Gossypol Promotes the Degeneration of Chicken Ovarian Follicles in Vitro

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% 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>
<thead>
<tr>
<th>Follicles</th>
<th>0</th>
<th>24</th>
<th>168</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primordial</td>
<td>66.7 (60/90)</td>
<td>71.4 (40/56)</td>
<td>65.4 (51/78)</td>
</tr>
<tr>
<td>Transitional</td>
<td>69.6 (16/23)</td>
<td>53.3 (8/15)</td>
<td>51.9 (14/27)</td>
</tr>
<tr>
<td>Primary</td>
<td>83.3 (10/12)</td>
<td>70.0 (7/10)</td>
<td>56.3 (9/16)</td>
</tr>
<tr>
<td>Secondary</td>
<td>68.2 (15/22)</td>
<td>42.9 (3/7)</td>
<td>57.1 (8/14)</td>
</tr>
<tr>
<td>Antral</td>
<td>69.2 (9/13)</td>
<td>78.1 (57/73)</td>
<td>84.6 (22/26)</td>
</tr>
<tr>
<td>Total</td>
<td>68.8 (110/160)</td>
<td>71.4 (115/161)</td>
<td>64.6 (104/161)</td>
</tr>
</tbody>
</table>

*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).

**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
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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