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Gossypol, a polyphenolic aldehyde from cotton plant, interferes with swine granulosa cell function

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Abstract

Gossypol is a polyphenol isolated from the seed, roots and stem of cotton plant (*Gossypium sp.*) It has been associated with adverse effects on female reproduction, but recently also shown having promising effects against several malignancies. Its mechanisms of action are however still not fully understood. This study was therefore conducted to investigate the effect of 5 or 25 μg/mL gossypol on swine granulosa cell steroidogenic activity, redox status and Vascular Endothelial Growth Factor (VEGF) production. Study demonstrated that gossypol significantly ($P < 0.001$) inhibited granulosa cell estradiol 17β and progesterone production, an effect that could be at least partially mediated by an increase ($P < 0.05$) of nitric oxide and superoxide anion production as a consequence of superoxide dismutase inhibition. Moreover, gossypol stimulates ($P < 0.001$) VEGF production. In conclusion, study has demonstrated effecs of gossypol on swine granulosa cell function in vitro. Effects on female swine fertility can not be excluded.

Keywords: Angiogenesis; Ovary; Free radicals; Steroids; Reproduction

1. Introduction

Gossypol ($C_{30}H_{30}O_8$) is an aldehyde that is produced in the pigment glands of the roots, leaves, stems and seeds of the cotton plant genus *Gossypium.* Gossypol and two additional tautomeric forms, the hemiacetal and phenolic ketonoid, have been demonstrated to account for numerous chemical and biological reactions associated with this compound [1]. During the early 1900s, toxicosis caused by excessive consumption of cotton products by non-ruminant animals was related to gossypol poisoning. In the 1960s, villagers of many Chinese rural areas had switched from cooking with soybean oil to crude cottonseed oil [2]. Several years later many couples experienced fertility problems [3]. A similar impairment of reproductive performances has been documented in dairy cows fed with cottonseed and its by-products. In particular, reduced rates of pregnancy and increased pregnancy loss have been related to high free gossypol diets [4]. A negative effect of gossypol on fertility has also been shown in non ruminant females such as rats and hamsters, possibly by a disruption of steroid hormone metabolism [5] as well as cytotoxicity in the embryo [6,7]. In the pig, symptoms of chronic ingestion of high levels of free gossypol in cottonseed meal include labored breathing, dyspnea, decreased growth rate and anorexia [8]. However, the effects of gossypol on swine reproduction have not been studied in detail. However, a few published data indicate that gossypol interferes with the expression of normal estrous cycle probably due to mechanisms that affect ovarian func-

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tion. Moreover, administration of this substance before implantation would inhibit pregnancy [9]. In order to gain more detailed knowledge on the effect of gossypol on female reproduction and to unravel its mechanisms of action, we examined its effects on swine granulosa cell proliferation and steroidogenesis, the main parameters of ovarian cell function. In addition, being aware of the fundamental role of reactive oxygen species in mediating follicular physiology, the effects of gossypol on granulosa cell redox status were examined. Finally, since we have previously demonstrated [10] that angiogenesis is essential for ovarian follicle development, we also tested a possible modulatory action on the production of the main proangiogenic factor, VEGF, by granulosa cells.

2. Materials and methods

All reagents used in this study were obtained from Sigma (St. Louis, MO, USA) unless otherwise specified.

2.1. Granulosa cell collection

Swine ovaries were collected at a local slaughterhouse from Large White cross-bred gilts, parity = 0. The stage of the cycle was unknown. Follicles were classified on a dimension-based fashion [11]. The ovaries were placed into cold PBS (4 °C) supplemented with penicillin (500 IU/mL), streptomycin (500 μg/mL) and amphotericin B (3.75 μg/mL), maintained in a freezer bag and transported to the laboratory within 1 h. After a series of washings with PBS and ethanol (70%) granulosa cells were aseptically harvested by aspiration of large follicles (> 5 mm) with a 26-gauge needle and released in medium containing heparin (50 IU/mL), centrifuged for pelleting and then treated with 0.9% prewarmed ammonium chloride at 37 °C for 1 min to remove red blood cells. Cell number and viability were estimated using a haemocytometer under a phase contrast microscope after vital staining with trypan blue (0.4%) of an aliquot of the cell suspension. Cells were seeded in culture medium (CM) M199 supplemented with sodium bicarbonate (2.2 mg/mL), bovine serum albumin (BSA 0.1%), penicillin (100 IU/mL), streptomycin (100 μg/mL), amphotericin B (2.5 μg/mL), selenium (5 ng/mL) and transferrin (5 μg/mL). Once seeded, cells were incubated in the presence or absence of gossypol (5 or 25 μg/mL) and maintained for 48 h at 37 °C under humidified atmosphere (5% CO_2). This procedure was identical for all experiments performed in this study.

2.2. Granulosa cell proliferation and steroid production

2.2.1. Cell proliferation

10^4 cell/well were seeded in 96-well plates in 200 μL CM. Cell proliferation was evaluated by 5-bromo-2′-deoxyuridine (BrdU) incorporation assay test (Roche, Mannheim, Germany). Briefly, after addition of 20 μL BrdU to each well after 44 h of incubation in the presence or absence of gossypol treatments, culture media were removed and a DNA denaturating solution was added in order to improve the accessibility of the incorporated BrdU for antibody detection. Thereafter, 100 μL anti-BrdU antibody were added to each well. After a 1.5 h incubation at room temperature, the immune complexes were detected by the subsequent substrate reaction. The reaction product was quantified by measuring the absorbance at a wavelength of 450 nm against 690 nm using a Spectra Shell Microplate reader (SLT Spectra, Milan, Italy). To test the viable cell number, absorbance was related to a standard curve prepared by culturing in quintuplicate granulosa cells at different plating densities (from 10^3 to 10^5/200 μL) for 48 h. The curve was repeated in four different experiments. The relationship between cell number and absorbance was linear (r = 0.92). Cell number/well was estimated from the resulting linear regression equation. The assay detection limit was 10^3 cell/well and the variation coefficient was less than 5%. The number of cells obtained was used for correcting hormones, VEGF production and redox status data.

2.2.2. Steroid production

10^4 cells/well were seeded in 96-well plates in 200 μL CM supplemented with androstenedione (28 ng/mL). Culture media were then collected, frozen and stored at –20 °C until progesterone (P4) and 17β estradiol (E2) determination by validated RIAs [12].

P4 assay sensitivity and ED50 were 0.24 and 1 nmol/l, respectively; E2 assay sensitivity and ED50 were 0.05 and 0.2 nmol/l. The intra- and inter-assay coefficients of variation were less than 12% for both assays.

2.3. Granulosa cell redox status

2.3.1. NO production

10^5 cells/200 μL CM were seeded in 96-well plates. NO was assessed by measuring nitrite levels in culture media by the microplate method based on the formation of chromophore after reaction with Griess reagent, which was prepared fresh daily by mixing equal volumes of stock A (1% sulfanilamide, 5% phosphoric acid)