicrograph of a liver section of rabbits given (40mg/kg. body weight) of tramadol intramuscularly for 30 days showing marked glycogen depletion of the liver hepatocytes. (PAS X400).

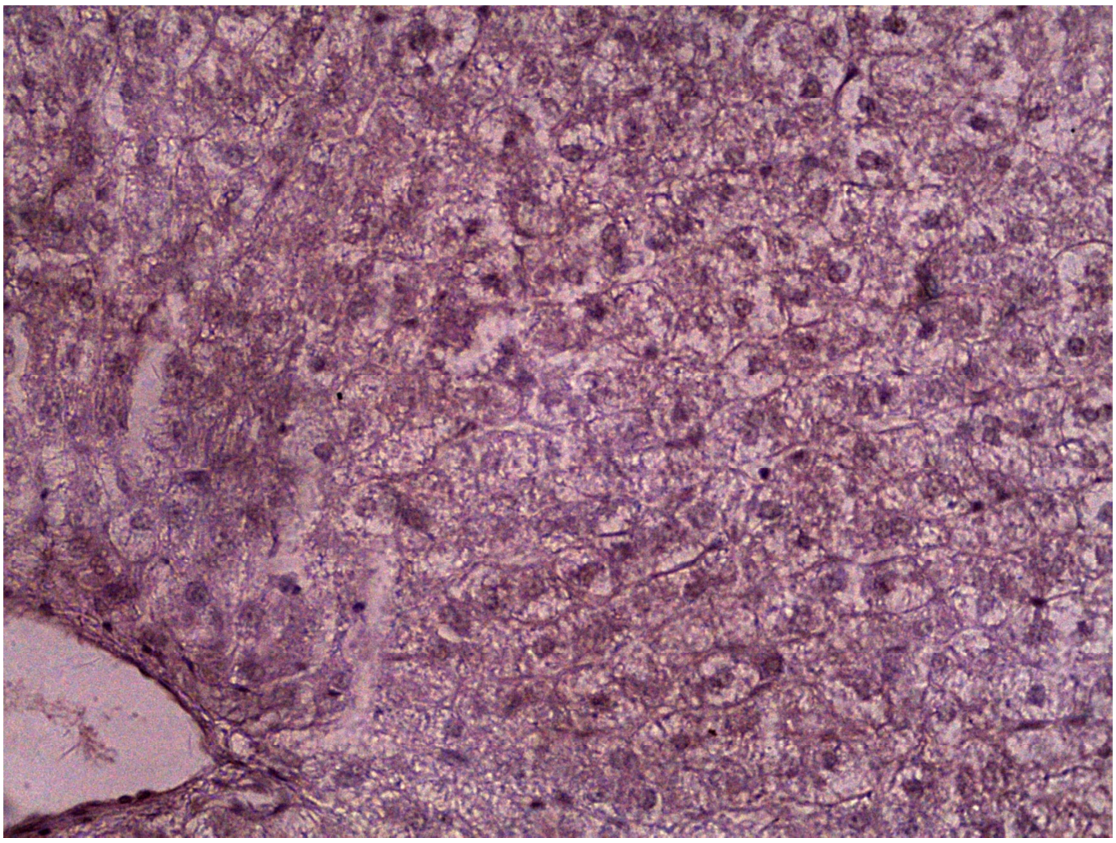

Figure 4.3.4: A photomicrograph of a liver section of a rabbit given (40mg/kg. body weight) of tramadol intramuscularly for 50 days (high dose) showing severe glycogen depletion especially in the peripheral lobular cells. (PAS X400).

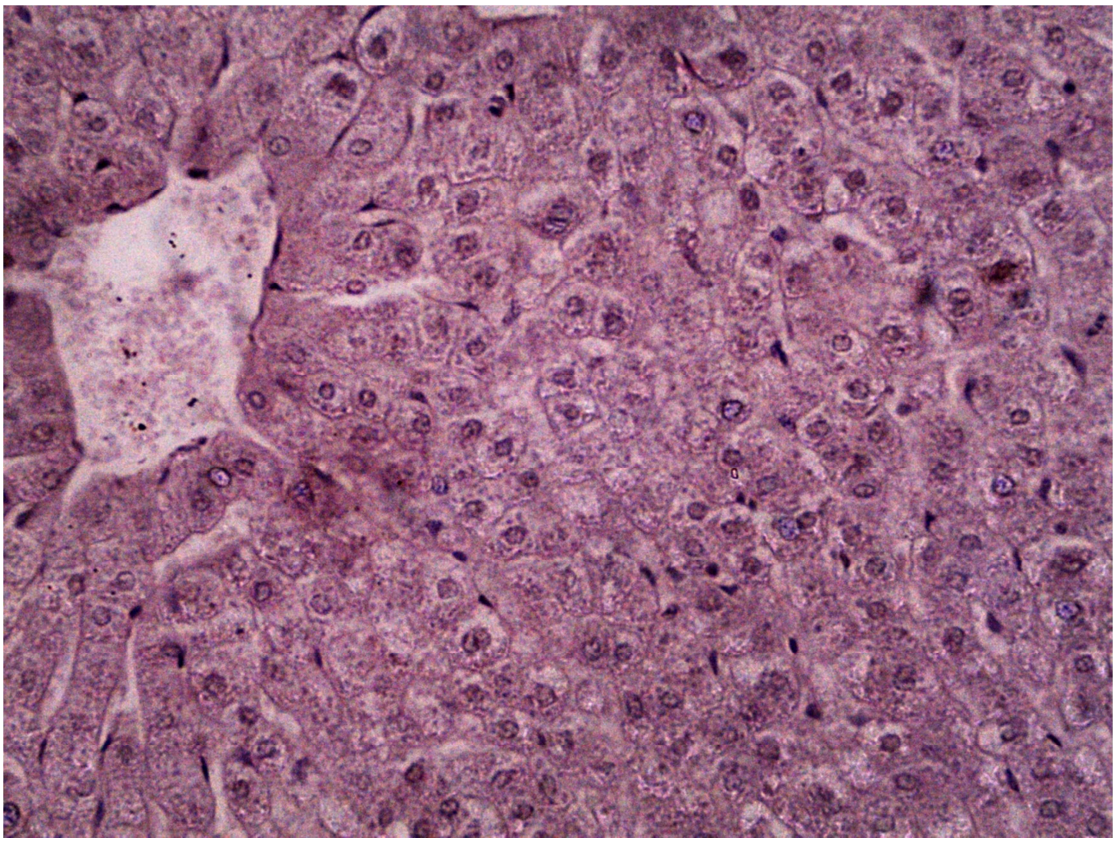

Figure 4.3.5: A photomicrograph of a liver section of a rabbit given (40mg/kg. body weight) of tramadol intramuscularly for 30 days and left for a recovery period (10 days) showing partial improvement in the glycogen content of liver hepatocytes especially the central area of hepatic cells radiating from the central vein. (PAS X400).

4.4 Histochemistry of liver total proteins

A. Control group

The protein contents of the liver cells of control rabbits (Figure 4.4.1) were demonstrated by the mercuric bromophenol blue method as blue granules against a light-blue ground cytoplasm, which indicate the presence of some soluble proteins. The protein granules were scattered all over the cytoplasm. The cells were limited by intensely stained plasma membranes. The nuclear envelopes and nucleoli as well as some chromatin elements were also positively stained. Kupffer cells were moderately stained with bromophenol blue; which reveals their moderate amount of protein materials.

B. Treated groups

The animals received tramadol (40mg/kg. body weight) for 10 days did not manifest significant obvious changes in both cytoplasmic and nuclear total protein contents of their hepatocytes (Figure 4.4.2). The cell and the nuclear membranes, were also stained lighter than the normal condition. The decrease in total protein contents became more pronounced on the 20^{th} day following the treatment.

Hepatocytes of rabbits treated with tramadol (40mg/kg. body weight) for 30 and 50 days demonstrated a severe reduction of protein contents in comparison to the hepatocytes of the control rabbits (Figure 4.4.3 and Figure 4.4.4). The cytoplasm exhibited a diffusely weak bromphenol blue reaction with some scattered fine and moderately sized granules lying mainly near the cell and nuclear membranes.

The protein contents and the general appearance of the hepatic cells were approximately restored after administration of tramadol and left rabbits for a recovery period (10 days), the majority of cells contained abundant well-stained proteinic granules in the cytoplasm. The staining affinity of the plasma membranes and the nuclear envelopes as well as the chromatin elements have clearly elevated than those of the previous case (Figure 4.4.5).

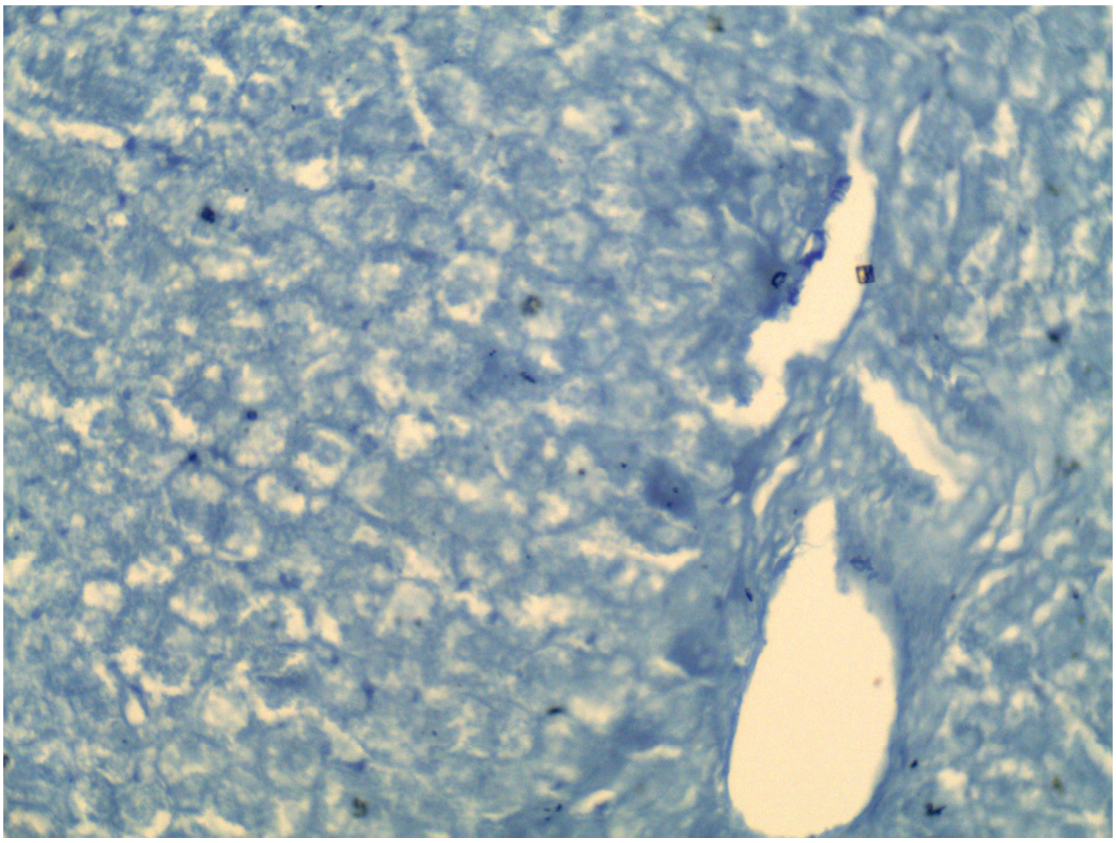

Figure 4.4.1: A photomicrograph of a liver section of rabbits in the control group showing the normal content of proteins in the liver cells as deeply blue colored. (MBB X400).

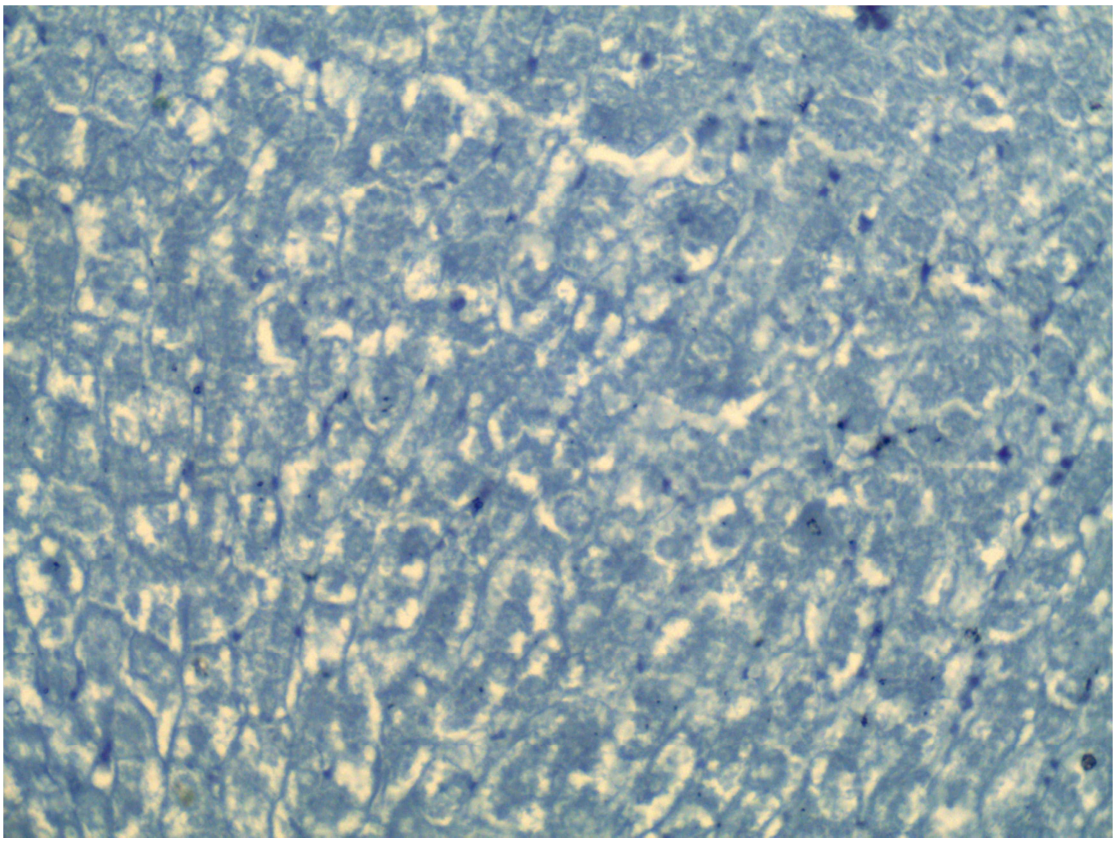

Figure 4.4.2: A photomicrograph of a liver section of a rabbit given (40mg/kg. body weight) of tramadol intramuscularly for 10 days (low dose) displaying the closely normal content of proteins in the hepatocytes. (MBB X400).

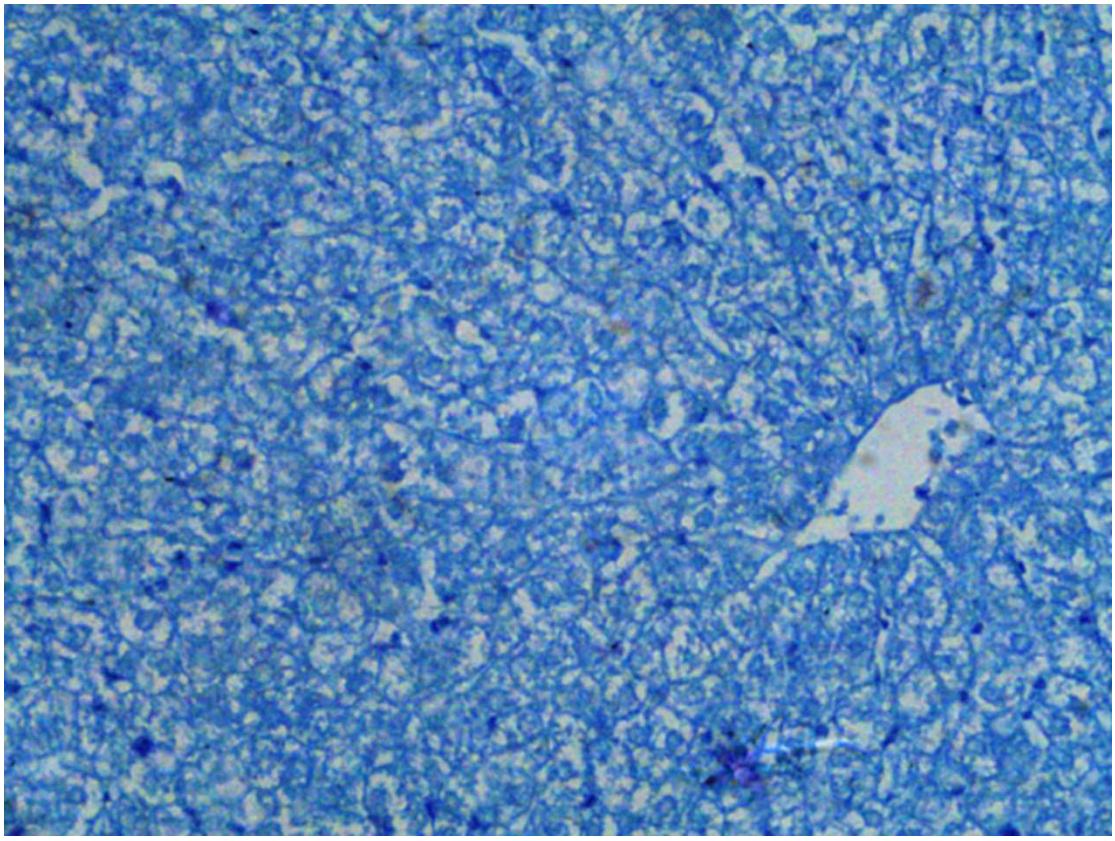

Figure 4.4.3: A photomicrograph of a liver section of rabbits given (40mg/kg. body weight) of tramadol intramuscularly for 30 days the showing marked depletion of protein contents in the liver cells. (MBB X400).

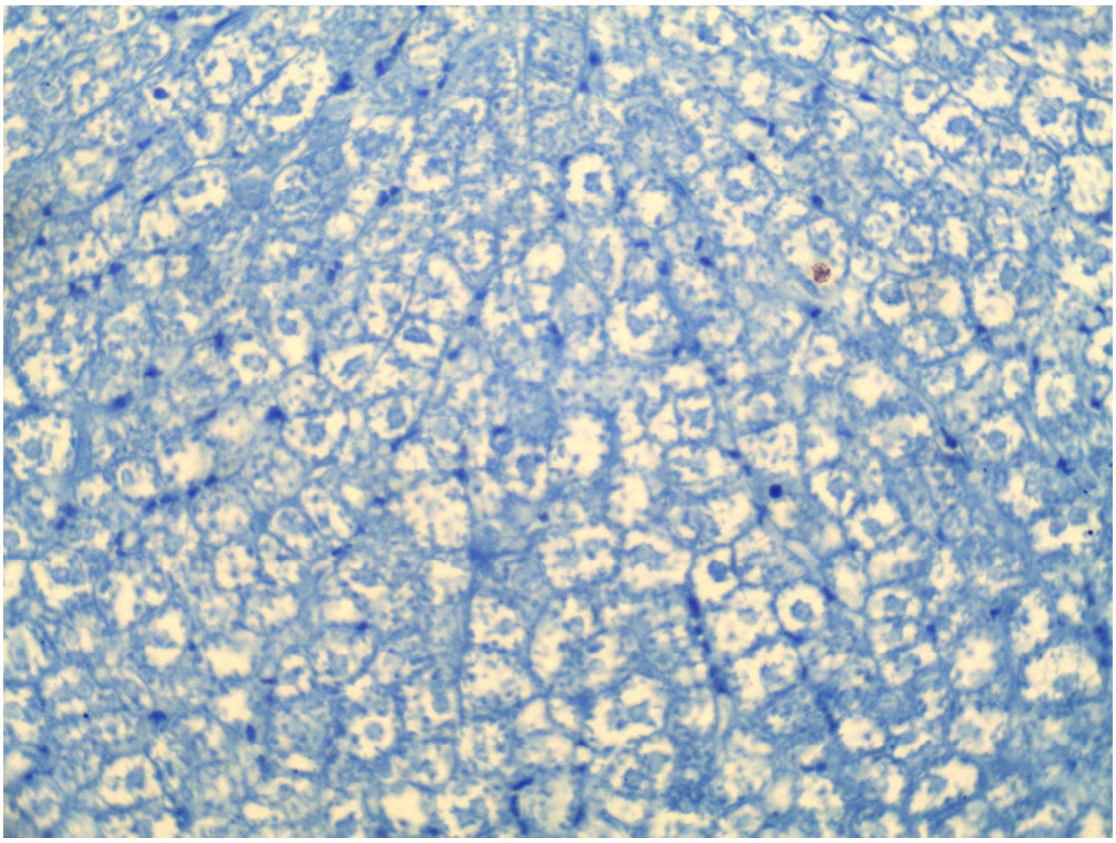

Figure 4.4.4: A photomicrograph of a liver section of rabbits given (40mg/kg. body weight) of tramadol intramuscularly for 50 days showing dramatically affected liver cells with the depletion of protein contents in the hepatocytes. (MBB X400).

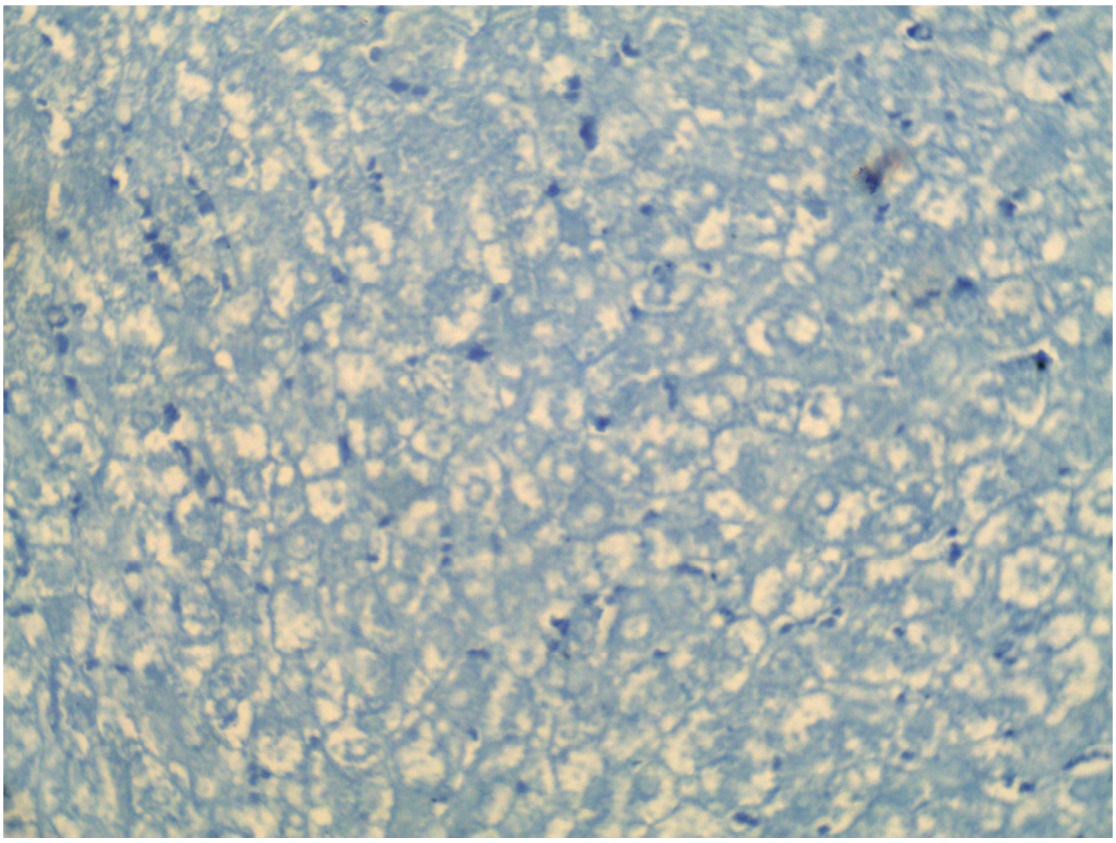

Figure 4.4.5: A photomicrograph of a liver section of a rabbit given (40mg/kg. body weight) of tramadol intramuscularly for 30 days and left for a recovery period (10 days) showing partial improvement in the amounts of protein in liver cells. (MBB X400).

4.5 Histochemistry of kidney polysaccharides

A. Control group

Examination rabbits kidney of control group stained with PAS technique showed the positive materials in the cortical tissues. Parietal and visceral walls of the Bowman's capsule, capillaries of the glomeruli, the basement membranes of the proximal and distal convoluted tubules and the brush borders of the proximal convoluted tubules exhibited strong positive reaction with PAS technique. The cytoplasm of the tubules was stained faintly white, while the nuclei showed PAS negative reaction (Figure 4.5.1).

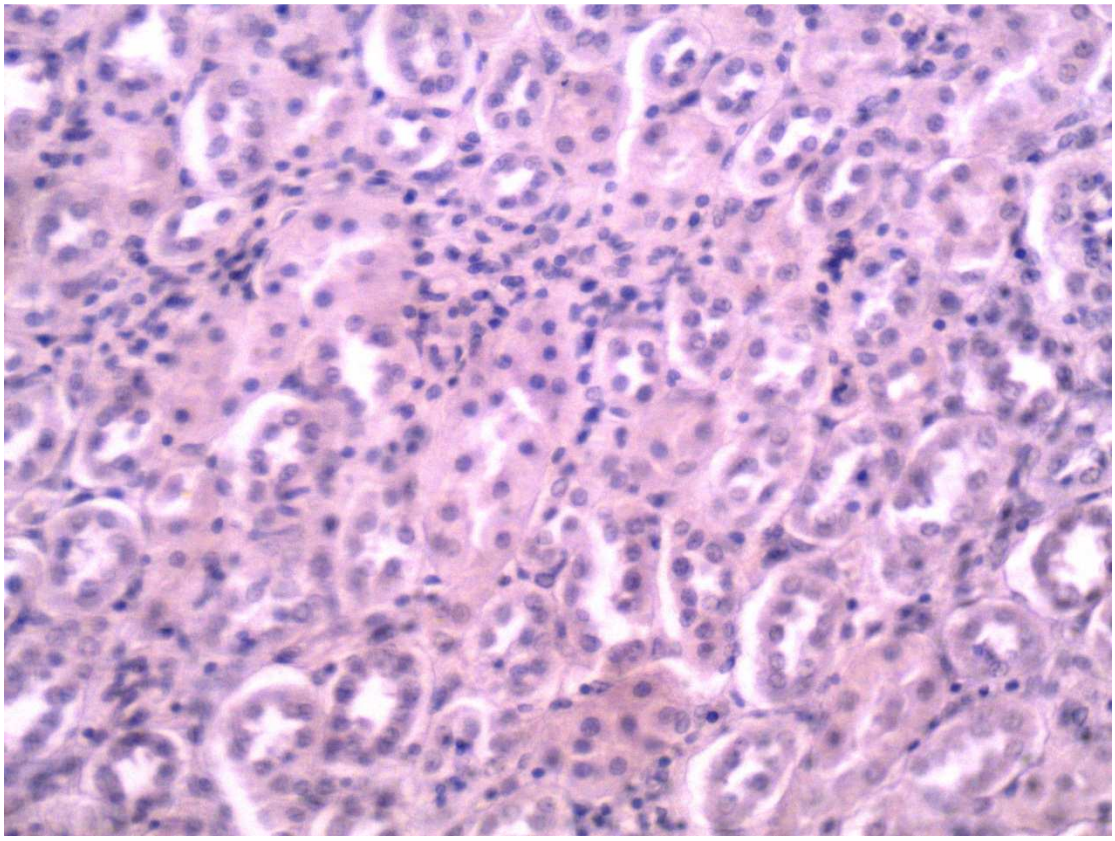

Figure 4.5.1: A photomicrograph of a kidney section of rabbits in the control group showing normal content of glycogen as deeply purple colored PAS-positive inclusions densely located in the cytoplasm of renal tubules. (PAS X400).

B. Treated groups

Examination of kidney sections of the rabbits treated with tramadol (40mg/kg. body weight) for 10 days revealed a normal PAS reaction in the glomeruli, Bowman's capsules, the basement membranes of the renal tubules and the brush borders of the proximal convoluted tubules. The stainability was slightly

less as in the normal one. Microscopic observation of kidneys of rabbits treated with tramadol for 30 days showed moderate diminution in polysaccharide (Figure 4.5.2) and marked diminution in PAS positive material in the renal corpuscles and tubules of rabbits treated for 50 days (Figure 4.5.3).

In the recovery group, rabbits treated with tramadol for 30 days and left for 10 days without tramadol administration, some cells of regenerated renal tubules restored their original picture familiar in the PAS-preparations revealing clear signs of improvement but still somewhat lesser than typical normal picture (Figure 4.5.4).

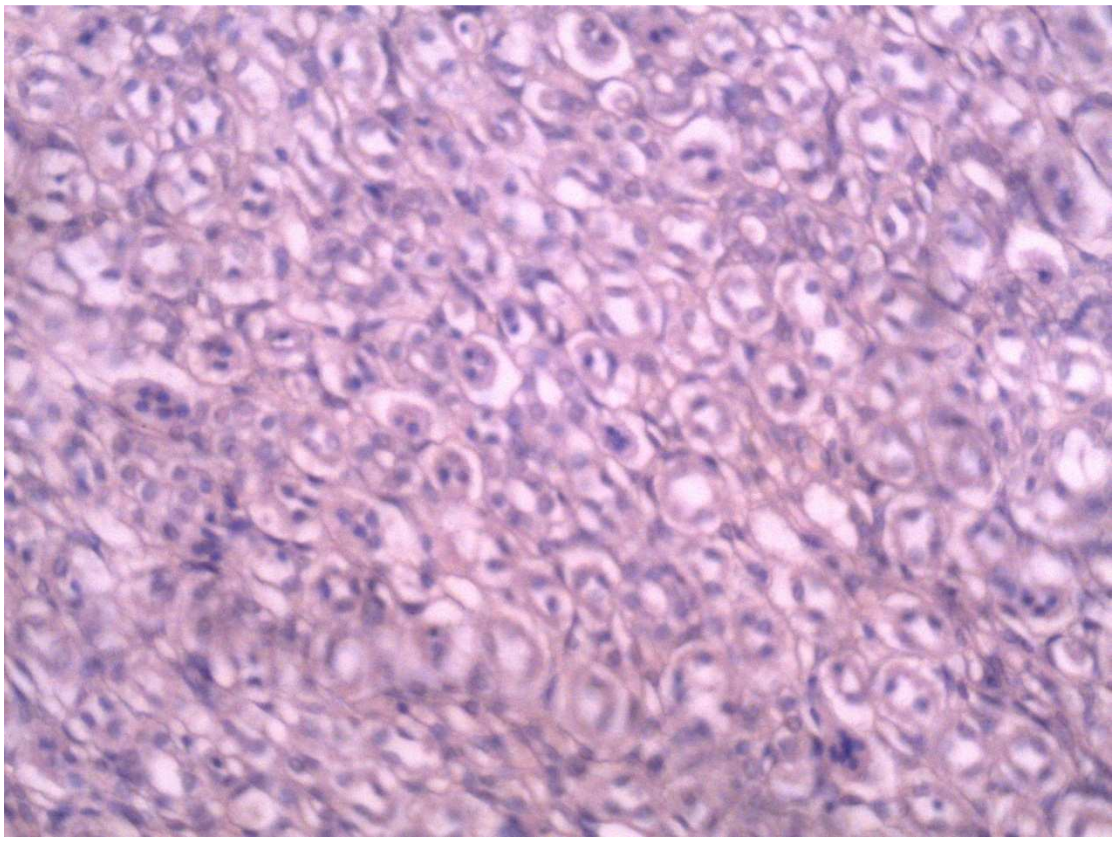

Figure 4.5.2: A photomicrograph of a kidney section of rabbits given (40mg/kg. body weight) of tramadol intramuscularly for 30 days showing moderate glycogen depletion in the renal tubules. (PAS X400).

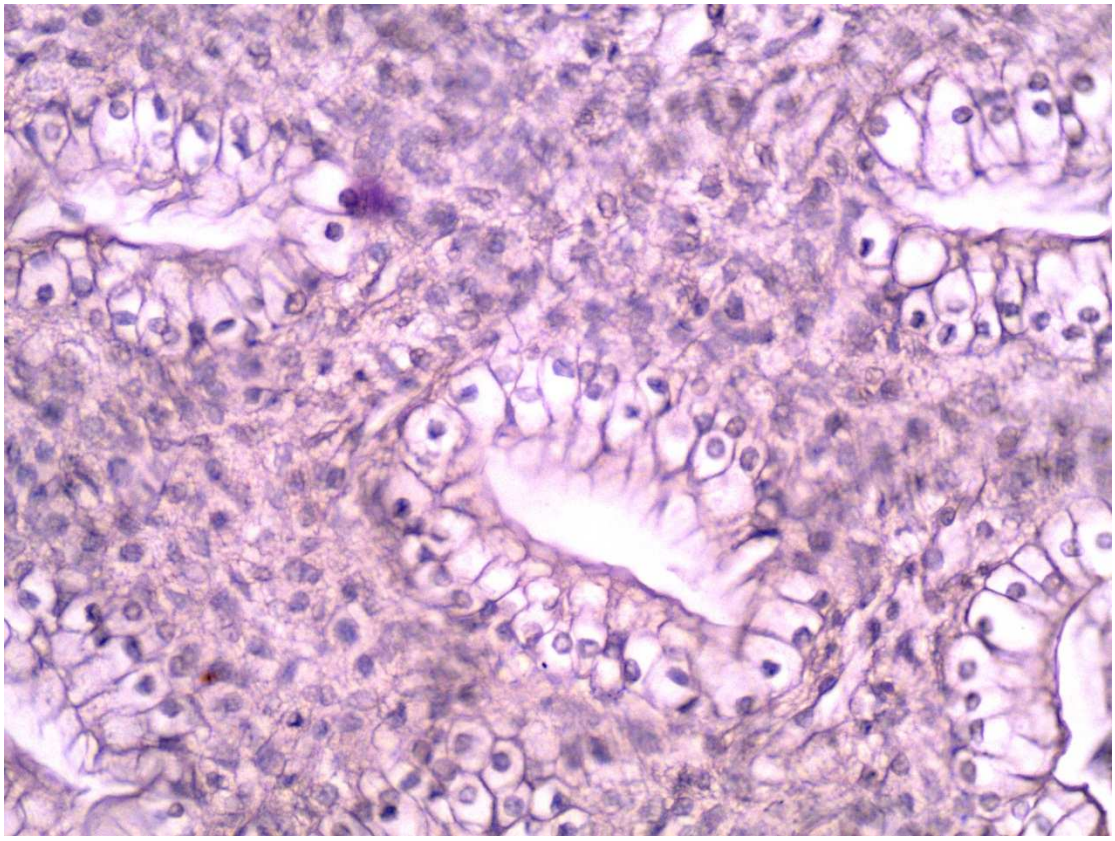

Figure 4.5.3: photomicrograph of a kidney section of rabbits given (40mg/kg. body weight) of tramadol intramuscularly for 50 days showing marked glycogen depletion in the renal tubules. (PAS X400).

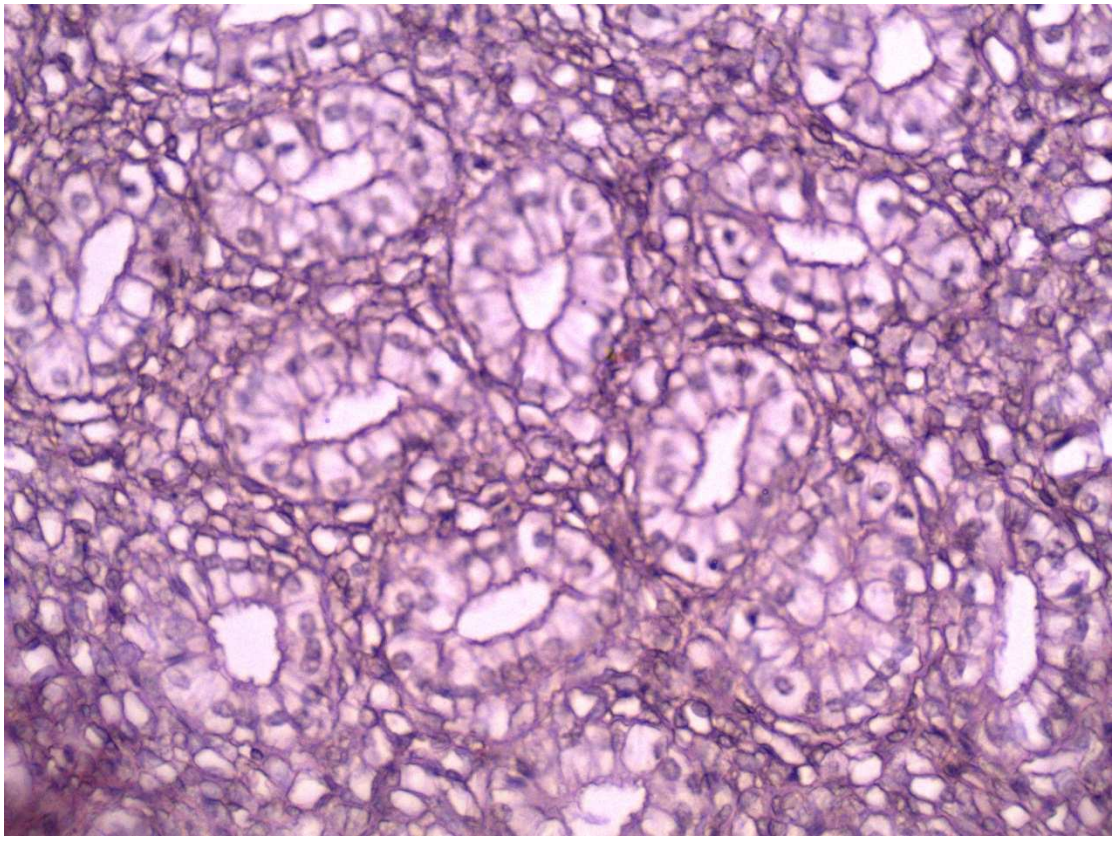

Figure 4.5.4: A photomicrograph of a kidney section of a rabbit given (40mg/kg. body weight) of tramadol intramuscularly for 30 days and left for a recovery period (10 days) showing partial improvement in the glycogen content of kidney especially the cubical epithelial cells of renal tubules. (PAS X400).

4.6 Histochemistry of kidney total proteins

A. Control group

The protein contents of the cortex of the control kidney of rabbits were demonstrated as blue granules against a light-blue ground cytoplasm scattered in the entire cytoplasmic region. The protein contents were also showed as dark blue color in the brush borders and the plasma membranes of the renal corpuscles and renal tubules. The glomeruli were positively stained and the nuclei contained positively stained chromatin. These features were noticed in rabbits of control group (Figure 4.6.1).

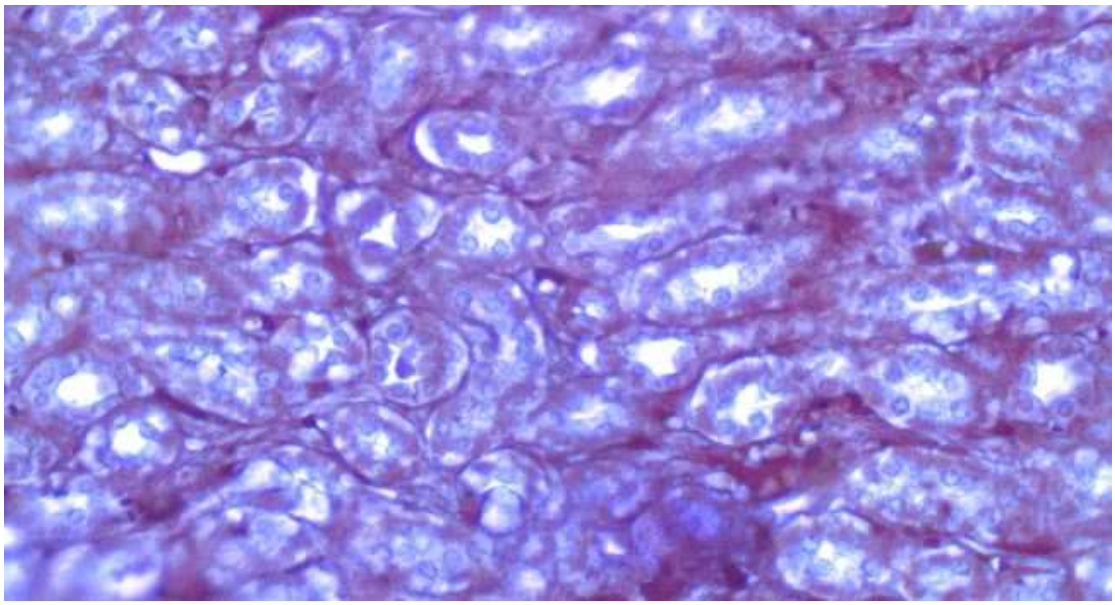

Figure 4.6.1: A photomicrograph of a kidney section of rabbits in the control group showing the normal content of proteins in the renal tubules as deeply blue colored. (MBB X400).

B. Treated groups

The animals receiving tramadol (40mg/kg. body weight) for 10 days did not manifest any obvious changes in the protein contents of their kidney cells. Examination of kidney sections of the rabbits injected with tramadol intramuscularly for 40 days manifested obvious changes in the protein contents of their kidney cells. The glomeruli and renal tubules have lost the most protein contents and became slightly less stainable than the control animal cells (Figure 4.6.2).

The protein contents as well as the general appearance of the renal tissues were approximately restored after administration of tramadol for 30 days and

left rabbits for a recovery period (10 days). The cells of the glomeruli were again densely stained nearly like those of the controls. Furthermore, the microscopic observation showed the cytoplasm of the proximal and distal tubules contained abundant protein granules (Figure 4.6.3).

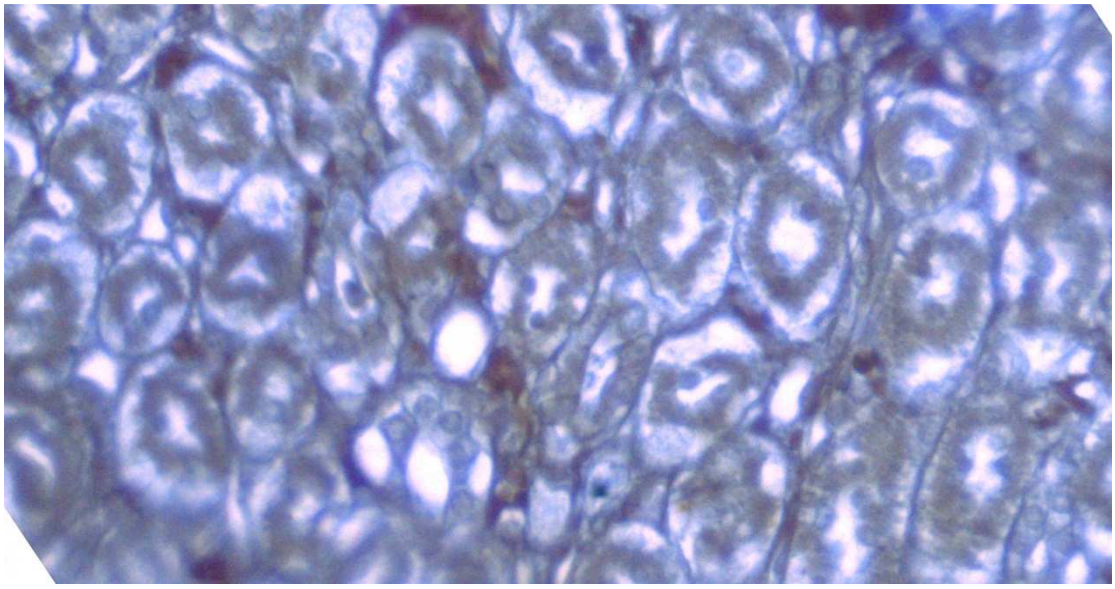

Figure 4.6.2: A photomicrograph of a kidney section of a rabbit given (40mg/kg. body weight) of tramadol intramuscularly for 40 days displaying mild protein depletion in the renal tubules. (MBB X400).

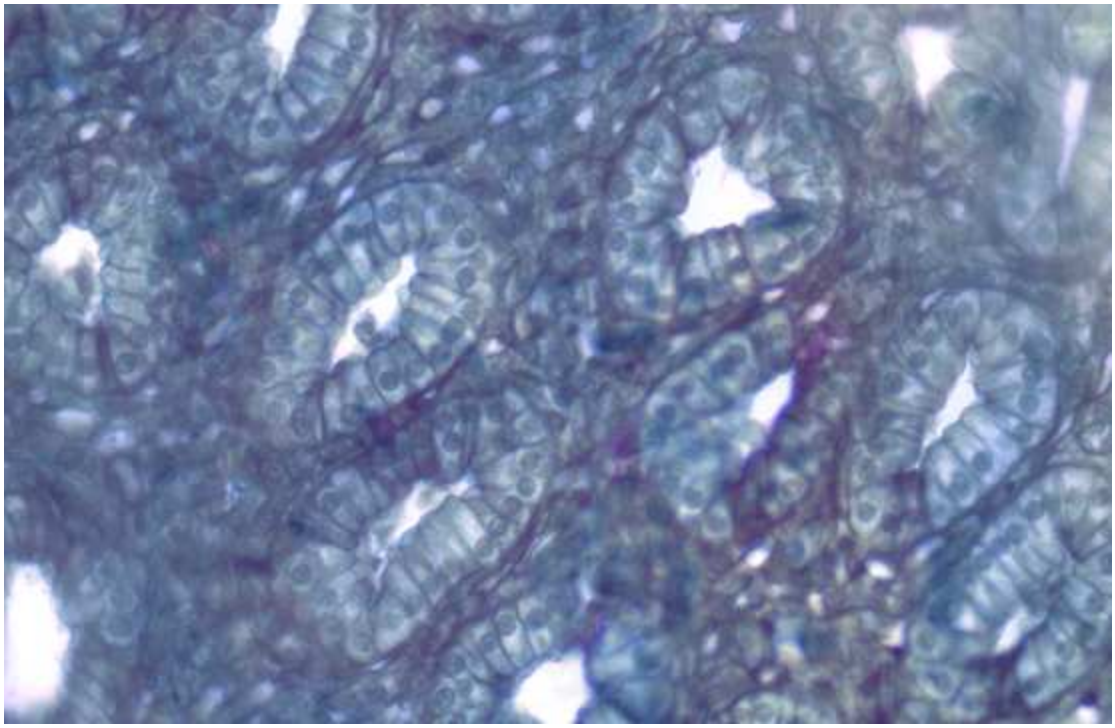

Figure 4.6.3: A photomicrograph of a kidney section of a rabbit given (40mg/kg. body weight) of tramadol intramuscularly for 30 days and left for recovery period (10 days) showing partial improvement in the amounts of protein in renal tubules. (MBB X400).

Chapter 5

DISCUSSION

5.1 Histopathology of liver

Although opioids are being widely used since very long time, their long-term effects especially at cellular level, are not clearly understood **(Atici *et al.,* 2005)**. However, such presently detected histopathological findings may be a attributed to the liver. The liver is the center for detoxifying any foreign compounds entering the body. So, it uniquely exposed to a wide variety of exogenous and endogenous products, such as environmental toxins and chemicals present in food or drinking **(Wight, 1982)**.

Comparing with the control group, light microscopy of the findings were revealed, congested blood vessels in the central vein and sinusoids, periportal fibrosis and fibroblastic cells proliferation, inflammatory cells infiltration, fatty changes, degeneration of some hepatocytes and vacuolation of liver cells especially in those treated with tramadol for a longer duration i.e. 30 days and more. In this study, the histopathological findings revealed normal histological structure in control group.

The congestion in the central vein and sinusoids present in rabbits treated for 10 days and more. This result was corresponded with that observed by **(Atici *et al.,* 2005 and Buhari *et al.,* 2012)**. Congestion in the central vein may be attributed to the harmful effect of tramadol on heart. It is well known that the mammalian heart is affected by opioids administration **(Tanush *et al.,* 2015)**.

The current study revealed that treatment with tramadol induced portal tract fibrosis with bile ductal dilatation and proliferation or clusters of inflammatory cells surrounding portal area. Evidently, the more striking sign of the liver tissue damage was well discerned at the portal area. The hepatic vein exhibited striking dilatation with thickened wall surrounded by an exuberant amount of the collagenous fibers. We attribute that the mechanism of the injury may be that the growth factors may be released into hepatic sinusoids to activate stellate cells and to initiate fibrogenesis. Similar results were reported by **Reeves and Friedman, (2002)**, who described hepatic fibrosis as the presence of excess collagen due to new fiber formation, laid down as part of the tissue repair response to chronic liver injury. They reported that the principal effecter of hepatic fibrogenesis is recognized as the hepatic stellate cell. In response to liver injury they undergo an activation process in which they become highly proliferative and synthesize a fibrotic matrix rich in type I collagen.

Also, **George *et al.* (2001)** examined the effects of dimethylnitrosamine (DMN) that can cause fibrosis of the liver. They observed collagen fiber deposition,

together with severe centrilobular necrosis, focal fatty changes, bile duct proliferation, bridging necrosis and fibrosis surrounding the central veins. A decrease in total protein and increase in DNA were also documented.

The appearance of inflammatory cells in the hepatic tissue due to tramadol administration may suggest that tramadol could interact with proteins and enzymes of the hepatic interstitial tissue interfering with the antioxidant defense mechanism and leading to reactive oxygen species (ROS) generation which in turn may imitate an inflammatory response **(Johar *et al.,* 2004)**. A remarkable cellular infiltration in the hepatic tissue of treated group present in this study. This supported by **Nwaopara *et al.* (2007)** whose studies suggested that abundance of leucocytes, in general, and lymphocytes, in particular, are a prominent response of body tissues facing any injurious impacts.

Zhang and Wang, (1984) suggested that the cytoplasmic vacuolation is mainly a consequence of considerable disturbance in lipid inclusions and fat metabolism occurring during pathological changes. Also, vacuolar degeneration has been regarded **by Durham *et al.* (1990)** to be an alteration produced to collect the injurious substances in the cells. In this study, the vacuolation of the cytoplasm of the liver cells appeared at first in the heaptocytes of the peripheral zone of the hepatic lobules, extending gradually toward the center. This may be due to the direction of the lobular blood supply. Vacuolation and damage of liver cells were noted by other investigators following treatment with different agents **(Yabe, 2000 and Samaranayake *et al.,* 2000)**.

The hepatic fatty change seen in the present work is due to Lipids accumulation in the hepatocytes when lipoprotein transport is disrupted. The appearance of lipid vacuoles within the hepatocytes of the treated groups might indicate tramadol interference with mitochondrial and microsomal function that leads to disruption of lipoprotein and lipids accumulation. Toxic effects of opioids at cellular level which may be explained by lipid peroxidation **(Lurie *et al*., 1995)**.

Biological membranes contain a large amount of polyunsaturated fatty acids, which are particularly susceptible to peroxidative attacks by oxidants resulting in lipid peroxidation. Therefore, lipid peroxidation has been used as an indirect marker of oxidant-induced cell injury. A significant increase in lipid peroxidation was reported in rats receiving an acute dose of cocaine **(Masini *et al*., 1997)**. Similarly lipid peroxides were found significantly increased among chronic heroin users **(Panchenko *et al*., 1999)**. Published patient surveys and case-reports support the efficacy and safety of long-term use of opioid analgesics in chronic non-malignant nociceptive and neuropathic pain **(Portenoy and Foley, 1986 and Turk *et al*., 1994)**.

The data of the present study showed that tramadol activates the phagocytic activity of the sinusoidal cells by increasing the number of Kupffer cells. This might be a result of increased autophagy throughout the hepatic tissue to help in removing the accumulated tramadol and its metabolites where lysosomes are involved in the intracellular breakdown into small metabolic products. The produced Kupffer cells hyperplasia might be correlated with the amount of injury to the hepatic tissue induced by tramadol intoxication and represents a defense mechanism of detoxification and might be contributed to hepatic oxidative stress **(Neyrinck, 2004)**.

Considerable alterations induced by tramadol intoxication were seen in the hepatocytes of treated groups including pyknotic and karyolitic nuclei. This might be due to increased cellular activity and nuclear interruption in the mechanism of tramadol detoxification. Some studies indicate that nuclear polymorphism is seen in hepatic dysplasia and carcinomatous lesion **(Zusman *et al.,* 1991)**. Binucleation seen in the results of the present study might represent a consequence of cell injury and is a sort of chromosomes hyperplasia which is usually seen in regenerating cells **(Gerlyng *et al.,* 2008)**.

5.2 Histopathology of Kidney

The present study indicated that tramadol induced marked histopathological alterations in the kidney tissues of rabbits such as tissue impairment, hydropic degeneration, expansion of the glomerular chamber and swelling of their lining epithelium, mononuclear cell infiltration, injured brush border of proximal convoluted tubules, necrotic lesions and pyknotic nuclei of the urinary tubules. Similar results, have been reported by **(Atici *et al.,* 2005 and Buhari *et al.,* 2012)**.

The mechanism of swelling starts as a decrease in O_2 levels which causes a drop in aerobic respiration. To maintain ATP levels, the cells must rely more on glycolysis. Glycolysis leads to lactic acid builds up, which causes the intracellular pH to drop. An acidic environment in the cell causes dysfunction of the Na^+/K^+ ATPas and consequent cell swelling due to an influx of Na^+ and H_2O. Persistent ischemia can lead to ca^{++} influx mitochondrial and lysosomal damage, and membrane damage **(Lieberthat *et al.,* 1998)**.

In the present investigation, many renal tubules of the rabbits kidneys showed marked hydropic degeneration and degenerative lesions under the effect of tramadol. This is justifiable since the renal tubules are particularly sensitive to toxic influences, in part because they have high oxygen consumption and vulnerable enzyme systems, and in part because they have complicated transport mechanisms that may be used for transport of toxins and may be damaged by such toxins. Also the tubules come in contact with toxic chemicals

during their excretion and elimination by the kidneys **(Tisher and Brenner, 1989)**.

The presence of necrosis may be related to the depletion of ATP, which finally leads to the death of the cells **(Shimizu *et al.,* 1996)**. Renal medullary necrosis occurs as a primary manifestation of renal disease. The mechanism of which is poorly understood, but it seems to involve a vascular change. Also, prostaglandin synthetase is found in the kidney, primarily in the medulla, and inhibition of this enzyme resulted in decreased production of prostaglandin E2 (PGE2) and loss of its vasodilatory effect on juxtamedullary arterioles. **(Date and Shastry, 1982)**. These findings supported by **Atici *et al.* (2005)** who reported that an evidence of renal damage such as tubular vacuolization , mononuclear cell infiltration and focal necrosis in addition to increase serum blood urea nitrogen (BUN) and creatinine levels in rats receiving long-term morphine administration was also reported

Tramadol application was also noticed in the present investigations to cause a tubular lesions in the kidney, being more apparent during the late days post-treatment. One possible mechanism for the tubular lesions was the direct toxic effect on the cell function **(Alden and Frith, 1992)**. Damage to the brush border and leakage of alkaline phosphates (ALP) and gammaglutamyle transferase (GGT) enzymes, which are associated with the brush border of the renal tubules, as a result of toxin binding to the brush border and considered as an early marker of toxic tubular insult **(Fadel and Larsen, 1994; Edelstein *et al.,* 1995 and Davies *et al.,* 1995)**.

Other possible mechanisms for the tubular lesions may involve reactive intermediates or oxidative stress, or both **(Alden and Frith, 1992)**. Biologically reactive intermediates are electron-deficient compounds (electrophiles) that bind to cellular electron-rich compounds, such as proteins and lipids. Mixed-function oxidases catalyze the formation of their toxic metabolites. Reactive intermediates bind covalently to critical cellular macromolecules and interfere with normal biologic activity. Oxidative stress is induced by increasing production of reactive oxygen specie (ROS), such as superoxide anion, hydrogen peroxide and hydroxyl radicals. ROS can induce lipid peroxidation, inactivate cellular enzymes, depolymerize polysaccharides, and induce deoxyribonucleic acid breaks and chromosome breakage **(Goldstein and Schnellmann, 1995)**.

The findings of the present investigation showed that exposure to tramadol resulted in progressive tubular, glomerular and interstitial histological alterations. Renal damage was also reported in experimental rats placed on a long term administration of morphine like agent Levo-alpha-acetylmethadol (LAAM) **(Borzelleca *et al.,* 1994)**. On the other hand, minimal histological

changes confined to the tubular cells were observed in rats receiving long-term tramadol administration **(Atici *et al.,* 2005)**.

5.3 Histochemistry of polysaccharides

In the present study, obvious alteration in the histochemical results of kidney and liver cells of the rabbits treated with 40 mg/kg body weight of tramadol was noted. Carbohydrate was found to undergo a remarkable diminution in all treated groups in comparison to the liver and kidney of the control rabbits. Such diminution exhibited time dependent characteristics. However, such presently decrease in carbohydrate content can be explained by **(Chen *et al.,* 1999)** who stated that initiation of lipid peroxidation, necrosis and subsequent impairment in cellular metabolism collectively altered the major cellular components, including protein, and glycogen.

In general, the reduction of carbohydrates components under the effect of tramadol could be due to the release of hydrolytic enzymes from the ruptured lysosomes under the toxic effect of the toxic agents **(Shalaby, 1985)**. The above detected depletion in glycogen inclusions supported by previous findings postulated by **Popp and Cattely, (1991)** that indicated that glycogen accumulation may be decreased as manifestation of toxicity, which is apparently due to impairment of enzymatic activity for glycogen catabolism or decrease in glycogen synthesis.

The data collected from the present investigation could suggest that depletion of liver and kidney glycogen which takes place under such conditions might be attributed to the effect of tramadol on glucose absorption or on the enzymes involved in the process of glycogenesis or/and glycolysis **(Jarrar and Taib, 2012)**. **Elyazji *et al.* (2013)** reported that there is a general increase in serum glucose levels in rabbits in response to tramadol administration.

Hepatocytes of the periportal zones were more affected than the perivenous hepatocytes which might indicate glycogenesis was more affected than glycolysis in the periportal hepatocytes which is metabolized by the perivenous cells that contain higher levels of glucokinase and pyruvate kinase during the post absorptive phase. The heterogeneous reduction in glycogen content between the same types of cells may indicate a difference in the overall release of glucose. Hepatocytes in the area surrounding the terminal afferent are mainly gluconeogenic, while those ones surrounding the terminal efferent venules are mainly glycolytic and lipolytic and are involved in biotransformation as general detoxification mechanism such mode of occurrence of these inclusions supported by the findings of **Jarrar and Taib, (2012)**.

On the contrary, a good support is provided to the present results. In this regard, **Abdel-Raheem *et al.* (1991)** indicated that marked declines occurred in the liver, kidney and brain glycogen contents as consequences of administration of heroin at fixed doses to adult albino rats. In addition, **Zahran, (1994)** found that heroin administration led to a duration and dose-dependent decrease of glycogen content in liver and kidney of rabbit. Such decrease was found to be also concomitant with marked hepatic and renal G-6-Pase declines. The possible interpretation of our results of carbohydrates depletion in the present study could be attributed to the toxication effects of tramadol on the liver cells; under pathological condition the cells lost their capacity to metabolize glycogen normally.

The results showed that treated rabbits with tramadol caused depletion of carbohydrates in the cytoplasm of renal tubules. This result was in correspondence with other studies reported by **Sakr *et al.* (2003)** due to the treatment of gibberellin to the rats, and **Abdeen *et al.* (1994), Sakr *et al.* (2002)** and **Elyazji *et al.* (2013)** due to the use of a variety of animals under different pathological conditions. Regarding the possible recovery of glycogen content in animal liver and kidney following drug withdrawal, no studies seem to have been carried out in this respect but the present investigations has shown that the restoration of carbohydrate contents was obviously shown in either the liver or kidney tissue of animals treated with tramadol for 30 days after a recovery period (10 days) compared to control animals.

5.4 Histochemistry of total proteins

In the present study, the daily administration of rabbits with the therapeutic dose of tramadol (40 mg/kg b.w) caused a remarkable reduction in the total proteins contents of the liver cells of treated rabbits in comparison to the liver of the control rabbits. Treatment of rabbits with tramadol for 30 days induced a marked decrease in the protein content of the urinary tubules. More reduction in proteins was manifested in the cells treated for 40 and 50 days, where the proteinic granules were clearly reduced in amount and stainability. In addition, the liver and kidney of animals received tramadol for 30 days and left for a recovery period (10 days) showed partial improvement in the total proteins contents in comparison to the treated animals.

The reduction of protein contents observed in this study may be attributed partially to the decrease of hepatic protein synthesis due to the hyperactivity of hydrolytic enzymes **(Sivaprasada *et al.,* 1983)**. Furthermore, this decrease in protein content can be explained by **(Chen *et al.,* 1999)** who stated that initiation of lipid peroxidation, necrosis and subsequent impairment in cellular metabolism collectively altered the major cellular components, including protein, and glycogen.

On the other hand, **Reid and Li, (2001)** found that reactive oxygen species may activate the ubiquitin proteasome pathway. Proteasomes are very large protein complexes located in the nucleus and the cytoplasm inside all eukaryotes **(Peters *et al.,* 1994)**. The main function of the proteasome is to degrade unneeded or damaged proteins by proteolysis. Proteasomes are part of a major mechanism by which cells regulate the concentration of particular proteins and degrade misfolded proteins. Proteins are tagged for degradation with a small protein called ubiquitin. The tagging reaction is catalyzed by enzymes called ubiquitin ligases. Once a protein is tagged with a single ubiquitin molecule, this is a signal to other ligases to attach additional ubiquitin molecules. The result is a polyubiquitin chain that is bound by the proteasome, allowing it to degrade the tagged protein **(Lodish *et al.,* 2004)**. Hence, the activation of the ubiquitin proteasome pathway may participate in Tramadol-induced proteins depletion.

In addition, **Palla *et al.,* (1987)**, postulated that in many kidney diseases, the permeability of the glomerular capillaries is increased leading to increased levels of excreted proteins. They added that any lesions produced in the kidney tubules will eventually cause dysfunction in the transport mechanism to and from the renal epithelium.

Experimental studies have also supported toxic effects of chronic use of opioids on liver proteins. **Hashiguchi *et al.* (1996)** studied the central effects of morphine and morphine-6-glucuronide on tissue protein synthesis. They found that morphine and M6G suppress tissue protein synthesis through central mechanisms, mediated by opiate-induced respiratory.

The lost proportions of protein inclusions recorded in the present materials was particularly restored approximately 10 days after the abstinence of (40 mg/kg body. weight) dosage of tramadol, but such restoration apparent to be relatively incomplete in rabbits previously treated for 30 or 40 days. None of such findings seen to have been reported in any other communications.

Chapter 6

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

- Histopthological changes in liver tissue were manifested in hepatocellular damage as degenerative and destruction of hepatocytes, congestion of sinusoids, inflammatory cells infiltration, necrosis and fibroblastic cells proliferation were showed in rabbits liver treated with tramadol for 30, 40 and 50 day. On the other hand, moderate inflammatory cells infiltration and fibroblastic cells proliferation observed in groups treated for 30 day. Also, karyolysis and complete pyknosis of many cells were noticed.

- The kidney of tramadol-treated rabbits showed abnormal features. Such as expansion of glomerulus, glomerular tuft atrophy, renal injury with tubular cell swelling, focal tubular necrosis and mild mononuclear cell infiltration were noticed. Also, degeneration in the epithelial cells lining the renal tubules at the cortical zone with pyknotic and karyolysed nuclei were noticed in kidney of rabbits treated for 30, 40 and 50 days.

- Histochemical observations of liver treated with tramadol for 10 days preserved the normal contents of glycogen. While Specimens treated with tramadol for 30 days showed mild glycogen depletion and rabbits treated for 50 days showed marked glycogen depletion. Rabbits treated with 30 and 50 days showed marked diminution in PAS positive material in the renal corpuscles and tubules. In a recovery group, some of the hepatocytes and regenerated renal tubules displayed a slight increase in their glycogen.

- The protein inclusions of animals received tramadol for 10 days did not manifest obvious changes, while hepatocytes of treated rabbits with tramadol for 30 and 50 days demonstrated a marked reduction of protein contents in comparison to control rabbits. Kidney sections of the rabbits injected with tramadol for 30 and 40 days manifested obvious changes in the protein contents. The glomeruli and renal tubules have lost the most protein contents and became slightly less stainable than the control group. On the other hand, the protein contents as well as the general appearance of the renal tissues were approximately restored after the recovery period.

6.2 Recommendations

- Enhancement of people awareness toward tramadol toxicity by launching educational programs and workshops on tramadol.
- Notification of health authorities on the result of this study to take decisions to prevent the misuse of tramadol
- Further studies are needed to investigate the impacts of tramadol on human health.

Chapter 7

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