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The effect of gossypol acetate on the fertility of boars

Mariana Torres[1,a] – Kayode Balogun[2,a] – Larissa Shirley[2] – André Furugen Cesar de Andrade[1] – Kara Stewart[2] – Keping Hu[3] – Xiaoying Wang[3] – Zoltan Machaty[2*]

[1]Department of Animal Reproduction, University of São Paulo, Pirassununga, SP, Brasil
[2]Department of Animal Sciences, Purdue University, West Lafayette, IN, USA
[3]Institute of Medicinal Plant Development, Beijing, China
*Correspondence: zmachaty@purdue.edu

[a] These authors contributed equally to this work and should be considered as co-first author.

SUMMARY

This study evaluated the effect of gossypol acetate, a potential antifertility compound, on semen quality and testicular histopathology of boars. Six boars were allotted into two experimental groups. Half of the animals were fed a diet that was supplemented with 2 mg of gossypol acetate per kg body weight per day for 9 weeks, control animals received a gossypol acetate-free feed. During the experimental period, semen was collected weekly from each boar and semen parameters were recorded. The animals were then euthanized, testicular samples were collected and histopathological examination of the testicular cells, as well as morphometrical analysis of the seminiferous tubules, were performed. The percentage of spermatozoa showing tail abnormalities increased significantly (P=0.017) in the semen of boars fed gossypol acetate-supplemented feed, while several other semen parameters deteriorated without showing statistical significance. Gossypol acetate supplementation also led to a decrease (P=0.042) in the number of spermatogonia in the seminiferous tubules and an increase (P=0.020) in the number of vacuoles in the seminiferous epithelium, consistent with changes seen in cases of male reproductive toxicity. In conclusion, gossypol acetate negatively affected a number of semen characteristics and also had detrimental effects on the histopathology of the testes.

Keywords: *gossypol, spermatozoa, testis*

INTRODUCTION

Gossypol is a polyphenol derived from the pigment glands in the stems, leaves, flower buds, roots, and seeds of the cotton plant (*Gossypium* sp.). It is a yellow substance that is part of the natural defense mechanism of the plant against predators as it provokes infertility in insects (Coutinho, 2002; Gadelha et al., 2014). During cotton processing, the cotton is ginned, the seeds are crushed and the oil is extracted, and the remaining cottonseed meal is often used in animal feed as a protein supplement. However, cottonseed meal contains a significant amount of gossypol, which at high concentrations can decrease fertility rates in ruminant and non-ruminant animals. Furthermore, gossypol can be toxic as gossypol poisoning has been reported to manifest in the form of acute respiratory distress, anorexia, apathy, impaired immune function and even death in the affected animals (Gadelha et al., 2014). Beneficial effects of the compound have also been described: gossypol and its derivatives were found promising as a treatment for breast cancer (Van Poznak et al., 2001), leukemia (Balakrishnan et al., 2008), and prostate cancer (Jiang et al., 2012) in humans.

In early China, gossypol was used as a male contraceptive. Later studies showed that although the usage of gossypol caused infertility that was irreversible in about 10% of the patients, for temporary contraception in men gossypol was effective. It had no deleterious effects on major biochemical parameters, endocrine function or libido (National Coordinating Group on Male Antifertility Agents, China, 1979). Gossypol has its antifertility effects at the level of spermatogenesis: it damages the germinal epithelium and reduces the number of germ cells as well as somatic cells present in the testes (Chenoweth et al., 2000; El-Sharaky et al., 2010). It was also shown to inhibit sperm motility, decrease sperm concentration, and induce specific mitochondrial injury to the sperm tail (Randel et al., 1992). Gossypol was also found to be not teratogenic: according to a study that tested its effects on rats, a 20-times increase in the dosage needed to cause infertility in men did not affect fetal development and the progress of pregnancy in gestating females (Beaudoin, 1985).

Different species have been used to investigate how gossypol influenced male reproduction. Results of these studies revealed that gossypol had various dose-dependent effects on animals. For example, feeding young pubertal brahman bulls with gossypol led to decreased sperm production and motility, and an increased abnormal sperm count (Chenoweth et al., 1994), while Hassan et al. (2004) found primary and secondary sperm abnormalities and an increased number of spermatozoa with proximal droplets in prepubertal bulls. However, after 28 days of feeding gossypol-free diets, these abnormalities were reversed. Similarly, Hahn et al. (1981) reported degeneration of spermatocytes in hamsters and rats treated with gossypol orally. Dose-dependent effects of gossypol on rats included a decreased sperm count and motility, increased number of abnormal sperm cells in the ejaculate, reduced levels of testosterone, LH and FSH in the serum as well as increased abnormal sperm count at 5, 10 and 20 mg kg^{-1} body weight day^{-1} (El-Sharaky et al., 2010). Others also reported tubular degeneration, reduced testosterone concentrations, and involutions of the ventral prostate and vesicular glands at gossypol

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doses of 10 mg kg^{-1} body weight day^{-1} (Gåfvels et al., 1984); and decreased spermatogenesis in addition to Sertoli cell and seminiferous tubules damage at 25 mg kg^{-1} body weight day^{-1} (Heywood et al., 1986). Interestingly, while most studies found various kinds of deleterious effects of gossypol on male reproduction in different species, Nunes et al. (2010) and Guedes and Soto-Blanco (2011) reported no changes in semen volume, sperm concentration, motility and morphology after feeding cottonseed meal (0.5 kg $animal^{-1}$ day^{-1}) for 120 days to sheep and goats. The objective of the present study was to test the effect of a low dose of gossypol acetate, a solvate of gossypol and acetic acid on semen quality and testicular histopathology of boars.

MATERIALS AND METHODS

Chemicals

The chemicals used during the experiments were purchased from Sigma Aldrich Chemical Company (St. Louis, MO, USA) unless otherwise indicated.

Animals and experimental treatments

The animals in this study were handled according to a protocol approved by the Purdue Animal Care and Use Committee (PACUC) of Purdue University. Six sexually mature boars (two Duroc and four Large White) weighing 266.11±11.39 kg were housed in individual pens at the Animal Science Research and Education Center (ASREC; Purdue University, West Lafayette, IN, USA). The nutritional intake was calculated for each boar individually based on the body weight and offered once a day (7:00 AM); the intake was 0.0094 kg feed kg^{-1} body weight day^{-1}. Water was provided ad libitum. Before the beginning of gossypol acetate treatment, semen was collected two times from each boar within a one-week interval. The groups were homogenized with regard to body weight, sperm concentration, and sperm motility. Feed was prepared separately for each treatment group. The feed of the control group was a corn/soybean meal diet containing no gossypol acetate, while that of the treatment group contained 2 mg of gossypol acetate kg^{-1} body weight mixed with the same base diet. The feed was individually weighed and offered daily for each boar to ensure they consumed the entire dose intended; the animals received the control and gossypol acetate-supplemented feed for 61 consecutive days.

Semen collection

During the experimental period, semen was collected weekly from each boar using the gloved-hand method. Before collection, the preputial diverticulum was cleaned by hand pressure and dried. The collected semen was immediately extended with an equal volume of Beltsville Thawing Solution (BTS; Minitube USA, Verona, WI, USA) and then transported to the laboratory in an isothermal container.

Sperm quality analysis

Upon arrival at the laboratory, the semen was kept in a 37 °C water bath for 10 minutes. Sperm total and progressive motility were evaluated by the Ceros® II sperm analyzer (v. 1.6.3; Hamilton Thorne Inc, Beverly, MA, USA). For the analysis, a 3 µL aliquot of semen was placed into a pre-warmed (37 °C) Leja® slide chamber (IMV Technologies USA, Brooklyn Park, MN, USA); 5 view fields were evaluated with at least 400 spermatozoa in total using phase-contrast microscopy. The NucleoCounter® SP-100 sperm cell counter (Chemometec®, Alerod, Denmark) was used to assess sperm concentration and viability (i.e., percentage of live cells). Prior to evaluation, 10 µL of each sample was extended in 1000 µL of either the S100 reagent (Reproduction Provisions®, Walworth, WI, USA) or semen extender as recommended by the manufacturer and loaded into an SP1-Cassette™ (Chemometec®). Viability assessment is based on the fact that the S100 reagent kills all sperm cells while the SP1-Casette contains propidium iodide, a fluorescent dye that can penetrate cells with damaged plasma membrane only. Finally, sperm morphology was assessed under a phase-contrast, bright-field microscope with 40x magnification. From each sample, 1 mL was fixed with 100 µl of 10% formalin for morphological evaluation, then 6 µL of this suspension was placed on a microscope slide, covered with a coverslip and 200 spermatozoa were classified as either morphologically normal or containing proximal droplets, distal droplets, distal midpiece reflexes, or head and tail abnormalities. Acrosome morphology was assessed using oil immersion at 100x magnification where 100 sperm cells were classified as having morphologically normal or abnormal acrosomes.

Testicular histopathology

At the end of the 61-day-long feeding period, the experimental animals were slaughtered and their testicles recovered. Tissue samples were collected from the bottom, middle, and top sections of the testicles and fixed in 10% neutral buffered formalin solution for 24 hours. They were then washed with running water, dehydrated using an ethyl alcohol series, cleared in xylene and embedded in paraffin wax. After embedding, the specimens were sectioned to a thickness of 4 µm using a Leica RM2235 microtome and stained with either hematoxylin-eosin or toluidine blue. Hematoxylin stains cell nuclei purple, while eosin stains the extracellular matrix as well as the cytoplasm pink; toluidine blue stains nucleic acids blue and polysaccharides purple while it also increases the sharpness of histology slide images. Fifteen tubular profiles (5 from each section of every testicle) were chosen randomly and measured for each animal. In the stained sections, the number of cells (including spermatogonia, spermatocytes, spermatids, spermatozoa, and Sertoli cells) and vacuoles were counted; the area, diameter, and perimeter of the seminiferous tubules, as well as the lumen, were measured using the Fiji software.

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Statistical analysis

During the evaluation of the results, two treatments (control and gossypol acetate) and eleven different periods (weeks) were considered. The homogeneity of variances was verified by the Shapiro-Wilk test; transformations (Log or arcsin) and removal of outliers were performed whenever necessary. The data were evaluated using PROC MIXED of the SAS software v9.4 (SAS Institute Inc., Cary, NC, USA) for repeated measures with the main effect of treatment, week and their interaction using a compound symmetry covariance structure. Semen quality parameters for two weeks prior to feeding gossypol acetate were averaged and included as covariates in the model. Testicular histopathology data were also evaluated using PROC MIXED of the same software. Results are shown as means±s.e.m. and means were considered significantly different at $P< 0.05$.

RESULTS AND DISCUSSION

Sperm quality

In response to 2 mg kg^{-1} body weight day^{-1} gossypol acetate, although sperm motility was not affected, numerically lower ejaculate volumes, sperm concentrations and total number of sperm cells in semen was recorded (*Table 1*). Tail abnormalities increased significantly (P=0.017), while the percentage of sperm with a proximal cytoplasmic droplet and head abnormalities were also numerically higher in these animals without showing statistical significance. The percentage of normal sperm cells in the semen and acrosome morphology remained unchanged, and the mean viability was >70%. The percentages of normal sperm cells in the semen, head abnormalities, distal midpiece reflexes and distal cytoplasmic droplets varied the most among weeks with increasing occurrence as the environmental temperatures increased during the experimental period (*Figure 1*). This increase was seen across treatments. The frequency of abnormal acrosomes (P=0.032) also varied among weeks, while the ejaculate volume had a treatment by week interaction (P=0.003) caused by random variation commonly seen in semen data.

Testicular histopathology

At the end of the 9-week experimental period, the number of spermatogonia in the seminiferous tubules was significantly lower (P=0.042) and the number of vacuoles in the seminiferous epithelium was significantly higher (P=0.02) in boars fed with gossypol acetate (*Table 2*, *Figure 2*). No other variable including the number of germ cells of different developmental stages, number of Sertoli cells, or the characteristics of the seminiferous tubules showed any significant difference between the treatment groups in response to dietary gossypol acetate supplementation. There was no difference between the mean value of these parameters measured in the various testicular regions (i.e., top, middle, bottom) of the gossypol acetate-fed animals (data not shown).

Table 1: **Semen quality data in control and gossypol acetate-fed boars**

Variables	Control (mean±s.e.m.)	Gossypol (mean±s.e.m.)	P-value		
			TRT	WEEK	TRT x WEEK
Volume (ml)	239.69±16.38	219.70±11.66	0.691	0.198	0.003
Motility (%)	69.04±2.84	67.35±3.19	0.883	0.318	0.462
Progressive motility (%)	63.49±2.89	61.89±3.10	0.836	0.203	0.565
Concentration (10^6/ml)	367.63±25.37	307.12±23.43	0.274	0.050	0.104
Total cells (x 10^9)	88.79±6.76	65.67±5.35	0.356	0.213	0.810
Viability (%)	73.66±0.36	73.81±0.27	0.758	0.110	0.954
Normal cell (%)	67.14±3.27	66.95±3.44	0.933	<0.001	0.666
Proximal droplet (%)	2.29±0.52	7.30±1.61	0.250	0.076	0.400
Distal droplet (%)	11.09±1.46	8.45±1.24	0.412	0.022	0.848
Distal midpiece reflex (%)	14.65±2.78	9.13±2.49	0.620	0.013	0.649
Tail abnormalities (%)	1.21±0.20[a]	2.66±0.29[b]	0.017	0.188	0.337
Head abnormalities (%)	1.96±0.30	2.64±0.50	0.404	0.003	0.359
Abnormal acrosomes (%)	2.43±0.52	2.20±0.48	0.613	0.032	0.821

[a, b]Values in a row with different superscripts are significantly different; TRT – Treatment; TRT x WEEK - Treatment by week interaction

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Figure 1: **Week effect of feeding boars with or without gossypol on various spermatozoa characteristics**

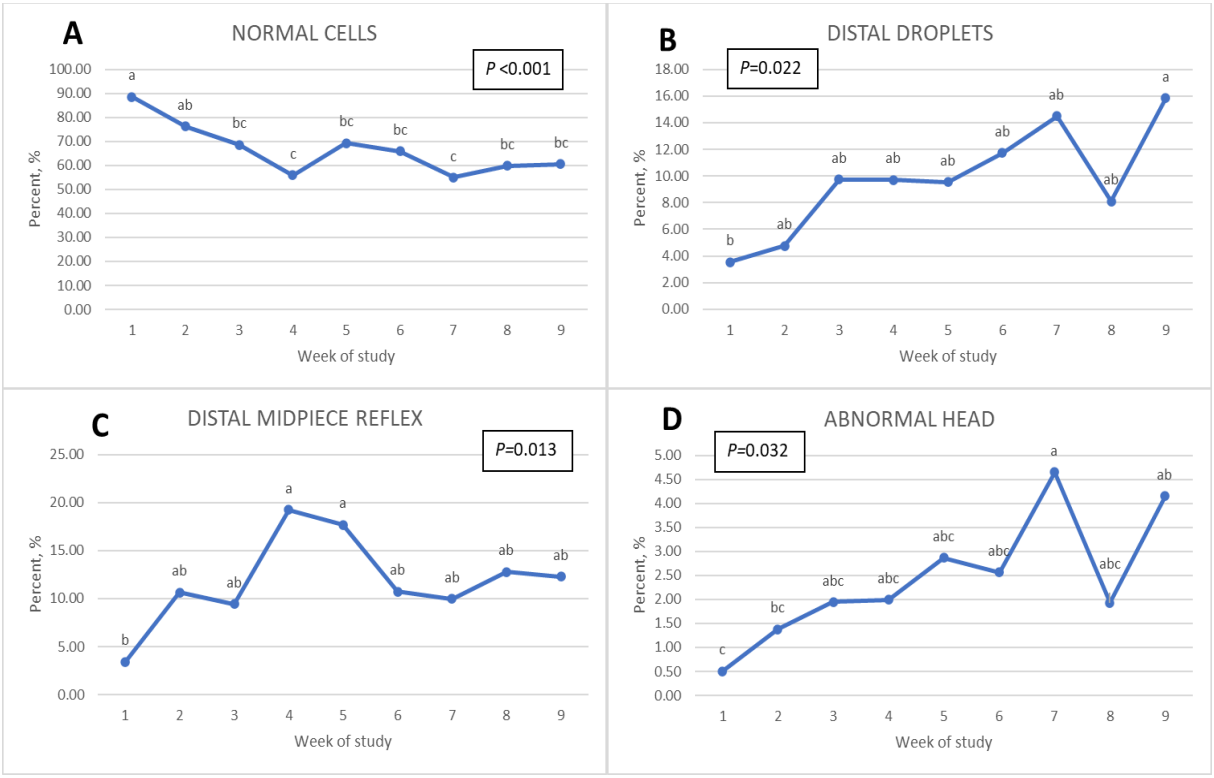

Table 2: **Histopathology of testicular samples of control and gossypol acetate-fed boars**

Variable	Treatment		P-value
	Control (mean±s.e.m.)	**Gossypol** (mean±s.e.m.)	
Spermatogonia (n)	61.13±1.99[a]	55.04±2.25[b]	0.042
Spermatocytes (n)	69.54±2.95	77.28±2.84	0.065
Spermatids (n)	158.86±6.12	169.25±7.29	0.257
Spermatozoa (n)	129.33±7.40	132.16±9.28	0.819
Seminiferous tubules perimeter (µm)	4042.83±64.84	4187.12±76.75	0.120
Seminiferous tubules area ($\mu m^2 \times 10^5$)	12.00±0.38	12.47±0.46	0.375
Seminiferous tubules diameter (µm)	1229.27±18.62	1227.03±23.72	0.953
Seminiferous tubules lumen perimeter (µm)	1610.04±99.40	1762.73±95.25	0.147
Seminiferous tubules lumen area ($\mu m^2 \times 10^5$)	1.10±0.11	1.20±0.11	0.321
Seminiferous tubules lumen diameter	359.27±20.28	396.57±21.61	0.120
Vacuoles (n)	1.65±0.27[a]	2.70±0.36[b]	0.020
Sertoli cells (n)	14.69±0.50	14.50±0.51	0.792

[a, b]Values in a row with different superscripts are significantly different

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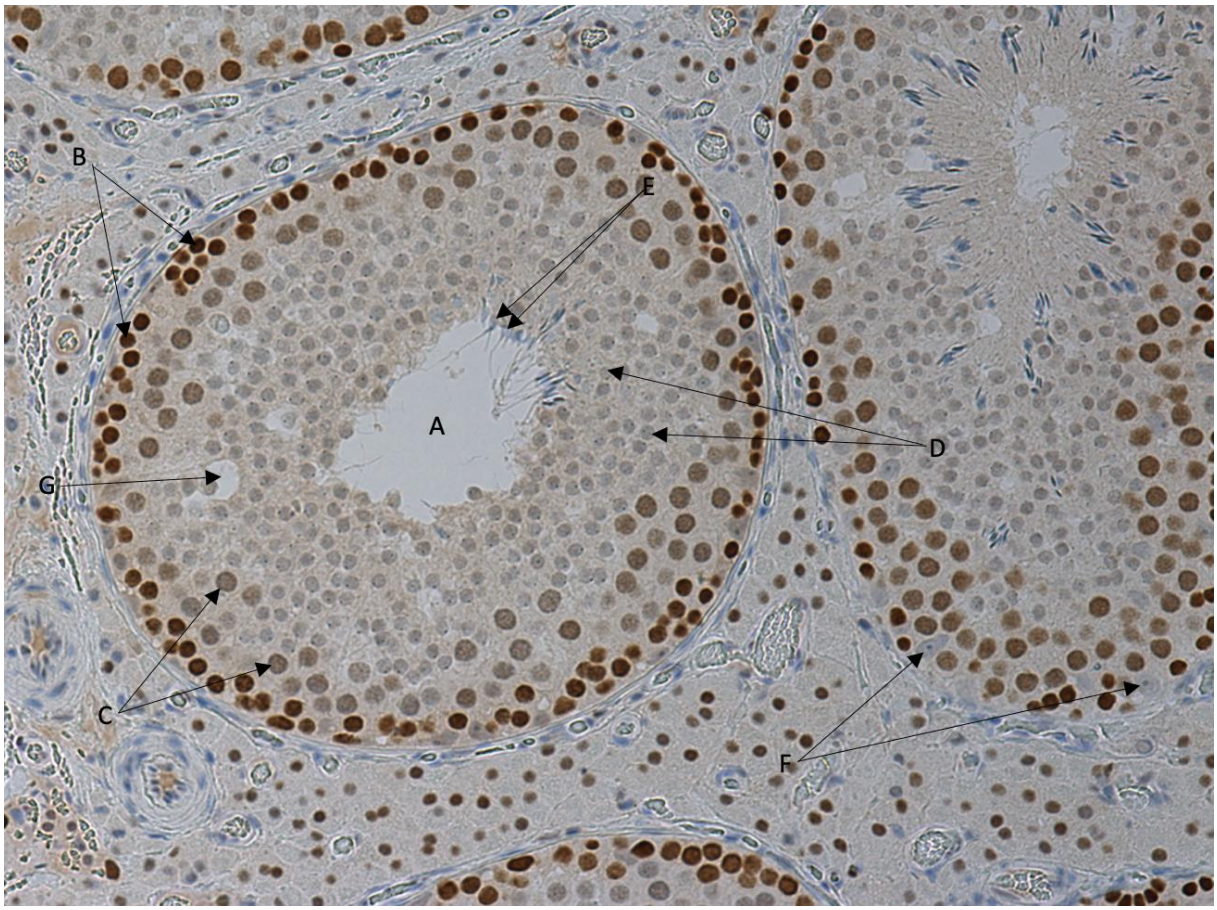
Figure 2: **Hematoxylin staining of a typical testicular sample showing germ cells, Sertoli cells and vacuoles**



A – Seminiferous tubule
B – Spermatogonia
C – Spermatocytes
D – Spermatids (round)
E – Spermatids (elongated)
F – Sertoli cell nucleus
G – Vacuole in Sertoli cell

Gossypol has been in the focus of attention of physiologists and animal nutritionists for decades with an initial interest centering around its toxicity. It was noted early on that livestock fed with cottonseed meal as a food supplement showed damage primarily to the heart, liver, and kidney (Morgan, 1989). Gossypol present in the pigment glands of the cotton plant was soon identified as the active ingredient. It was determined that it affected primarily monogastric animals whereas ruminants could tolerate higher levels because gossypol bound to proteins in the rumen. Subsequent studies revealed that gossypol inhibited the activity of enzymatic systems of the mitochondrial electron transport chain and thereby disconnected respiration and oxidative phosphorylation; it also decreased antioxidant concentrations in cells and caused damage to biological membranes by promoting the formation of reactive oxygen species (Kovacic, 2003). In addition, it was discovered in China in the 1960's that cooking with crude cottonseed oil caused infertility in men; these individuals showed symptoms such as reduced testicular size, azoospermia or oligospermia, and impotency (Segal, 1985). A close examination of the situation eventually showed that the culprit, again, was gossypol. This was followed by a great number of studies using purified gossypol, gossypol acetate (gossypol acetic acid), or gossypol formic acid that characterized the antifertility effect, site and mechanism of action, and pharmacokinetics of gossypol, as a result of which gossypol was suggested as a male contraceptive agent (Wu, 1972; Tang et al., 1980; Xue, 1980).

The first clinical trial started in 1972 found that based on sperm examination gossypol was over 99.7% effective in preventing fertilization (Zavos and Zarmakoupis-Zavos, 1996). The effects of gossypol on male reproduction was then investigated in various species including bulls (Chenoweth et al., 1994; Hassan et al., 2004), hamsters (Hahn et al., 1981), rats (Gåfvels et al., 1984; Heywood et al., 1986; El-Sharaky et al., 2010), rabbits (Chang et al., 1980), goats (Nunes et al., 2010), sheep (Guedes & Soto-Blanco, 2011) and

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humans (Coutinho et al., 2000). These studies revealed that at the beginning of a gossypol treatment, the drug attached to sperm stored in the epididymis and blocked their motility, later it also acted at the level of the testes and interfered with spermatogenesis (Segal, 1985). However, limited information is available about how gossypol influences the fertility of boars. In this study we fed boars with 2 mg gossypol acetate kg^{-1} body weight day^{-1} and examined semen quality as well as histological characteristics of the testes in the experimental animals. Because spermatogenesis in swine lasts approximately 40 days (França et al., 2005) gossypol acetate was fed to the boars for a period that exceeds this time period (61 days), so that its effect on the formation of new spermatozoa could also be assessed.

Gossypol is known to have a dose-dependent inhibitory effect on sperm motility (Stephens et al., 1983; Singla and Garg, 2013), an effect that may partly be attributed to gossypol's property to decrease the concentration of antioxidants and promote the formation of reactive oxygen species (El-Mokadem et al., 2012; Santana et al., 2015). Gossypol was reported to induce oxidative damage in unsaturated lipids (peroxidation), the consequence of which is membrane damage to spermatozoa (Kanwar et al., 1990; Bernard et al., 2001; Santana et al., 2015). This leads to a loss of motility as shown in rabbit and mouse spermatozoa (Jones and Mann, 1977; Alvarez and Storey, 1982; 1985). Gossypol also inhibits glucose uptake in spermatozoa. Spermatozoa take up glucose by means of carrier molecules (glucose transporters, GLUTs) located in their plasma membranes and use it as an energy source for motility. However, experiments focusing on gossypol's mechanism of action revealed that it was general membrane damage rather than specific inactivation of glucose transport proteins that was responsible for the marked decrease in glucose uptake (Kanwar et al., 1990). Gossypol also reduced ATP production by suppressing oxygen consumption and inhibiting ATPase activity in Spisula spermatozoa (Ueno et al., 1988). Sperm motility requires a large amount of adenosine triphosphate (ATP) molecules, which are produced via glycolysis or the oxidative phosphorylation pathway in the mitochondria (Calamera et al., 1982; du Plessis et al., 2015). Therefore, a hindrance to glucose uptake or ATP production will impair total sperm motility, which is crucial for fertilization. In primates, gossypol caused aplasia within the sperm mitochondrial sheath (Randel et al., 1992), while it was also shown that inhibition of mitochondrial metabolism leads to reduced sperm motility indicating that mitochondrial activity is critical for sperm motility (Guo et al., 2017). Finally, gossypol reportedly blocks cAMP formation in sperm cells (Zavos and Zarmakoupis-Zavos, 1996). As cAMP controls the flagellar beat in mammalian sperm (Esposito et al., 2004), a drop in cAMP levels leads to decreased sperm motility. In our experiments gossypol acetate had a negligible effect on sperm motility, a possible reason for this may be the relatively low dose (2 mg kg^{-1} body weight day^{-1}) it was given to the experimental animals.

In addition to its impact on motility, gossypol was reported to affect sperm production. Low sperm concentration has been linked to damage caused by gossypol to the germinal epithelium, resulting in a decrease in spermatogenesis and a reduction in total number of spermatozoa in various species (Shi et al., 1987; Santana et al., 2015). Others (Xue, 1985; El-Sharaky et al., 2010) also reported low sperm count and an increase in abnormal sperm morphology in male albino rats treated with gossypol acetate for two weeks, while Tanyildizi and Bozkurt (2004) found similar effects in cattle. Sperm tail abnormalities were higher in the semen of boars fed with gossypol acetate in our study. Tail lesion is a form of such abnormalities caused by gossypol and has been described as segmental aplasia of the mitochondrial sheath that is observed from late-stage spermatids through epididymal spermatozoa of mammals (Randel et al., 1992). Sperm concentration and total sperm number were numerically lower in the boars that received gossypol acetate supplementation in their feed, and some of the morphological abnormalities were also higher in the ejaculates of these animals. However, probably because of the low dose at which gossypol acetate was administered, these changes were statistically not significant. Gossypol had been reported to be effective as a reversible contraceptive in men when administered orally at 15 mg day^{-1}, during a 16-week period (Coutinho et al., 2000). Oral administration of gossypol acetate at 20 and 10 mg kg^{-1} day^{-1} to rats and hamsters, respectively, induced the onset of infertility in hamsters after 4 weeks and in rats after 6 weeks of treatment, with a reported increase in the number of dead spermatocytes post treatment (Hahn et al., 1981). In addition, cottonseed meal supplementation that provided 8.2 g of gossypol day^{-1} to 500 kg Brahman bulls was enough to induce adverse effects on sperm morphology after 3–4 weeks (Chenoweth et al., 1994). All these show that the effective dosage of gossypol to influence fertility varies from species to species.

Gossypol is known to have a harmful effect on the seminiferous epithelium, i.e., germ cells of different developmental stages as well as Sertoli cells. In our study, gossypol acetate reduced the number of spermatogonia present in the seminiferous tubules, while it had no negative impact on the number of germ cells of more advanced developmental stages. The number of Sertoli cells was not affected either. Sertoli cells are critical for the normal development of male germ cells and it is known that any agent that affects the viability and function of Sertoli cells have profound effects on spermatogenesis (D'Cruz et al., 2010). In primary cultures, gossypol acetate at a dose of 3.0 μg mL^{-1} was toxic to rat Leydig and Sertoli cells (Lan et al., 1992). However, as our results indicate, an adequate Sertoli cell population can still maintain a normal number of spermatocytes and spermatids in the seminiferous tubules even when the spermatogonial number is reduced. In addition, a great number of

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spermatogonia are present in the seminiferous tubules and even under normal circumstances many of them die by apoptosis during the mitotic phase of spermatogenesis (Shaha et al., 2010; Wang et al., 2017), this may be another reason why a smaller spermatogonial population can be sufficient to generate a normal number of spermatozoa during sperm production.

Another harmful effect of gossypol acetate that histological analysis of the testes revealed in the present study was an increase in the number of vacuoles inside of the seminiferous epithelium. Seminiferous tubule vacuolation was reported to occur in cases of male reproductive toxicity with vacuoles appearing within or between Sertoli cells in response to chemical administration. Vacuolation of Sertoli cells is a sign of morphologic injury to Sertoli cells or alternatively, vacuoles may represent spaces left behind by missing degenerated germ cells (Creasy, 2001). This finding is consistent with the result of other reports where degenerative and even necrotic effects in the seminiferous tubules and the entire testes were reported in different species including hamsters (Srivastava et al., 1989) and rats (Kalla et al., 1990) and after the administration of gossypol through different routes. Vacuolation and a reduction in the size of the seminiferous epithelium is a sign of degeneration that have been linked to a decrease in spermatogetic activity (El-Sharaky et al., 2010). In a similar manner, accumulation of immature germ cells in the lumen and an increase in intracellular gaps due to the disruption in cell-cell contacts in the seminiferous epithelium has also been reported in rats that received gossypol (Saleh et al., 2018). However, we have not detected these changes in our experiments.

CONCLUSIONS

Dietary supplementation of gossypol acetate at 2 mg kg^{-1} body weight day^{-1} negatively affected the number of spermatogonia present in the testes and had a harmful effect on the morphology of the seminiferous tubules as well as that of the spermatozoa. These changes would probably have been even more pronounced had a higher dose of gossypol acetate been administered. The results support earlier findings that gossypol can adversely affect fertility and may be useful as a male contraceptive if administered at the appropriate dose.

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