Potential protective effect of puncturevine (*Tribulus terrestris, L.*) against xylene toxicity on bovine ovarian cell functions

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Summary

The action of the medicinal plant *Tribulus terrestris* (TT) on bovine ovarian cell functions, as well as the protective potential of TT against xylene (X) action, remain unknown. The aim of the present in vitro study was to elucidate the influence of TT, X and their combination on basic bovine ovarian cell functions. For this purpose, we examined the effect of TT (at doses of 0, 1, 10, and 100 ng/mL), X (at 20 μg/mL) and the combination of TT + X (at these doses) on proliferation, apoptosis and hormone release by cultured bovine ovarian granulosa cells. Markers of proliferation (accumulation of PCNA), apoptosis (accumulation of Bax) and the release of hormones (progesterone, testosterone and insulin-like growth factor I, IGF-I) were analyzed by quantitative immunocytochemistry and RIA, respectively. TT addition was able to stimulate proliferation and testosterone release and inhibit apoptosis and progesterone output. The addition of X alone stimulated proliferation, apoptosis and IGF-I release and inhibited progesterone and testosterone release by ovarian cells. TT was able to modify X effects: it prevented the antiproliferative effect of X, induced the proapoptotic action of X, and promoted X action on progesterone but not testosterone or IGF-I release. Taken together, our observations represent the first demonstration that TT can be a promoter of ovarian cell functions (a stimulator of proliferation and a suppressor of apoptosis) and a regulator of ovarian steroidogenesis. X can increase ovarian cell proliferation and IGF-I release and inhibit ovarian steroidogenesis. These effects could explain its anti-reproductive and cancer actions. The ability of TT to modify X action on proliferation and apoptosis indicates that TT might be a natural protector against some ovarian cell disorders associated with X action on proliferation and apoptosis, but it can also promote its adverse effects on progesterone release.

Keywords: xylene, *Tribulus terrestris*, proliferation, apoptosis, hormones, ovarian granulosa cells.

Introduction

Puncture vine (*Tribulus terrestris* L.) (TT) is a popular medicinal plant containing many biologically active molecules, steroidal saponins, flavonoids, flavanol glycosides, alkaloids, and tannins, with antioxidant, anti-inflammatory and phytoestrogen properties, which define its physiological and medicinal effects [1-6]. TT is considered a traditional stimulator of masculine sexual desire [4]. In addition, its diuretic, antiurolithic, immunomodulatory, antidiabetic, hypolipidemic, cardiotonic, neurostimulatory, hepatoprotective, anti-inflammatory, analgesic, antispasmodic, antibacterial, anthelmintic, larvicidal, and anticariogenic and anticancer activities have been reported [2,3,5-7]. There is evidence of the stimulatory action of TT on rodent and porcine ovarian cell functions. Oral treatment of mice with aqueous extracts of TT can increase the weight of their reproductive organs (ovaries, oviducts, uterus; Abid, 2010) and the number of growing ovarian follicles [8]. A similar experiment in rats showed the ability of TT to promote estradiol and testosterone release, uterine and vaginal development [9], ovarian weight and the length of the estrous cycle [10]. Administration of TT increased the testosterone levels in women's plasma [11].

Our previous in vitro study [12] showed the ability of *Tribulus terrestris* extract to directly affect porcine ovarian granulosa cells and to promote the accumulation of both proliferation and apoptosis markers. These effects could be explained by the anti-androgen [13] and estrogenic [9] properties of TT phytoestrogens, which influence numerous reproductive events [14]. The action of TT on ovarian hormone release has not yet been studied. Furthermore, the influence of TT on reproductive functions in other species has not been investigated, although some ruminants of economic importance, such as cows, consume this plant, and TT could be potentially useful for improving reproduction.

TT can not only affect female reproductive functions but also mitigate or prevent reproductive disorders such as ovarian cancer [15], ovarian cysts [16], polycystic ovarian syndrome [10,13,17], and oligo/anovular infertility [18]. A number of female reproductive disorders could be induced by oil-related environmental contaminants, including xylene (X). X can induce aberrations in reproductive hormones, ovarian functions, fecundity and embryo death, as well as ovarian carcinogenesis. These adverse effects could be due to the direct influence of X on oxidative and inflammatory processes, the proliferation:apoptosis ratio and the secretory activity of ovarian cells [19]. For example, inhalation of X reduced progesterone and estradiol levels in rat blood but not their production by rat ovaries [20]. On the other hand, in in vitro studies, X increased progesterone and testosterone (but not insulin-like growth factor I, IGF-I) release by cultured murine ovaries [21]. The addition of X reduced the viability of cultured porcine granulosa cells and their proliferation and the release of progesterone and estradiol while increasing apoptosis [22]. In cultured bovine granulosa cells, X stimulated proliferation, apoptosis and IGF-I release but inhibited progesterone and testosterone release [22,23].

Some of these X effects were mitigated, prevented and even reversed by cell co-treatment with extracts of some medicinal and functional food plants, such as buckwheat (*Fagopyrum esculentum*), rooibos (*Aspalathus linearis*), vitex (*Vitex agnus-castus*) [22], and the plant constituent quercetin [23], with antioxidant or phytoestrogen properties [24-26]. TT contains a number of molecules with these properties (including glucosides of quercetin) [2-6]. These properties indicate that TT could not only affect female reproductive processes but also be a natural protector against their disorders. However, it remains unknown whether TT can prevent adverse effects of X on female reproductive processes.

The aims of the present study were:

(1) To examine the action of TT on basic bovine ovarian cell functions (proliferation, apoptosis, release of IGF-I and steroid hormones) playing a key role in the control of reproduction and fecundity [14].

(2) To validate the available knowledge concerning the direct action of X on these functions.

(3) To examine the ability of TT to mitigate X action on bovine ovarian cell functions.

For this purpose, we examined the effect of TT (at doses of 0, 1, 10, and 100 μg/mL), X (at 20 μg/mL) and the combination of TT + X (at these same doses) on proliferation, apoptosis and hormone release by cultured bovine ovarian granulosa cells.

Material and methods

Preparation, Processing and Culture of Ovarian Granulosa Cells

Ovaries from Holstein breed cattle aged 3–5 years at the follicular stage of the estrous cycle were obtained from slaughterhouses of the Research Institute of Animal Production in Nitra and Stara Myjava. The ovaries were individually stored in a thermos with a physiological solution at room temperature and processed within 6 hours of slaughter. The ovaries and ovarian cells were processed as described previously [12,22,23,27,28]. Ovarian granulosa cells were isolated by aspiration with a syringe from medium size (3–5 mm) follicles. After aspiration and isolation of the granulosa cells, these cells were washed in sterile DMEM/F12 1:1 medium (BioWhittaker TM, Verviers, Belgium), resuspended in the same medium supplemented with 10% fetal calf serum (South America Origin, Biowest) and 1% antibiotic–antimycotic solution (Sigma, St. Louis, MO, USA). We counted the cells by using Buerker´s chamber (Sigma) according the guide of manufacturer and adjusted the cell concentration to the required concentration (10^6 cells/mL^{-1}

medium). The cell suspension was diluted with culture medium and cultured in 24-well culture plates (Nunc™, Roskilde, Denmark, 1 mL of suspension/well, RIA), and cells for immunocytochemistry were cultured in 16-well chamber slides (Nunc Inc., International, Naperville, USA, 200 µl/well, for immunocytochemistry) at 37.5 °C and 5% CO_2 in culture plates (1 mL/well). After 4 days of culture, when the cells had formed a continuous monolayer covering 75% of the surface, the medium was replaced with fresh medium with 10% fetal calf serum and 1% antibiotic-antimycotic. The control group was treated with X (AppliChem GmbH, Darmstadt, Germany, 20 µg/mL) but without TT, and the other groups had the addition of X (AppliChem GmbH) and extract of TT leaves (Changsha Sunfull Bio-tech. Co, Hunan, China; 1, 10 and 100 µg of 95% extract/mL). These concentration of additives corresponded the doses of X (Sirotkin et al., 2017, 2021; Tarko et al., 2018) and TT (Sirotkin et al., 2020) which were efficient in the previous in vitro experiments on similar models. Moreover, the dose of TT used in the present experiments are comparable with the TT doses used previously in medicinal practice (Lorand *et al.* 2010; Chhatre *et al.* 2014; Shahid *et al.* 2016; Abarikwu *et al.* 2020; Martimbianco *et al.* 2020; Parham *et al.* 2020; Verma *et al.* 2021) and animal *in vivo* experiments [10]. TT extract was first suspended in dimethyl sulfoxide (DMSO) (AppliChem GmbH) 2 days before the experiments to produce stock solutions of 1 mg/mL. Thereafter, this stock solution of TT extract was dissolved in culture medium immediately before its addition to the cell culture, such that the final concentration of DMSO did not exceed 0.001%. Previous studies have not revealed any substantial effects of 0.001% DMSO on ovarian cell function and viability (data not shown). X was dissolved in the incubation medium immediately before the experiments. After 48 hours of incubation, we removed the medium from the culture plates via a syringe and stored it at –70 °C until analysis by radioimmunoassay (RIA). A monolayer of cells on the chamber slides was fixed with 4%

paraformaldehyde in PBS (phosphate buffered saline) for 10 minutes and stored at +4 °C until immunocytochemical analysis.

Quantitative immunocytochemistry

The presence of markers of proliferation (PCNA) and apoptosis (Bax) was detected by immunocytochemistry [29]. After washing and fixation, the cells were incubated in blocking solution (1% goat serum (from Santa Cruz Biotechnology, Inc., Santa Cruz, USA) in phosphate buffered saline (PBS) at room temperature for 1 hour to block nonspecific binding of the antiserum. The cells were then incubated with monoclonal antibodies against either a marker of proliferation (PCNA) or a marker of apoptosis (Bax) (Santa Cruz Biotechnology, Inc., dilution 1:500 in PBS) for 1 hour at room temperature. For the detection of the primary binding site of antibodies against PCNA and Bax, cells were incubated with a secondary swine anti-mouse IgG labeled with horseradish peroxidase (Servac, Prague, Czech Republic, 1:1000 dilution) for 1 hour. Positive signals were visualized by staining with DAB substrate (Roche Diagnostics GmbH, Mannheim, Germany) for 1 h. After DAB staining, the cells on the chamber slides were washed in PBS and then covered with a drop of fixation medium (DAKO, Glostrup, Denmark) and a coverslip. The presence and localization of the PCNA- and Bax-positive cells was detected by peroxidase-DAB (brown staining). The cells processed without the primary antibody were used as a negative control. The ratio of DAB–HRP–stained cells to the total cell number was calculated.

Radioimmunoassays (RIAs)

The concentration of insulin-like growth factor I (IGF-I) was determined using RIA in 25 μL of incubation medium after extraction previously validated for use in culture medium as described

158 before [30]. The sensitivity of the assay was 0.3 ng/mL. The cross-reactivity of the antiserum with
159 IGF-II was <1.9% and <0.01% with insulin, proinsulin, EGF, oxytocin and steroid hormones,
160 respectively. The inter- and intra-assay coefficients of variation were <8.2% and 3.4%,
161 respectively.

162 The concentrations of progesterone, testosterone and estradiol were determined via RIA in 25 µL
163 samples of incubation medium previously validated for use in culture medium.

164 **Progesterone** concentrations were measured using RIA as described by Prakash, Meyer,
165 Schallenberger and Van de Wiel [31]. Rabbit antiserum against progesterone, obtained from the
166 Research Institute for Animal Production, Schoonoord, Netherlands, displayed cross-reactivity of
167 <0.1% with 17 β-estradiol, dihydrotestosterone, testosterone, and 17 β-hydroxyprogesterone. The
168 assay sensitivity was 12.5 pg/mL, and the inter- and intra-assay coefficients of variation did not
169 exceed 3.3 and 3.0%, respectively.

170 **Testosterone** was assayed using RIA according to the method described by Münster [32]. The
171 sensitivity of the assay was 10 pg/mL. The antiserum displayed cross reactivity of <96% with
172 dihydrotestosterone, <3% with androstenedione, <0.01% with progesterone and estradiol, <0.02%
173 with cortisol, and <0.001% with corticosterone. The inter- and intra-assay coefficients of variation
174 were 12.3 and 6.8%, respectively.

175 **Estradiol** concentrations were evaluated by RIA according to Münster [32] by using antisera
176 against steroids (produced in the Institute of Animal Science, Neustadt, Germany) with an assay
177 sensitivity of 5 pg/mL. The cross-reactivity of the estradiol antiserum was < 2% to estrone, < 0.3%
178 to estriol, < 0.004% to T and <0.0001% to P4 and cortisol. The inter- and intraassay coefficients
179 of variation did not exceed 16.6% and 11.7%, respectively.

180

Statistical analysis

Each experimental group was represented by four culture wells or one chamber slide well. The proportions of cells containing specific immunoreactivity were calculated from inspection of at least 1000 cells per chamber. Assays of hormone levels in the incubation media were performed in duplicate. The rates of substance secretion were calculated per 10^6 cells/day. Significant differences between the control and experimental groups were evaluated by using two-way ANOVA and paired t-tests using statistical software SigmaPlot 11.0 (Systat Software, Erkrath, Germany). The data are expressed as means ± SEM. Differences from the control at $P<0.05$ were considered significant.

Results

Proliferation

X (at 20 μg/mL) when given alone stimulated proliferation (accumulation of PCNA). TT (at 1 ng/mL but not at 10 ng/mL or 100 ng/mL) also stimulated proliferation. Moreover, when given together with X, TT significantly ($P<0.05$) suppressed (at a TT dose 100 ng/mL, but not at 1 ng/mL and 10 ng/mL) the stimulatory effect of X on proliferation (Fig. 1A).

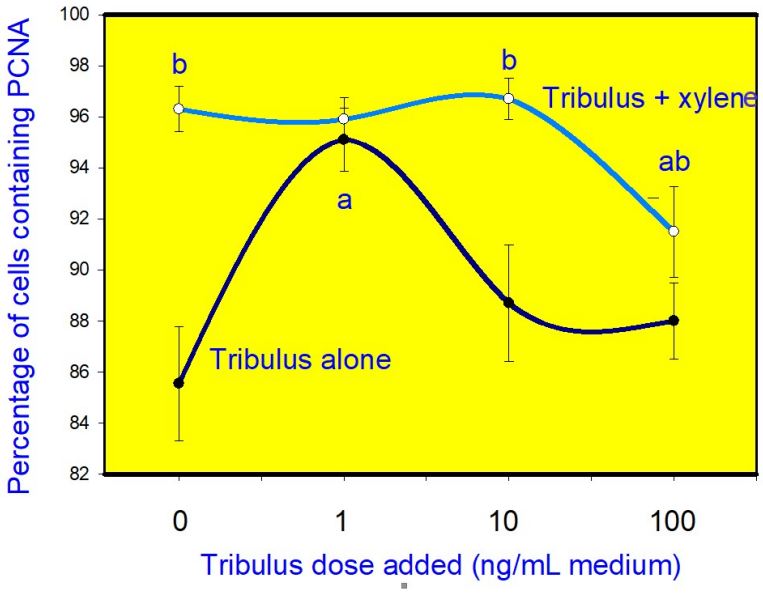

Fig. 1A. The effect of the extract of puncture vine (*Tribulus*) (0, 1, 10, or 100 ng/mL), xylene (20 µg/mL) and their combination (*Tribulus* + xylene) on proliferation (expression of PCNA). The values are mean ± SEM. a – effect of *Tribulus* – significant ($P<0.05$) differences between cells cultured with and without *Tribulus terrestris* extract, b – effect of xylene – significant ($P<0.05$) differences between the corresponding groups of cells cultured with and without xylene. SEM - standard error of the mean.

Apoptosis

X (at 20 µg/mL) when given alone did not change the accumulation of the apoptosis marker Bax. TT (at doses of 10 ng/mL but not at 1 ng/mL and 100 ng/mL) inhibited apoptosis. Moreover, when given together with X, TT evoked the stimulatory effect of xylene (at doses of 1 and 10 ng/mL but not at 100 ng/mL) on apoptosis (Fig. 1B).

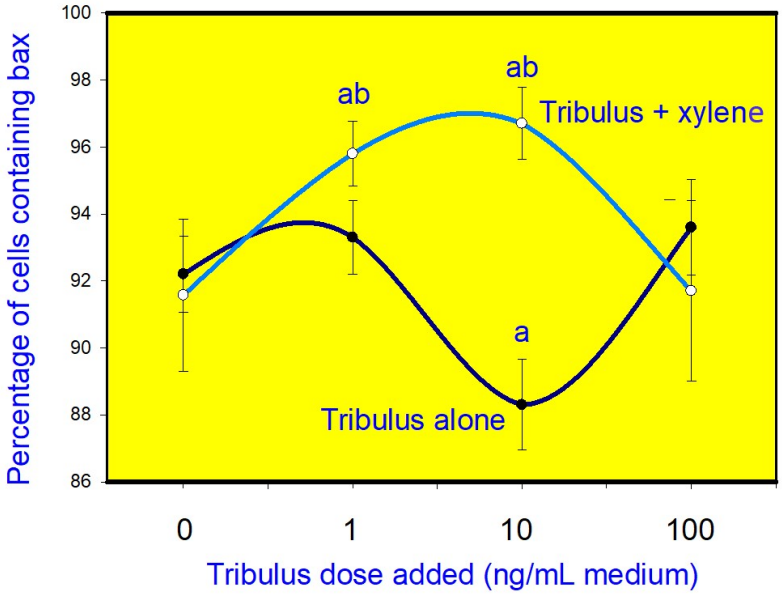

Fig. 1B. The effect of the extract of puncture vine (*Tribulus*) (0, 1, 10, or 100 ng/mL), xylene (20 µg/mL) and their combination (*Tribulus* + xylene) on apoptosis (expression of Bax). The values are mean ± SEM. a – effect of *Tribulus* – significant (P<0.05) differences between cells cultured with and without *Tribulus terrestris* extract, b – effect of xylene – significant (P<0.05) differences between the corresponding groups of cells cultured with and without xylene. SEM - standard error of the mean.

Hormone release

IGF-I

X (at 20 µg /mL) stimulated the release of IGF-I. TT (1 ng/mL, 10 µg/mL and 100 ng/mL) did not change the IGF-I output. Moreover, when given together with X, TT (at doses of 1 ng/mL, 10 ng/mL, and 100 ng/mL) did not modify the stimulatory effect of X on IGF-1 release (Fig. 1C).

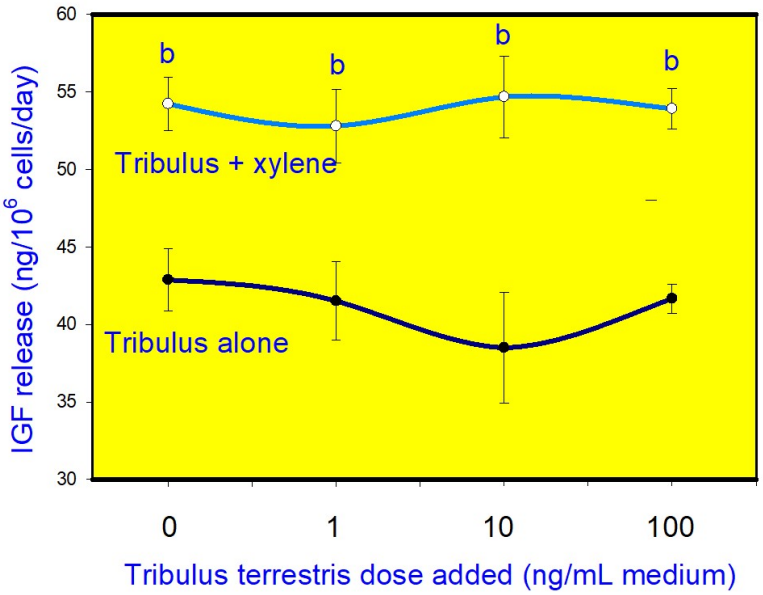

Fig. 1C. The effect of the extract of puncture vine (*Tribulus*) (0, 1, 10, or 100 ng/mL), xylene (20 µg/mL) and their combination (*Tribulus* + xylene) on the release of IGF-I. The values are mean ± SEM. a – effect of *Tribulus* – significant ($P<0.05$) differences between cells cultured with and without *Tribulus terrestris* extract, b – effect of xylene – significant ($P<0.05$) differences between the corresponding groups of cells cultured with and without xylene. SEM - standard error of the mean.

Progesterone

X when given alone (at 20 µg /mL) and TT alone (1 ng/mL and 10 ng/mL, but not at 100 ng/mL) inhibited progesterone release. Moreover, when given together with X, TT promoted the inhibitory effect of X (at doses of 1 ng/mL, 10 ng/mL and 100 ng/mL) on progesterone release (Fig. 1D).

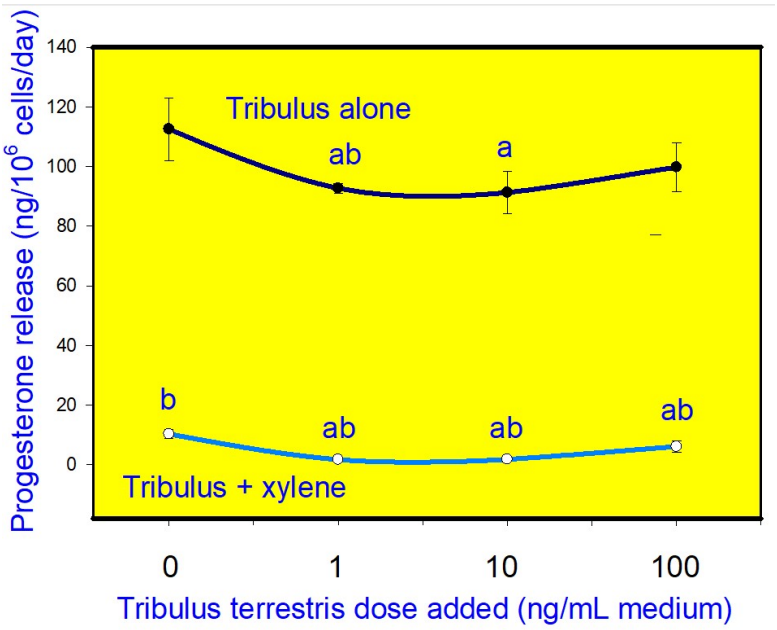

Fig. 1D. The effect of the extract of puncture vine (*Tribulus*) (0, 1, 10, or 100 ng/mL), xylene (20 µg/mL) and their combination (*Tribulus* + xylene) on the release of progesterone. The values are mean ± SEM. a – effect of *Tribulus* – significant ($P<0.05$) differences between cells cultured with and without *Tribulus terrestris* extract, b – effect of xylene – significant ($P<0.05$) differences between the corresponding groups of cells cultured with and without xylene. SEM - standard error of the mean.

Testosterone

X (20 µg /mL) inhibited testosterone release. TT (at a dose of 100 ng/mL but not at 1 ng/mL or 10 ng/mL) stimulated testosterone release. Moreover, when given together with X, TT did not modify (at doses of 1 ng/mL and 10 ng/mL) the effect of X on testosterone release, but cells cultured with X together with TT at dose of 100 ng/mL, released testosterone above the control level (Fig. 1E).

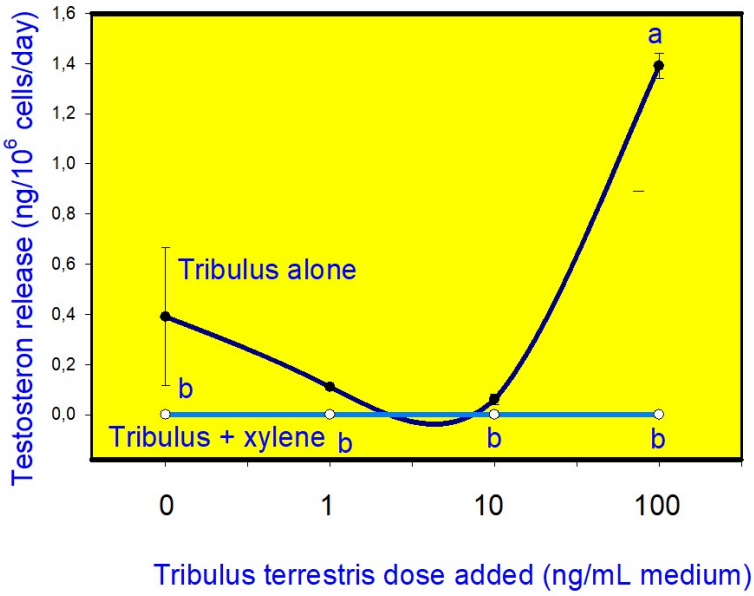

Fig. 1E. The effect of the extract of puncture vine (*Tribulus*) (0, 1, 10, or 100 ng/mL), xylene (20 µg/mL) and their combination (*Tribulus* + xylene) on the release of testosterone. The values are mean ± SEM. a – effect of *Tribulus* – significant ($P<0.05$) differences between cells cultured with and without *Tribulus terrestris* extract, b – effect of xylene – significant ($P<0.05$) differences between the corresponding groups of cells cultured with and without xylene. SEM - standard error of the mean.

Estradiol

In all of the collected samples of the incubation medium, the measured concentrations of estradiol were below the RIA detection limit.

Discussion

The creation of a monolayer, the presence of a proliferation marker and the production of IGF-I and steroid hormones indicate that the tested bovine granulosa cells were in good condition and suitable for analysis and testing of both the negative and positive behavior of both T and X. Ovarian

cell luteinization is characterized by high production of progesterone and low or no production of estradiol. The high production of progesterone and the release of estradiol under the detection limit observed in our experiments indicate that the cells after culture underwent luteinization. Furthermore, the present observations demonstrated that both TT and X directly affected ovarian cells and their basic functions, namely, proliferation, apoptosis and the release of hormones. These parameters are considered to be both markers and regulators of ovarian functions and fecundity [14].

Does *Tribulus terrestris* affect ovarian cell functions?

Our observations demonstrated the ability of TT to promote the proliferation and release of testosterone and to inhibit apoptosis and the release of progesterone but not IGF-I release by bovine ovarian cells. These observations are in line with previous reports on the ability of TT to promote rodent reproductive processes *in vivo* [9,10,33]. Furthermore, the present observations of TT action on bovine granulosa cells partially correspond to previous observations on porcine cells. In porcine cells TT promoted both the proliferation and apoptosis, indicating that TT can increase ovarian cell turnover [12]. The ability of TT to promote proliferation and suppressed apoptosis in bovine granulosa cells indicates that in cows TT can increase not only ovarian cell turnover but also the ovarian cell number. The available data suggest that TT can promote mammalian reproduction by promoting ovarian cell proliferation and regulating apoptosis, which in turn can result in the promotion of ovarian follicular growth and development. TT action on bovine granulosa cells in our experiments was associated with changes in steroid hormones and IGF-I. Previously, increases in plasma testosterone and estradiol levels were observed in rats [9] and women [18] treated with TT.

The TT effects on ovarian cell proliferation and apoptosis in our experiments might be mediated by suppression of the release of progesterone, a known suppressor of ovarian cell proliferation and follicullogenesis [14]. It should not be excluded that TT can also promote ovarian functions through stimulation of testosterone, a precursor of estrogens, which can promote ovarian cell proliferation and follicular and oogenesis [14], although the lack of substantial estradiol production in our experiments is not in agreement with this hypothesis. Another possible mediator of plant action on ovarian cell proliferation and apoptosis could be IGF-I. It, like TT, can promote proliferation and suppress apoptosis of ovarian cells in various species [14]. Nevertheless, the lack of an effect of TT on IGF-I release in our experiments does not correspond to this hypothesis. Therefore, TT action on bovine ovarian cell proliferation could be mediated by progesterone, but not by testosterone, estradiol or IGF-I.

The intracellular mechanisms of TT action on ovarian cell functions also require further elucidation. Several TT constituents possess the ability to affect steroid hormone receptors and to block reactive oxygen species, which are causes of apoptosis [1-6]. One such candidate in TT with phytoestrogenic and antioxidant properties could be quercetin [7]. Nevertheless, it is unlikely that the stimulatory effects of TT on ovarian cell functions observed in our experiments are due to the presence of quercetin because our previous similar studies showed not a stimulatory but an inhibitory action of this phytoestrogen on these parameters in porcine [12,23,27,28] and bovine [27] ovarian cells. A number of other TT constituents with phytoestrogenic and antioxidant action can also affect ovarian cell functions [1,17], but their role in mediating TT action on the ovary has not yet been demonstrated.

Therefore, the TT constituents and the possible role of steroid hormone receptors and oxidative processes in mediating TT action on the ovary require experimental validation.

Taken together, our observations demonstrate mainly the stimulatory action of TT on basic ovarian cell functions, which can promote ovarian folliculogenesis and affect ovarian steroidogenesis. These observations provide new evidence that TT may be useful for the promotion of both animal and human female reproduction and it could be used to treat reproductive disorders inducing infertility. For example, TT could be promising for the treatment of ovarian cancer (which is characterized by reduced apoptosis[15]) or signs of polycystic ovarian syndrome (increased androgen production and retarded ovarian follicular growth and development, [10,13,17]. Furthermore, dietary TT can promote reproductive processes in cows and other phytophagous farm animals, which can be useful in their production. Nevertheless, the areas, conditions and protocols of TT application require validation with adequate *in vivo* studies.

Does xylene affect ovarian cell functions?

Our results showed that X addition stimulated the proliferation but not the apoptosis of bovine ovarian granulosa cells. These observations are opposite to the character of X action on porcine granulosa cells reported previously, where X suppressed both proliferation and apoptosis [22]. Furthermore, they only partially confirm our previous observation of the stimulatory action of X on both proliferation and apoptosis in cultured bovine granulosa cells [22,23].

Moreover, in the present experiments, we observed an inhibitory effect of X on the release of progesterone and testosterone output and increased IGF-I release by bovine granulosa cells. These observations are partially consistent with previous reports concerning the inhibitory action of X on rat blood progesterone levels [20], production of progesterone by cultured porcine granulosa cells [22], and both progesterone and testosterone release by cultured bovine granulosa

cells [12,23]. Furthermore, they are in line with the previous observations of the ability of X to stimulate bovine granulosa cell IGF-I release [23].

Therefore, the present experiments confirmed previous reports concerning the ability of X to promote ovarian cell proliferation, to affect apoptosis, to suppress progesterone and to stimulate ovarian IGF-I release. On the other hand, they indicated some differences of the X action on ovarian cells among different species and even in experiments performed on the same model. These differences could be due to variability in the resistance to X among different species and even among different animals.

The functional interrelationships between X-dependent processes might be hypothesized. For example, progesterone and testosterone are known regulators of ovarian cell proliferation and apoptosis [14]. Therefore, it is possible that the changes in ovarian cell proliferation and apoptosis could be a consequence of changes in ovarian steroid hormone release. Furthermore, IGF-I is a potent promoter of ovarian cell proliferation and an inhibitor of apoptosis [14]. The increase in both IGF-I release and proliferation in X-treated cells suggests that X can promote granulosa cell proliferation via stimulation of IGF-I output. On the other hand, increased IGF-I release in our experiments was not associated with any changes (present experiments) or even an increased [22,23] accumulation of apoptosis markers in bovine granulosa after X additions. These observations indicate that X action on bovine ovarian cell apoptosis, in contrast to proliferation, is probably mediated by signaling molecule(s) other than IGF-I.

The characteristics and mechanisms of X action on ovarian cells and factors influencing this action require further investigation. Nevertheless, the available information suggests that the adverse effect of X on female reproduction could be due to the direct action of this environmental contaminant on ovarian cells and its ability to alter cell proliferation, apoptosis, steroid and peptide

391 hormone release – the processes determining ovarian folliculogenesis and fecundity [14].
392 Furthermore, steroid hormones [34] and IGF-I [35] are important promoters of cancer in
393 reproductive and nonreproductive organs and are characterized by increased cell proliferation and
394 survival [36,37]. Therefore, the ability of X to induce malignant transformation could be due to X
395 action on these hormonal stimulators of cell proliferation. This direct influence of X on ovarian
396 cells observed in our and previous experiments should be taken into account before exposing
397 laboratory animals, farm animals and humans to this contaminant. Furthermore, understanding the
398 characteristics and mechanisms of X action on the ovary could be helpful for the prevention,
399 mitigation and treatment of its adverse effects on reproduction.

400

401 *Does Tribulus terrestris modify the effect of xylene on ovarian cell functions?*

402 Addressing this question is important from a practical viewpoint because the application
403 of medicinal or food plants could be an easy way to protect female reproductive processes from
404 the adverse effects of X. In vitro studies demonstrated that extracts of some plants, such as
405 buckwheat, rooibos, Vitex agnus-castus [22] and their constituent quercetin [23] could mitigate,
406 prevent and even invert the influence of X on some parameters of porcine ovarian cells. On the
407 other hand, none of these additives were able to eliminate all of the X effects. One of the purposes
408 of the present studies was to examine whether TT could do so.

409 In the performed experiments, TT was able to prevent the effects of X on proliferation and
410 induce the proapoptotic action of X to promote its effect on progesterone, but it did not modify the
411 effect of T on IGF-I or testosterone release. In the available literature, we failed to find any
412 information about the protective effect of TT against the action of X or other environmental

413 contaminants. Therefore, our observations represent the first evidence that TT can modify X action
414 on ovarian cell functions.

415 Understanding the physiological significance of X and TT action on various ovarian cell
416 parameters requires further study. Nevertheless, some hypotheses concerning the role of these
417 molecules in the control of ovarian functions and ovarian cancer development might be suggested.
418 Malignant transformation is characterized by upregulation of cell proliferation and downregulation
419 of apoptosis. The ability of X to promote ovarian carcinogenesis could be the primary cause of its
420 carcinogenic action. In this case, prevention of the pro-proliferative action of X could be the first
421 mechanism of the protective effect of TT against ovarian cancer induced by the pro-proliferative
422 action of X. Furthermore, TT can induce the ability of X to promote ovarian cell apoptosis. It
423 should not be excluded that TT can prevent ovarian cancer by a second mechanism, inducing the
424 proapoptotic effect of X. Furthermore, TT can block the ability of X to suppress ovarian cell
425 proliferation, which can be a cause of X-induced infertility (Sirotkin and Harrath, 2014).
426 Therefore, TT can be not only a stimulator of reproductive processes but also a natural protector
427 against the anti-reproductive action of X. On the other hand, the ability of TT to promote the
428 inhibitory action of X on the release of progesterone indicates that TT can not only prevent but
429 also promote the anti-reproductive effect of X. The functional interrelationships between processes
430 influenced by TT and X could be supposed. For example, progesterone can be a promoter of
431 ovarian cell apoptosis [14].

432 It also remains to be determined what TT constituents and their biochemical properties are
433 responsible for their reproductive and protective effects. TT contains steroidal saponins,
434 flavonoids, flavanol glycosides, alkaloids, and tannins with antioxidant, anti-inflammatory and
435 phytoestrogen properties, which can be responsible for its physiological, medicinal and protective

436 effects [1-6]. Detection of such constituents could help in understanding the mechanisms and areas
437 of application of TT as a putative biostimulator and protector of female reproduction.

438 Therefore, the reproductive effects of both TT and X require additional studies.
439 Nevertheless, the present observations represent the first demonstration that TT can be a promoter
440 of ovarian cell function (a stimulator of proliferation and a suppressor of apoptosis) and a regulator
441 of ovarian steroidogenesis. X can increase ovarian cell proliferation and IGF-I release and inhibit
442 ovarian steroidogenesis. These effects could explain the anti-reproductive and carcinogenic action
443 of X. The ability of TT to modify X action on proliferation and apoptosis indicates that TT might
444 be a natural protector against some ovarian cell disorders associated with X action on proliferation
445 and apoptosis, but it can also promote the adverse effect of X on progesterone release. On the other
446 hand, the protective action of TT should be verified by further *in vivo* studies.

447

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