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Gossypol Promotes the Degeneration of Chicken Ovarian Follicles in Vitro

■Author(s)

Gadelha ICN^I
Lima MA^I
Melo MM^{II}
Soto-Blanco B^{II}

^I Laboratory of Molecular Biology of Reproduction, Department of Animal Sciences, Universidade Federal Rural do Semi-Árido (UFERSA), Mossoró, RN, Brazil.

^{II} Laboratory of Toxicology, Department of Veterinary Clinics and Surgery, Veterinary School, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil.

■Mail Address

Corresponding author e-mail address
Benito Soto-Blanco
DCCV-EV-UFGM, Avenida Presidente
Antônio Carlos, 6627
31275-013. Belo Horizonte, MG, Brazil
Tel: +31 3409.2255
Email: benito.blanco@pq.cnpq.br

■Keywords

Cottonseed, female reproduction, gossypol, ovaries, reproductive toxicology.

ABSTRACT

Gossypol, a phenolic compound produced by the pigment glands of cotton, is known to affect male reproduction. In females, exposure to gossypol has been associated with interference with the estrous cycle and embryo development. In laying hens fed cottonseed, gossypol was found to reduce egg production and weight and to cause discoloration of the egg yolk and/or albumen. It is probable that gossypol directly affects ovarian follicles, but this has not been confirmed experimentally yet. Thus, the aim of the present study was to determine if gossypol affected cultivated ovarian follicles of the chicken. Ovarian follicles of adult female chickens were cultivated with different concentrations of gossypol (0, 5, 10 and 20 µg/mL) and classified according to the stage of development as primordial, transitional, primary, secondary or antral, and as viable or atretic. The percentages of viable and atretic follicles in the ovaries cultivated for 24 hours in the presence of gossypol were significantly different from those of the controls for all types of follicles except for secondary follicles, but in all types after cultivation for 7 days. The percentage of viable follicles was higher than that of atretic follicles in ovaries cultivated without gossypol. In contrast, ovaries cultivated with gossypol showed a predominance of atretic follicles. Gossypol increased the proportion of atretic follicles at all stages of development in cultivated chicken ovaries. Thus, gossypol may affect ovarian follicular viability and maturation, which might interfere with female fertility.

INTRODUCTION

Cottonseeds are used as a protein source in animal feeds, but their use is limited by the presence of gossypol (Nagalakshmi *et al.*, 2007; Gadelha *et al.*, 2014a). Gossypol (2,2-bis(8-formyl-1,6,7-trihydroxy-5-isopropyl-3-methylnaphthalene)) is a phenolic compound produced by the pigment glands in cotton stems, leaves, seeds and flower buds (Rogers *et al.*, 2002; Kenar, 2006; Alexander *et al.*, 2008; Soto-Blanco, 2008). This compound is known to affect male reproduction, inhibiting spermatogenesis and reducing spermatozoid motility and viability (Randel *et al.*, 1992; Fornes *et al.*, 1993; Brocas *et al.*, 1997; Yuan & Shi, 2000; El-Sharakly *et al.*, 2010). These effects were dose- and time-dependent, but were reversed when gossypol was no longer ingested (Randel *et al.*, 1992; Hassan *et al.*, 2004).

The exposure to gossypol has been associated with interference with the estrous cycle of female rodents (Gu & Anderson, 1985; Adeyemo *et al.*, 2007; Gadelha *et al.*, 2014a) and with pregnancy and early embryonic development in cattle (Randel *et al.*, 1992, 1996; Brocas *et al.*, 1997; Gadelha *et al.*, 2011), and embryo implantation in rats (Lin *et al.*, 1991). The ovaries of heifers fed cottonseed meal contained



fewer large follicles (>5 mm) than those of heifers fed soybean meal (Randel *et al.*, 1996). Gossypol was also responsible for reduced populations of viable ovarian follicles and larger populations of atretic follicles in rats (Gadelha *et al.*, 2014b) and sheep (Câmara *et al.*, 2015). *In-vitro* studies established that gossypol affected granulosa cell functioning in pigs (Basini *et al.*, 2009), bovine ovarian steroidogenesis (Gu *et al.*, 1990; Lin *et al.*, 1994), and bovine oocyte cumulus expansion and nuclear maturation (Lin *et al.*, 1994).

In laying hens fed cottonseed, gossypol was found to reduce egg production (Fitzsimmons *et al.*, 1989; Panigrahi *et al.*, 1989) and egg weight (Panigrahi *et al.*, 1989), and to cause discoloration of the egg yolk and/or albumen (Panigrahi *et al.*, 1989; Panigrahi & Hammonds, 1990; Panigrahi, 1992; Panigrahi & Plumb, 1996; Lordelo *et al.*, 2007). The complete cessation of egg production may occur (Fitzsimmons *et al.*, 1989). It is possible that gossypol directly affects ovarian follicles, but this has not been confirmed experimentally. Thus, the aim of the present study was to determine if gossypol affects cultivated ovarian follicles of the chicken.

MATERIAL AND METHODS

This study was approved by the Institutional Animal Care and Use Committee at the Universidade Federal Rural do Semi-árido (UFERSA) (CEUA-UFERSA 31/2014, process 23091.003576/2014-66).

The experimental procedure for ovarian follicles culture was based on an earlier study (Câmara *et al.*, 2015). The ovaries were obtained from four adult Bantam chickens. One ovary was fixed in Carnoy's solution (60% ethanol, 30% chloroform, and 10% glacial acetic acid) for 12 hours (*in-situ* control) for histological analysis. The other ovaries were cultivated in 24-well cell culture plates containing 1 mL of culture medium, consisting of alpha minimum essential medium (α -MEM) Eagle supplemented with 2 mM glutamine, 2 mM hypoxanthine, 1.25 mg/

mL bovine serum albumin-BSA, 50 μ g/mL ascorbic acid (Sigma, Sigma-Aldrich, St Louis, MO, USA), and antibiotics (100 μ g/mL of penicillin and 100 μ g/mL of streptomycin, Gibco, Grand Island, NY, USA). Four concentrations of (+/-)-gossypol acetic acid (G4382, Fluka, Buchs, Switzerland) were tested: 0, 5, 10 and 20 μ g/mL. The tested doses were based on an earlier study that evaluated the *in-vitro* and *in-vivo* effects of gossypol on sheep (Câmara *et al.*, 2015). The plates were incubated at 39 °C in 5% CO₂ (Panasonic CO₂ Incubator MCO-18AC, Leicestershire, UK) for 24 hours or 7 days. The culture medium was replaced every 48 hours. After incubation, the ovaries were fixed and processed for histological analysis using hematoxylin and eosin staining.

At least 160 follicles per treatment were counted and classified according to Pedersen & Peters (1968). The follicles were classified according to the stage of development as primordial (one layer of flattened granulosa cells), transitional (one layer of both flattened and cuboidal granulosa cells), primary (one layer of cuboidal granulosa cells), secondary (two layers of cuboidal granulosa cells), or antral (containing an antral cavity). The follicles were also classified as viable or atretic. The viable follicles presented a regular shape and well-organized granulosa cells, without signs of atresia. The atretic follicles were characterized by the presence of a retracted oocyte, a pyknotic nucleus, a discontinuous basement membrane, and disorganized granulosa cells.

The obtained data were statistically analyzed using R software (version 3.0.3) (R Development Core Team, 2008). The frequencies of viable and atretic follicles were compared using Fisher's exact test. A significance level of p values < 0.05 was adopted.

RESULTS

The effect of the period of cultivation on the follicle populations of chicken ovaries is shown in Table 1. The follicle populations of freshly collected chicken ovaries

Table 1 – Viable ovarian follicle populations (%) of chicken ovaries cultivated for 0, 24 and 168 hours.

Follicles	Hours of cultivation			P^a
	0	24	168	
Primordial	66.7 (60/90)	71.4 (40/56)	65.4 (51/78)	n.s.
Transitional	69.6 (16/23)	53.3 (8/15)	51.9 (14/27)	n.s.
Primary	83.3 (10/12)	70.0 (7/10)	56.3 (9/16)	n.s.
Secondary	68.2 (15/22)	42.9 (3/7)	57.1 (8/14)	n.s.
Antral	69.2 (9/13)	78.1 (57/73)	84.6 (22/26)	n.s.
Total	68.8 (110/160)	71.4 (115/161)	64.6 (104/161)	n.s.

^aFisher's exact test; n.s.: non-significant ($p>0.05$)



and those cultivated for 24 hours or for 7 days were not significantly different.

The percentages of viable and atretic follicles in ovaries cultivated for 24 hours in the presence of gossypol were significantly different from those of the controls for all of the types of follicles, except for secondary follicles (Table 2). The percentage of viable follicles was higher than that of atretic follicles in the ovaries cultivated without gossypol. In contrast, ovaries cultivated with gossypol showed a predominance of atretic follicles.

After cultivation for 7 days, the percentages of viable and atretic ovarian follicles of all types were significantly different (Table 2). A predominance of viable follicles in ovaries cultivated without gossypol and a predominance of atretic follicles in ovaries cultivated with gossypol were determined (Figure 1).

DISCUSSION

We found that chicken ovaries cultivated with gossypol at all of the tested concentrations showed an increased proportion of atretic follicles at all stages of development.

Several studies demonstrated reduced egg production in laying hens fed diets with various minimal levels of free gossypol, including 126 mg/kg (Fitzsimmons *et al.*, 1989), and 130 mg/kg (Panigrahi *et al.*, 1989). The complete cessation of egg production may occur when hens are fed 1,008 mg free gossypol/kg of feed (Fitzsimmons *et al.*, 1989). Egg weight was

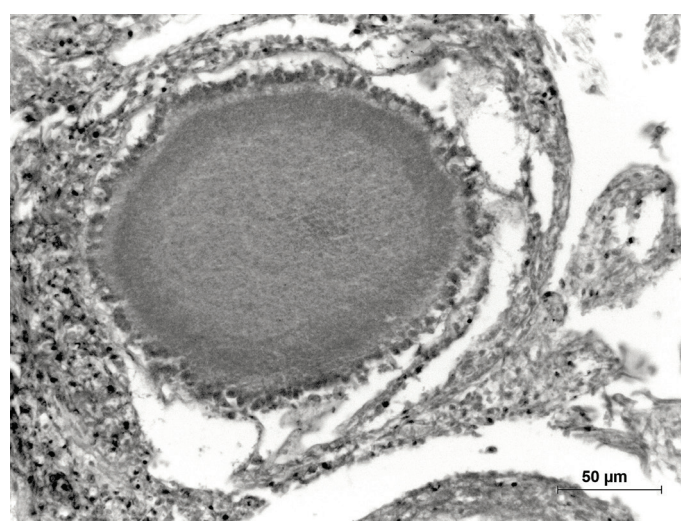


Figure 1 – Atretic antral follicle from a chicken ovary cultured with 5.0 µg/mL gossypol for 7 days.

reduced by the exposure to free gossypol in the feed at 255 mg/kg (Panigrahi *et al.*, 1989). Other described adverse effects of gossypol exposure are discoloration of the egg yolk and/or of the albumen (Panigrahi *et al.*, 1989; Panigrahi & Hammonds, 1990; Panigrahi, 1992; Panigrahi & Plumb, 1996; Lordelo *et al.*, 2007). The results of the present study evidence that the reported effects of gossypol on egg production and egg quality may be due to its direct damage of the ovaries.

In our study, gossypol affected ovarian follicle cells, increasing the proportion of atretic follicles at all stages of development in cultivated chicken ovaries, consistently with the observations in rats (Gadelha *et al.*, 2014b) and sheep (Câmara *et al.*, 2015). Damage

Table 2 – Viable ovarian follicle populations (%) of chicken ovaries cultivated with gossypol at different concentrations (0, 5, 10, or 20 µg/mL) for 24 hours or 7 days.

Follicles	Duration of culture	Gossypol (µg/mL)				P ^a
		0	5	10	20	
Primordial	24 hours	71.4 (40/56)	52.7 (29/55)	48.7 (37/76)	37.0 (34/54)	0.0032
	7 days	65.4 (51/78)	44.6 (33/74)	33.3 (27/81)	29.8 (25/84)	<0.0001
Transitional	24 hours	53.3 (8/15)	36.7 (11/30)	21.1 (8/38)	5.1 (2/39)	0.0003
	7 days	51.9 (14/27)	30.0 (6/20)	20.6 (7/34)	10.0 (2/20)	0.0106
Primary	24 hours	70.0 (7/10)	26.3 (5/19)	27.3 (6/22)	0	0.0460
	7 days	56.3 (9/16)	23.1 (3/13)	31.3 (5/16)	0	0.0335
Secondary	24 hours	42.9 (3/7)	34.8 (8/23)	9.1 (1/11)	30.0 (6/20)	n.s.
	7 days	57.1 (8/14)	10.5 (2/19)	0	7.4 (2/27)	0.0006
Antral	24 hours	78.1 (57/73)	48.5 (16/33)	40.0 (8/20)	40.4 (23/57)	<0.0001
	7 days	84.6 (22/26)	0	24.1 (7/29)	0	<0.0001
Total	24 hours	71.4 (115/161)	43.1 (69/160)	35.9 (60/167)	29.3 (51/174)	<0.0001
	7 days	64.6 (104/161)	26.8 (44/164)	27.2 (46/169)	16.6 (29/175)	<0.0001

^aFisher's exact test; n.s.: non-significant ($p>0.05$)



to primordial ovarian follicles permanently disrupts female reproduction because these cells develop into primary, secondary, and antral follicles in a continuous and nonreversible process (Hirshfield, 1997). The observed viability of primordial ovarian follicles in our study implies that chickens fed gossypol might experience early reproductive senescence due to the reduction of the ovarian reserve of follicles.

The results of the present study also show that the ovarian follicle damage was promoted directly by gossypol, but not by any products of its biotransformation. The mechanism for ovarian toxicity of gossypol is not fully understood, but may be due to its cytotoxic and apoptotic activities (Atmaca *et al.*, 2009; Cengiz *et al.*, 2010; Wang *et al.*, 2013). The cytotoxic effect may be a result of the generation of reactive oxygen species, leading to oxidative stress (Kovacic, 2003). In addition, gossypol can inhibit glucose 6-phosphate dehydrogenase, causing a decrease in NADPH production, which is required for the actions of glutathione peroxidase, an important component of the cellular antioxidant system (El-Mokadem *et al.*, 2012).

Gossypol is also known to interfere with intercellular communication (Hervé *et al.*, 1996) and the transport of ions across membranes (Cheng *et al.*, 2003; El-Sharaky *et al.*, 2010). Furthermore, it inhibits calcium influx and Mg-ATPase Ca-Mg-ATPase activity in the plasma membranes of spermatozooids (Breitbart *et al.*, 1989; El-Sharaky *et al.*, 2010), but an increase in the intracellular calcium concentration has also been reported (Cheng *et al.*, 2003).

Other potential mechanism underlying the toxicity of gossypol includes the interference with cell energy metabolism. Indeed, it was observed that this compound blocks the production, release, and use of ATP by spermatozooids (Ueno *et al.*, 1988), promoting ultrastructural changes in mitochondria (Chenoweth *et al.*, 2000; Romualdo *et al.*, 2002). In addition, gossypol inhibited steroidogenic activity in cultivated porcine granulosa cells (Basini *et al.*, 2009). The results from the present study show that cultured chicken ovarian follicles may be an excellent tool for future studies aiming to elucidating the cellular mechanism underlying the reproductive toxicity of gossypol.

In conclusion, gossypol increases the proportion of atretic follicles at all stages of development in cultivated chicken ovaries. Thus, gossypol may affect ovarian follicular viability and maturation, which might interfere with female fertility. The probable mechanism of the ovarian toxicity involves the interaction of direct cytotoxic effects and interference in steroidogenesis.

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