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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-UFMG, Avenida Presidente Antônio Carlos, 6627
31275-013. Belo Horizonte, MG, Brazil
Tel: +31 3409.2255
Email: benito.blanco@pq.cnpq.br

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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-bi(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-Sharaky *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 µg/mL ascorbic acid (Sigma, Sigma-Aldrich, St Louis, MO, USA), and antibiotics (100 µg/mL of penicillin and 100 µg/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 µg/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% CO_2 (Panasonic CO_2 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.

[a]Fisher'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).

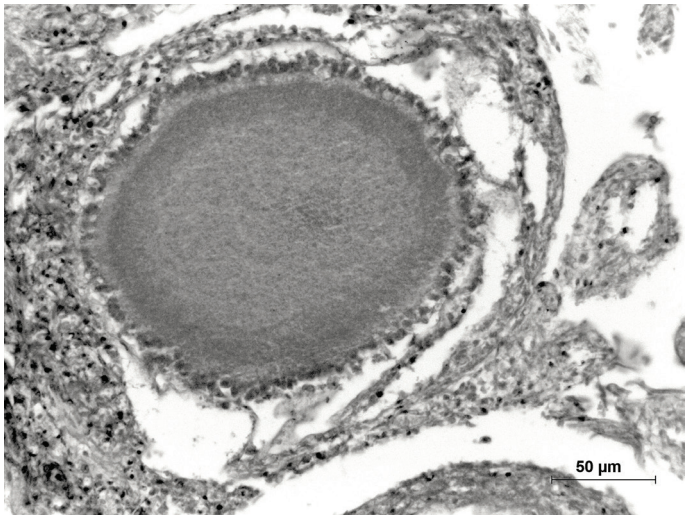

Figure 1 – Atretic antral follicle from a chicken ovary cultured with 5.0 µg/mL gossypol for 7 days.

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 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

[a]Fisher'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 spermatozoids (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 spermatozoids (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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REFERENCES

Adeyemo GO, Longe OG, Adejumo DO. The reproductive performance of breeder cocks fed cottonseed meal cake-based diets. International Journal of Poultry Science 2007;6(2):140-144.

Alexander J, Benford D, Cockburn A, Cravedi J, Dogliotti E, Domenico A Di, et al. Gossypol as undesirable substance in animal feed. The EFSA Journal 2008;908:1-55.

Atmaca H, GorumLu G, Karaca B, Degirmenci M, Tunali D, Cirak Y, et al. Combined gossypol and zoledronic acid treatment results in synergistic induction of cell death and regulates angiogenic molecules in ovarian cancer cells. European Cytokine Network 2009;20(3):121-130.

Basini G, Bussolati S, Baioni L, Grasselli F. Gossypol, a polyphenolic aldehyde from cotton plant, interferes with swine granulosa cell function. Domestic Animal Endocrinology 2009;37(1):30-36.

Breitbart H, Mayevsky A, Nass-Arden L. Molecular mechanisms of gossypol action on sperm motility. International Journal of Biochemistry 1989;21(10):1097-1102.

Brocas C, Rivera RM, Paula-Lopes FF, McDowell LR, Calhoun MC, Staples CR, et al. Deleterious actions of gossypol on bovine spermatozoa, oocytes, and embryos. Biology of Reproduction 1997;57(4):901-907.

Câmara ACL, Gadelha ICN, Borges PAC, Paiva SA, Melo MM, Soto-Blanco B. Toxicity of gossypol from cottonseed cake to sheep ovarian follicles. PLoS ONE 2015;10(11):e 0143708.

Cengiz E, Karaca B, Kucukzeybek Y, GorumLu G, Gul M, Erten C, et al. Overcoming drug resistance in hormone- and drug-refractory prostate cancer cell line, PC-3 by docetaxel and gossypol combination. Molecular Biology Reports 2010;37(3):1269–1277.

Cheng JS, Lo YK, Yeh JH, Cheng HH, Liu CP, Chen WC, et al. Effect of gossypol on intracellular Ca2+ regulation in human hepatoma cells. Chinese Journal of Physiology 2003;46(3):117-122.

Chenoweth PJ, Chase CC, Risco CA, Larsen RE. Characterization of gossypol-induced sperm abnormalities in bulls. Theriogenology 2000;53(5):1193-1203.

El-Mokadem MY, Taha TA, Samak MA, Yassen AM. Alleviation of reproductive toxicity of gossypol using selenium supplementation in rams. Journal of Animal Science 2012;90(9):3274-3285.

El-Sharaky AS, Newairy AA, Elguindy NM, Elwafa AA. Spermatotoxicity, biochemical changes and histological alteration induced by gossypol in testicular and hepatic tissues of male rats. Food and Chemical Toxicology 2010;48(12):3354-3361.

Fitzsimmons RC, Newcombe M, Moul IE. The long-term effects of feeding ground and whole cottonseed to laying hens. Canadian Journal of Animal Science 1989;69(2):425-431.

Fornes MW, Barbieri AM, Burgos MH. Sperm motility loss induced by gossypol: relation with OH. scavengers, motile stimulators and malondialdehyde production. Biochemical and Biophysical Research Communications 1993;195(3):1289-1293.

Gadelha ICN, Rangel AH do N, Silva AR, Soto-Blanco B. Efeitos do gossipol na reprodução animal. Acta Veterinaria Brasilica 2011;5(2):129-135.

Gadelha ICN, Fonseca NBS, Oloris SCS, Soto-Blanco B. Gossypol toxicity from cottonseed products. The Scientific World Journal 2014a;2014:231635.

Gadelha ICN, Macedo MF de, Oloris SCS, Melo MM, Soto-Blanco B. Gossypol promotes degeneration of ovarian follicles in rats. The Scientific World Journal 2014b;2014:986184.

Gu Y, Anderson NO. Effects of gossypol on the estrous cycle and ovarian weight in the rat. Contraception 1985;32(5):491-496.

Gu Y, Lin YC, Rikihisa Y. Inhibitory effect of gossypol on steroidogenic pathways in cultured bovine luteal cells. Biochemical and Biophysical Research Communications 1990;169(2):455-461.

Hassan ME, Smith GW, Ott RS, Faulkner DB, Firkins LD, Ehrhart EJ, et al. Reversibility of the reproductive toxicity of gossypol in peripubertal bulls. Theriogenology 2004;61(6):1171-1179.

Hervé JC, Pluciennik F, Bastide B, Cronier L, Verrecchia F, Malassiné A, et al. Contraceptive gossypol blocks cell-to-cell communication in human and rat cells. European Journal of Pharmacology 1996;313(3):243-255.

Hirshfield AN. Overview of ovarian follicular development: considerations for the toxicologist. Environmental and Molecular Mutagenesis 1997;29(1):10-15.

Kenar JA. Reaction chemistry of gossypol and its derivatives Journal of the American Oil Chemists' Society 2006;83(4):269-302.

Kovacic P. Mechanism of drug and toxic actions of gossypol: focus on reactive oxygen species and electron transfer. Current Medicinal Chemistry 2003;10(24):2711-2718.

Lin YC, Rajamahendran P, Rikihisa Y. Inhibition of rat embryo implantation in the gossypol-treated uterine horn. Theriogenology 1991;35(4):769-777.

Lin YC, Coskun S, Sanbuissho A. Effects of gossypol on in vitro bovine oocyte maturation and steroidogenesis in bovine granulosa cells. Theriogenology 1994;41(8):1601-1611.

Lordelo MM, Calhoun MC, Dale NM, Dowd MK, Davis AJ. Relative toxicity of gossypol enantiomers in laying and broiler breeder hens. Poultry Science 2007;86(3):582-590.

Nagalakshmi D, Rao SVR, Panda AK, Sastry VRB. Cottonseed meal in poultry diets: a review. J Poultry Science 2007;44(2):119-134.

Panigrahi S. Effects of treating cottonseed meal with a solution of ferrous sulphate on laying hen performance and discolourations in eggs. Animal Feed Science and Technology 1992;38(2-3):89-103.

Panigrahi S, Hammonds TW. Egg discolouration effects of including screw-press cottonseed meal in laying hen diets and their prevention. British Poultry Science 1990;31(1):107-120.

Panigrahi S, Plumb VE. Effects on dietary phosphorus of treating cottonseed meal with crystalline ferrous sulphate for the prevention of brown yolk discolouration. British Poultry Science 1996;37(2):403-411.

Panigrahi S, Plumb VE, Machin DH. Effects of dietary cottonseed meal, with and without iron treatment, on laying hens. British Poultry Science 1989;30(3):641-651.

Pedersen T, Peters H. Proposal for a classification of oocytes and follicles in the mouse ovary. Journal of Reproduction and Fertility 1968;17(3):555-557.

R Development Core Team. R: A language and environment for statistical computing [Internet]. Vienna: R Foundation for Statistical Computing; 2008.

Randel RD, Chase CC, Wyse SJ. Effects of gossypol and cottonseed products on reproduction of mammals. Journal of Animal Science 1992;70(5):1628-1638.

Randel RD, Willard ST, Wyse SJ, French LN. Effects of diets containing free gossypol on follicular development, embryo recovery and corpus luteum function in Brangus heifers treated with bFSH. Theriogenology 1996;45(5):911-922.

Rogers GM, Poore MH, Paschal JC. Feeding cotton products to cattle. Veterinary Clinics of North America, Food Animal Practice 2002;18(2):267-294.

Romualdo GS, Klinefelter GR, Kempinas WG. Postweaning exposure to gossypol results in epididymis-specific effects throughout puberty and adulthood in rats. Journal of Andrology 2002;23(2):220-228.

Soto-Blanco B. Gossipol e fatores antinutricionais da soja. In: Spinosa HS, Górniak SL, Palermo Neto J, editores. Toxicologia aplicada à medicina veterinária. Barueri: Manole; 2008. p. 531-545.

Ueno H, Sahni MK, Segal SJ, Koide SS. Interaction of gossypol with sperm macromolecules and enzymes. Contraception 1988;37(3):333-341.

Wang J, Jin L, Li X, Deng H, Chen Y, Lian Q, et al. Gossypol induces apoptosis in ovarian cancer cells through oxidative stress. Molecular bioSystems 2013;9(6):1489-1497.

Yuan YY, Shi QX. Inhibition of hamster sperm acrosomal enzyme by gossypol is closely associated with the decrease in fertilization capacity. Contraception 2000;62(4):203-209.