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Original article

Puncturevine (*Tribulus terrestris L.*) affects the proliferation, apoptosis, and ghrelin response of ovarian cells

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ABSTRACT

The objective of our study was to examine the direct effects of the medicinal plant *Tribulus terrestris* L. (puncturevine) on the basic functions of ovarian cells, including their proliferation, apoptosis, and response to the physiological hormonal stimulator ghrelin. In the first series of experiments, porcine ovarian granulosa cells were cultured with or without puncturevine extracts at concentrations of 0, 1, 10, or 100 μg/ml. In the second series of experiments, these cells were cultured with ghrelin at concentrations of 0, 1, 10, or 100 ng/ml, either alone or in combination with puncturevine (10 μg/ml). The expression levels of the proliferation marker PCNA and the apoptosis marker bax were analyzed *via* quantitative immunocytochemical methods. Puncturevine was found to stimulate the accumulation of both proliferation and apoptotic markers. Additionally, ghrelin alone could promote the proliferation and apoptosis of ovarian cells. The presence of puncturevine reversed ghrelin-stimulated apoptosis and instead induced apoptotic inhibition. However, puncturevine did not modify the proliferation-inducing effect of ghrelin. These observations demonstrated that (1) puncturevine directly promotes cell proliferation and apoptosis, turnover, of ovarian cells; (2) ghrelin is involved in the regulation of ovarian cell apoptosis and proliferation, consistent with existing evidence; (3) puncturevine antagonizes and even reverses the effects of the hormonal regulator, ghrelin, on ovarian cell apoptosis, but not proliferation; and (4) puncturevine affects not only the basic functions of ovarian cells but also their responses to upstream hormonal regulators.

1. Introduction

The subtropical plant *Tribulus terrestris L.*, belonging to the *Zygophyllaceae* family, is variously known by the common names puncturevine, bullheads, goat head, small caltrop, Gokshur, and Gokharu. It has been used for thousands of years in Indian (Ayurvedic), Sudanese, and Chinese folk medicine. Presently, its popularity as an energizer for improving the sexual and physical performances of males is growing around the world. The saponins, flavonoids, glycoside alkaloids, acids, and tannins present in puncturevine can improve male sexual functions, libido, and fertility; promote diuresis; and prevent and treat cardiovascular, neurodegenerative, immune, inflammatory, and metabolic disorders. Furthermore, puncturevine possesses antioxidant, antibacterial, anthelmintic, analgesic, anti-inflammatory, anti-spasmolytic, neuroprotective, hepatoprotective, anti-aging, antidotal, and anticancer properties. In addition, it promotes the absorption of other drugs (refer to [1–3] for detailed reviews). In contrast to the documented action of puncturevine on male reproductive functions [4,5], the known influences on the female reproductive system are limited to the ability of this plant to promote sexual desire [6,7], FSH output and ovulation [8] in infertile women, while the cellular mechanism of this effect remains largely unknown. Rabbits fed puncturevine extract exhibited differences in the expression of oocyte-derived factors, bone morphogenetic protein 15, and growth differentiation factor 9, which are presumed to participate in controlling follicular development and ovulation [9]. Puncturevine prevents the development of ovarian cysts and polycystic ovarian syndromes in rats [10–12] and women [8]. These observations indicate that puncturevine can possibly affect female reproductive functions; however, the studies conducted to date have not demonstrated whether this plant affects reproductive functions in healthy females, whether the effects on ovarian functions are mediated directly or via*via* upstream hypothalamo-hypophysial and/or peripheral hormonal regulators or whether puncturevine can affect ovarian responses to hormonal regulators.

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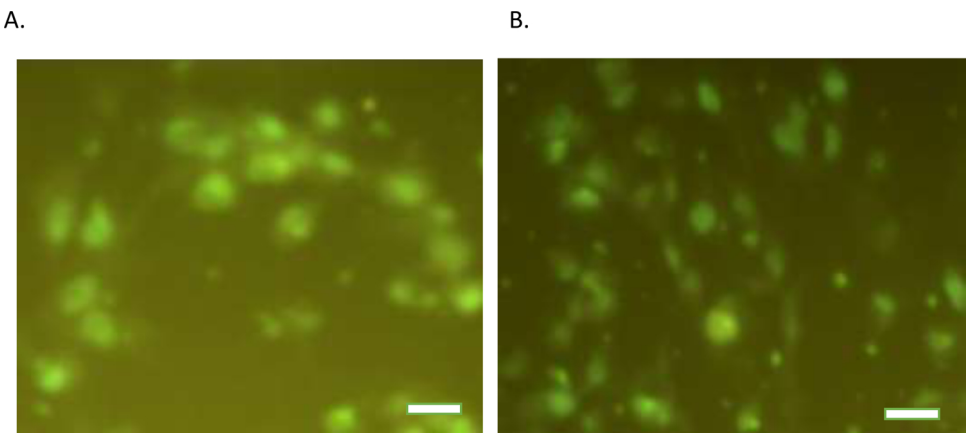

Fig. 1. Fluorescence images of porcine granulosa cells containing PCNA (A) and bax (B). Immunocytochemistry images showing fluorescence isothiocyanate (FITC; green) staining. Scale bars: 1 cm = 20 μm.

A novel and potent, but insufficiently studied, hormonal regulator of female reproductive functions is ghrelin. Its direct influence on basic ovarian functions, including cell proliferation, apoptosis, and the release of hormones, has been documented [13,14]. However, the interactions of ghrelin with natural products or components of medicinal plant extracts have yet to be examined.

The objective of our study was to examine the direct influence of puncturevine on the elementary functions of ovarian cells, including proliferation and apoptosis, as well as their responses to the physiological hormonal stimulator ghrelin. For this purpose, we analyzed the proliferation and apoptosis of porcine ovarian granulosa cells cultured with or without puncturevine extracts, ghrelin, and a combination of both.

2. Materials and methods

2.1. Isolation and culture of granulosa cells

Granulosa cells were isolated from the ovaries of noncycling pubertal gilts from animals aged approximately 180 d that were slaughtered at a local abattoir and processed as described previously [15,16]. After transfer to the laboratory, the ovaries were rinsed several times with sterile 0.9 % NaCl and 95 % alcohol. The granulosa cells were aspirated with a syringe and sterile needles from follicles with diameters of 3 − 5 mm, and then, the cells were isolated by centrifugation for 10 min at 200 × g, subsequently rinsed with sterile DMEM/F12 1:1 medium (BioWhittaker™, Verviers, Belgium), followed by resuspension in the same medium, supplemented with 10% fetal calf serum (BioWhittakerTM) and a 1% antibiotic-antimycotic solution (Sigma, St. Louis, MO, USA), at a final density of 10^6 cells/ml. Aliquots of the cell suspension were then dispensed into 16-well chamber slides (Nunc Inc., International, Naperville, USA, 200 μl/well) and incubated at 37.5 °C in 5% CO_2 in humidified air until a monolayer with a confluence of 60–75% was formed after 3–5 d, at which point the medium was renewed. After replacing the medium, the cells were cultured either in the presence of 95 % extract of puncturevine (*T. terrestris L.*) (Changsha Sunfull Bio-Tech., Co., Hunan China) at concentrations of 0, 1, 10, and 100 μg/ml or cultured with or without biological-grade human ghrelin (Sigma-Aldrich Inc., St. Louis, MO, USA) at concentrations of 0, 1, 10 and 100 ng/ml, alone or in combination with 10 μg/ml puncturevine extract (Changsha Sunfull Biotech. Co,). The molecules were dissolved in the culture medium immediately prior to their addition to the cells. The cells used as controls were cultured without either puncturevine or ghrelin, while the blank control consisted of the cell-free medium. After 2 d of culture, the medium was aspirated and stored at −18 °C in preparation for the radioimmunoassay (RIA) analysis. The cells remaining at the bottom of the wells were rinsed with ice-cold PBS (pH 7.5), fixed in paraformaldehyde (4 % in PBS, pH 7.2–7.4) for 60 min, and kept at 4 °C in preparation for the immunocytochemical analysis. The number and viability of the cells were determined by Trypan blue staining and a hemocytometer. Cell viability ranged between 70 and 80 %, and no statistically significant differences were observed in these indices between the control and experimental groups.

2.2. Quantitative immunocytochemistry

This analysis was performed as previously described [15,16]. Following washing and fixation, the cells were incubated in a blocking solution (1% goat serum in phosphate-buffered saline [PBS]) at +20 °C for 1 h to prevent the nonspecific binding of the antiserum. The cells were subsequently incubated in the presence of monoclonal antibodies at dilutions of 1:500 in PBS against PCNA (a marker of proliferation) [17] and bax (a marker of cytoplasmic apoptosis) [18], which were purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, USA. Following 2 h of incubation at +20 °C, the cells were stored overnight at 4 °C. To detect the binding sites of the primary antibodies, the cells were incubated with secondary swine antibody against mouse IgG, labeled with horseradish peroxidase (Sevac, Prague, Czech Republic, dilution 1:1000) or porcine secondary antibody labeled with fluorescein isothiocyanate (FITC, Sevac, dilution 1: 500), for 1 h. The FITC-labeled secondary antibody was detected by fluorescence microscopy, whereas the secondary antibody labeled with horseradish peroxidase was stained with the 3,3′diaminobenzidine (DAB) substrate (Roche Diagnostics GmbH, Manheim, Germany) and then visualized under a light microscope. The number of stained cells and the location of the intracellular molecules were determined by the brown coloration of the DAB peroxidase or the green fluorescence emitted by the FITC label, and the ratio of stained cells to the total number of cells was calculated.

2.3. Statistical analyses

The results represent the data summarized from three experiments performed on separate days with distinct groups of granulosa cells that were obtained from 10 to 20 animals. All experimental groups were represented by three wells containing granulosa cells. The proportion of cells with a specific immunoactivity was calculated from at least 1000 cells per well, and the percentage of cells containing antigens was determined after estimating the cell numbers. Significant differences between the experimental and control groups were evaluated with one- or two-way analysis of variance (ANOVA) followed by Duncan's test using the software Sigma Plot 11.0 (Systat Software, GmbH, Erkrath, Germany); a one-tailed P value < 0.05 was considered to be significant.

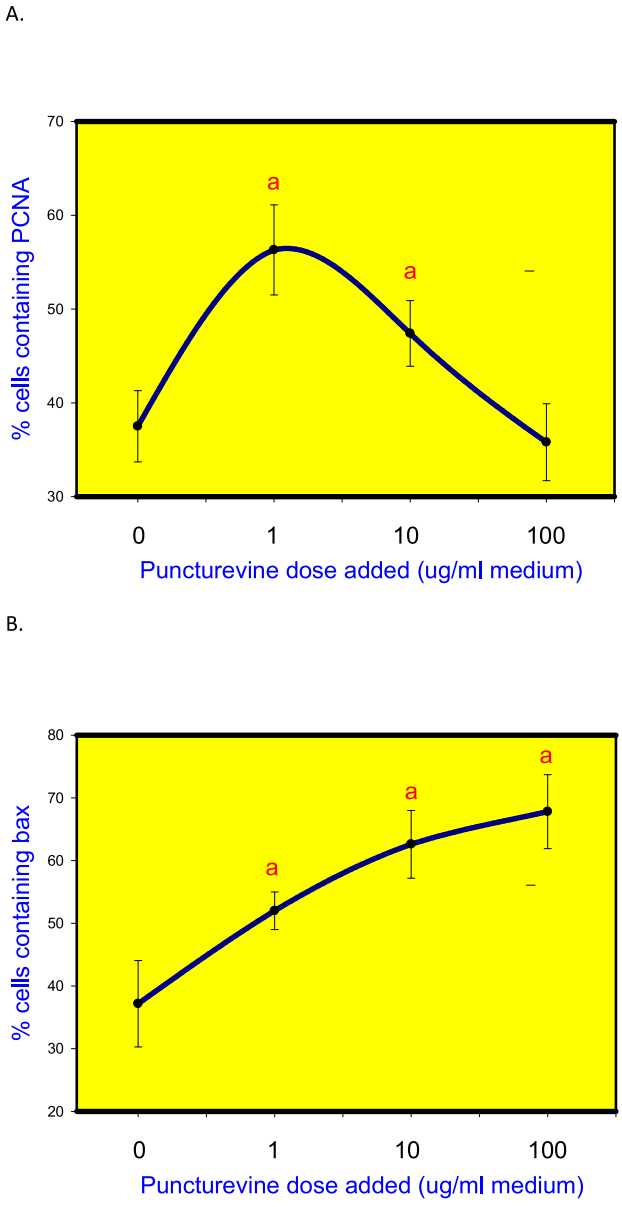

Fig. 2. The effect of the addition of puncturevine (*Tribulus terrestris* L.), at concentrations of 0, 1, 10, and 100 μg/ml, on the expression of the proliferation marker PCNA (A) and the apoptosis marker bax (B) in cultured porcine ovarian granulosa cells. Data derived from quantitative immunocytochemical analysis. a: Significant differences ($P < 0.05$) were observed between cells cultured with and without (0 μg/ml) puncturevine.

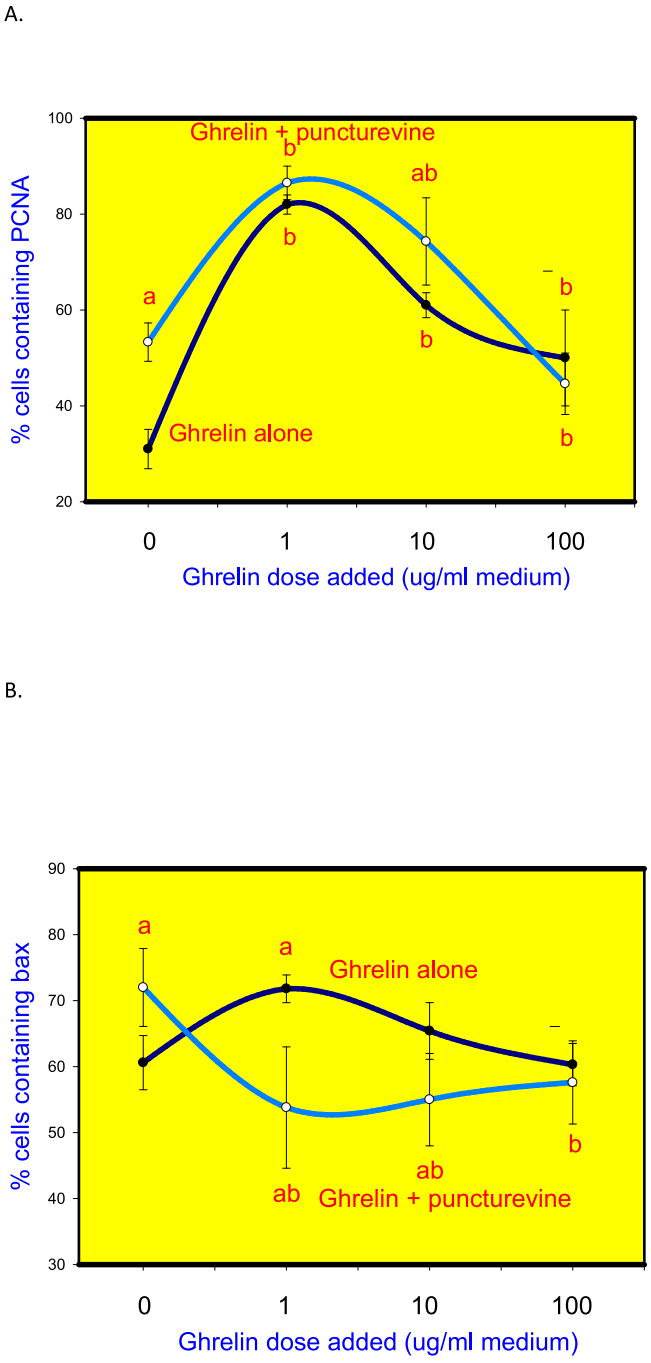

Fig. 3. The effect of the addition of ghrelin at concentrations of 0, 1, 10 and 100 ng/ml, either alone or in combination with 10 μg/ml puncturevine (*Tribulus terrestris* L.), on the expression of the proliferation marker PCNA (A) and the apoptosis marker bax (B) in cultured porcine ovarian granulosa cells. Data derived from quantitative immunocytochemical analysis. a: Effect of puncturevine: significant differences ($P < 0.05$) were observed between the corresponding groups of cells cultured with and without puncturevine. b: Effect of ghrelin: significant differences ($P < 0.05$) were observed between cells cultured with and without ghrelin.

3. Results

The cultured porcine ovarian cells contained both PCNA and bax. PCNA was located primarily in the nuclei and to a lesser extent in the cytoplasm, while bax was predominantly located in the cytoplasm in the majority of cells and in the nuclei of some of the cells (Fig. 1).

The first series of experiments demonstrated that the addition of the puncturevine (*T. terrestris* L.) extract increased the percentage of cells containing PCNA (after addition of the extract at doses of 1 or 10 μg/ml, Fig. 2A) and bax (at all doses of the extract) (Fig. 2B).

The increase in the population of PCNA- and bax-positive cells following the addition of the puncturevine extract at a dose of 10 μg/ml was confirmed by a second series of experiments (Fig. 3A, B, ghrelin at dose 0 ng/ml). In these experiments, ghrelin, when added alone, increased the population of PCNA-positive cells at all doses (Fig. 3A) and the population of bax-positive cells at a dose of 1 ng/ml. Cotreatment with puncturevine did not substantially modify the effect of ghrelin on PCNA accumulation (Fig. 3A). In contrast to the observations made when ghrelin was added alone, the proportion of bax-positive cells decreased when puncturevine extract was present in the medium with ghrelin (Fig. 3B).

4. Discussion

Trypan blue staining and cell counting did not reveal differences in the number and viability of cells among the different groups of cells after 2 d of treatment (refer to Materials and Methods). Nevertheless, the presence of both PCNA (a marker of the S-phase of the cell cycle) [17] and bax (a marker of cytoplasmic apoptosis) [18] was observed in the cultured cells (refer to Results), which indicated that these cells underwent both proliferation and apoptosis. Furthermore, the results demonstrated that the addition of both puncturevine (*T. terrestris* L.) and ghrelin influenced these processes.

The effect of puncturevine on the ovarian cells observed in our study is the first demonstration of the direct action of this plant on ovarian cells. The finding suggested that the influence of puncturevine on the fundamental processes previously observed in ovarian cells [8–11], could be due to the direct effects of its constituents on ovarian cells rather than its action on the upstream hormonal regulators (hypothalamo-hypophyseal-ovarian axis).

The effect of the puncturevine extract on PCNA and bax levels indicated its influence on two fundamental ovarian functions, namely, proliferation and apoptosis. The stimulatory action of puncturevine on both of these processes suggested that it could promote ovarian cell turnover, which defines the growth, development, selection, and finally the fecundity of ovarian follicles [14]. While puncturevine treatment at the highest dose (100 μg/ml) maximally stimulated the expression of bax, it had no effect on PCNA. Therefore, the administration of puncturevine at the highest dose can increase the rates of apoptosis/proliferation to favor apoptosis and thus suppress ovarian functions.

The stimulatory action of ghrelin on the accumulation of both PCNA and bax confirms the results of previous studies [13,14] that reported the direct regulation of ovarian cell proliferation and apoptosis by ghrelin. The nature and significance of the effect of ghrelin on proliferation and apoptosis require further study. Nevertheless, the ability of ghrelin to stimulate proliferation and the lack of its influence on apoptosis at moderate and high doses suggests that it promotes proliferation and reproduction while inhibiting apoptosis. These observations are consistent with our previous observations on the effects of ghrelin on the proliferation and apoptosis of porcine granulosa cells [15,16].

The similarity between the effects of ghrelin and puncturevine on ovarian cells suggests their potential interrelationship in the control of ovarian functions. This hypothesis might be confirmed by the observation that the addition of puncturevine antagonizes and even reverses the effect of ghrelin on apoptosis, but not proliferation, of ovarian cells. Although the mechanisms behind the interrelationships remain to be elucidated, the observation suggests that puncturevine affects not only the fundamental functions of ovarian cells *per se* but also the cellular responses to upstream hormonal regulators. The functional interrelationships between puncturevine and hormones suggest that the effect of the plant extract may depend on hormone release and that hormones can potentially promote or suppress the effect of this medicinal plant. On the other hand, it is premature to offer any practical recommendations concerning human treatment by puncturevine with or without hormones because the results of animal *in vitro* experiments are not necessarily similar to the results of *in vivo* applications in human patients. Extracts of puncturevine need to be medically applied to investigate whether the plant extract can directly affect basic ovarian functions *in vivo*. Understanding the nature and mechanism of action of puncturevine on the female reproductive system requires further *in vitro* and *in vivo* studies. Nevertheless, our observations demonstrated that (1) puncturevine directly promotes proliferation and apoptosis, that is, turnover, of ovarian cells; (2) ghrelin is involved in the regulation of proliferation and apoptosis, a finding consistent with existing reports; (3) puncturevine antagonizes and even reverses the effects of the hormonal regulator ghrelin on ovarian cell apoptosis but not proliferation; and (4) puncturevine affects not only the basic functions of ovarian cells but also their responses to upstream hormonal regulators.

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.repbio.2019.12.009.

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