International Scholars Journals

African Journal of Pig Farming ISSN 2375-0731 Vol. 3 (9), pp. 001-006, September, 2015. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

DNA damage and decrease of cellular oxidase activity in piglet sertoli cells exposed to gossypol

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Accepted 5 January, 2015

The study was designated to explore the toxic effects of gossypol on piglet sertoli cells. Sertoli cells were isolated from piglet testes using a two-step enzyme digestion and followed by differential plating. Piglet sertoli cells were cultured and classified into five groups, that is, group A, the control without gossypol, group B with 2.5 µg/ml gossypol, group C with 5 µg/ml gossypol, group D with 10 µg/ml gossypol and group E with 20 µg/ml gossypol. We found that sertoli cells' growth was inhibited by gossypol at dose 2.5 µg/ml when compared with the control group. The oxidase activity of sertoli cell also decreased at 2.5 µg/m gossypol. Moreover, DNA damage of sertoli cells was observed at 5 µg/ml gossypol. Putting this into consideration, our study suggests that exposure of gossypol to sertoli cells leads to an inhibition of sertoli cell growth and oxidase activity of sertoli cells at a low concentration, whereas gossypol results in DNA damage of sertoli cells at a higher concentration.

Key words: Gossypol, sertoli cells, oxidase, DNA damage.

INTRODUCTION

Gossypol is a yellow polyphenolic compound isolated from the seed of cotton plants of the Malvaceae family. It exists in two forms, free and bound. Free gossypol is the major toxic ingredient of cottonseed meal, which is a source of protein for animal feed and its protein content is as high as 33.21 to 45.09%. However, the toxicity of gossypol has limited the use of cottonseed meal. Gossypol is generally known as a male anti-fertility by suppressing spermatogenesis (Dodou et al., 2005) and potential anticancer agent, which can induce the apoptosis of human breast cancer cells (Ye et al. 2010). Cytotoxic gossypol and its derivatives have been used for male contraception in humans (Coutinho, 2002). Ten micrograms per day dose of gossypol, causes azoosper-mia and deleterious changes in monkey's testicular morphology, especially in semi-iniferous tubules (Sharma et al., 1999). In bulls, gossypol (8 mg/kg per day) induces testicular degeneration and significantly increases in primary and secondary sperm abnormalities and significant degeneration of germ cells between type B spermatogonia and pachytene primary spermatocytes (Cerelli and Johnson, 1999). In rats, gossypol with 15 mg/kg per day significantly decreases sperm concentration and increases the number of abnormal sperms (Romualdo et al., 2002). This effect maybe caused by gossypol, which disrupts the important cell functions (Ojha et al., 2006). Recently, gossypol has been shown to impair the gap junctional intercellular communication in the cultured Sertoli cells (Zhou et al., 2008).

In summary, animal experiments showed that gossypol damages animal spermatogenic epithelium, leads to abnormality and death of sperm, or even depletion of no sperm. Thus, gossypol causes reduced reproductive capacity or male infertility. This study was designated to probe the toxic effects of gossypol on animal male repro-

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Abbreviations: SOD, Superoxide dismutase; **GSH-Px,** glutathione peroxidase; **MDA,** malondialdehyde; **FCS,** fetal calf serum; **DNTB,** 5,5'-dithio-bis (2-nitrobenzoic acid) or Ellman's reagent.

duction using piglet sertoli cells *in vitro* as experimental model.

MATERIALS AND METHODS

Piglet testes

Testes were obtained from 3 to 4 weeks old piglets (Changsha, Hunan, China), placed in ice-cold phosphate-buffered saline (PBS) with 600 IU/ml penicillin-streptomycin and sent to the laboratory within 2 h.

Isolation, culture, and identification of testicular sertoli cells of piglets

Testicular capsule was removed under sterile conditions and semi-niferous tubules were isolated from piglet testis using mechanical dissociation and a one step enzymatic digestion with 1 g/l collagenase and 2.5 g/ trypsin, pursuant to the procedure as described previously (Dym et al., 1995; He et al., 2007) with minor modifications. Cell mixture containing male germ cells and sertoli cells were obtained using the second enzymatic digestion, collagenase IV, hyaluronidase and trypsin and sertoli cells were further separated from germ cells by differential plating according to the procedure described previously (He et al., 2007), Dirami et al., 1999). For differential plating, germ cells and sertoli cells were placed into tissue culture dish in the Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with 10% fetal calf serum (FCS) for 3 h at 34°C. Sertoli cells were attached to the culture plates, whereas male germ cells remained in suspension and were removed. Cell viability of sertoli cells was determined with 0.4% trypan blue exclusion assay.

Freshly isolated piglet sertoli cells were plated at a density of 2×10^6 cells/ml in DMEM/F12 supplemented with 10% FCS in a humidified incubator with 5% CO_2 and 100% humidity for 24 h. Sertoli cells were identified by oil red O staining and Fas ligand (FasL) expression as assayed by immunocytochemistry when 80 to 90% of the dish was confluent with cells.

Experimental groups and MTT assay

Piglet sertoli cells were classified into five groups, which are: group A, the control without gossypol; group B with 2.5 µg/ml gossypol; group C with 5 µg/ml gossypol; group D with 10 µg/ml gossypol; group E with 20 µg/ml gossypol. After culture for 24 h, the prolife-ration of sertoli cells were determined using MTT assays with quadruplicate, according to the procedure as previously described (Mosmann, 1983).

Determination of malondialdehyde (MDA) level, superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activity of sertoli cells

Malondialdehyde (MDA) level in the sonicated sertoli cells was measured by the thiobarbutiric acid method (Heath and Packer, 1968) and was presented as nmol per mg of protein. The activity of superoxide dismutase (SOD) in the sonicated sertoli cells was measured by the xanthine oxidase method (McCord and Fridovich, 1969) and presented as units per mg of protein. Activity of glutathione peroxidase (GSH-Px) in the sonicated sertoli cells was measured by the 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB) reaction test (Flohe and Gunzler, 1984) and presented as units per mg of protein. All the procedures were followed with the instruction on the kits from Nanjing Jiancheng Company (China).

Determination of DNA damage of sertoli cells by gossypol

Quantitation of DNA damage in sertoli cells were performed using a single-cell microgel electrophoresis technique under alkaline conditions (Singh et al., 1988) with certain modifications: the time of lysis, denature and electrophoresis were changed to 2 h, 1 h and 40 min, respectively. Sertoli cells were observed for epifluorescence under Fluophot microscope and 400 cells were counted in each group (4 parallel glass slides per group). Being excited by ultraviolet light, the nuclear DNA and the migration of DNA in orange-red DNA image (the comet tail) was clearly observed.

Statistical analysis

All the data were presented as mean ± SEM, statistically significant differences ($p < 0.05$) and extremely significant differences ($p < 0.01$) were determined among various groups by analysis of variance (ANOVA) and Tukey post-test using SPSS 12.0 statistical software.

RESULTS

Isolation, identification and culture of piglet sertoli cells

Piglet sertoli cells were isolated by a 2-step enzymatic digestion with trypsin and collagenase and followed by differential plating. Cell viability was up to 90% as assayed by trypan blue exclusion. After 24 h of culture, sertoli cells were attached to dish and formed a large columnar or irregular appearance with an elongated cellular body. Sertoli cells were connected with each other and showed mosaic-like arrangement and intercellular irregular protrusions. On both sides of cells, there were several prominences and strong refraction. Phagocytosed objects, varying sizes of vacuoles and the cell nucleus were observed in the cytoplasm of the cells. Oil red O staining showed that, red lipid droplets occurred near the nucleus or at the two poles of cytoplasm (Figure 1a). After 72 h of culture, high expression of FasL in sertoli cells was observed by immunocytochemistry (Figure 1b).

Effects of gossypol on proliferation of piglets sertoli cells

Piglets' sertoli cells were treated with different concentrations of gossypol for 24 h and the effect of gossypol on the proliferation of sertoli cells was assessed by MTT assay (Table 1). Table 1 showed that, with the increased concentration of gossypol, the sertoli cell proliferation decreased gradually. Compared with the control group A, cell growth inhibition in group B was significant difference ($P < 0.05$), while groups C, D and E showed extremely significant difference ($P < 0.01$) when compared with

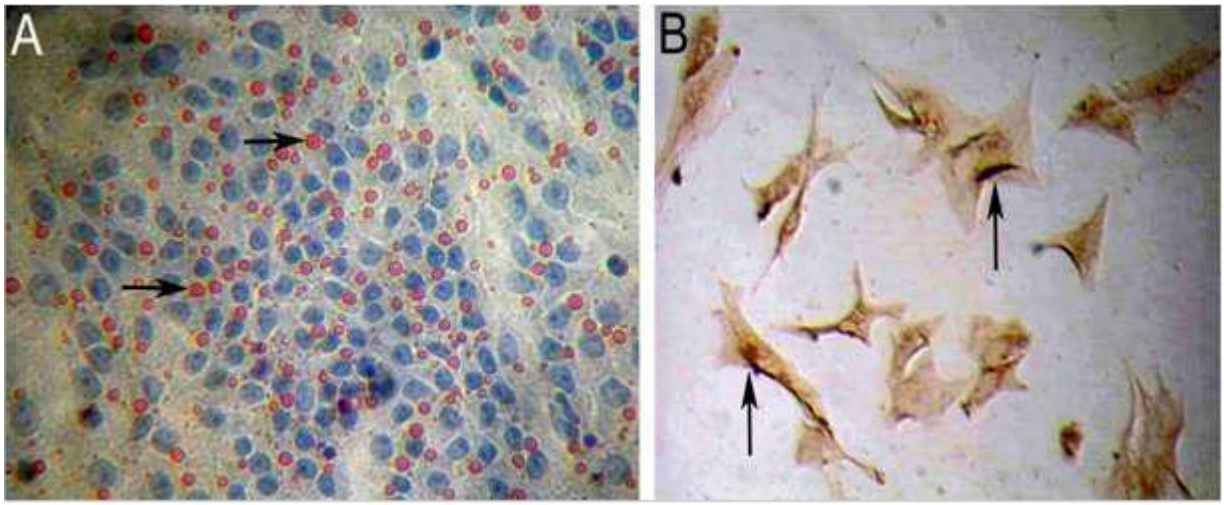

Figure 1. Identification of the isolated piglet sertoli cells: (A) The oil red O staining showed that red lipid droplets (arrows) were presented near the nucleus or at the two poles of cytoplasm of the isolated piglet cells, which confirmed the identity of piglet sertoli cells. Cell nuclei were counterstained with hematoxylin; (B) Immunocytochemistry revealed that the isolated piglet cells were positive for FasL (arrows), further verifying the identity of piglet sertoli cells. Magnification: (A) and (B) 400 ×.

Table 1. Cell proliferation of piglet sertoli cells by MTT assay.

Group	OD value at 490 nm	Inhibition of proliferation (%)
Control (A)	0.66 ± 0.06	0
B	0.46 ± 0.03*	30.59
C	0.40 ± 0.05**	39.27
D	0.39 ± 0.01**	41.01
E	0.30 ± 0.02**	54.95

Compared to the control group, "*" indicates significant difference ($p < 0.05$), "**" indicates extremely significant difference ($p < 0.01$).

group A. The results suggest that, the effect of gossypol on growth inhibition of sertoli cells is in a dose-dependent manner.

Effects of gossypol on antioxidant enzymes activities

Superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) are two important enzymes in the antioxidant defense system. Malondialdehyde (MDA) is regarded as a major marker of lipid peroxidation in tissue. After expo-sure to gossypol, MDA content, SOD and GSH-Px activities of sertoli cells were measured and the data was shown in Table 2. Compared with the control group A, the increase of malondialdehyde content in group B was significantly different ($P < 0.05$). And the increase of MDA content in groups C, D and E was extremely significant ($P < 0.01$). These results indicate that, the increase of MDA content of sertoli cells by gossypol is also in a dose-dependent manner.

The decrease of SOD and GSH-Px activity was observed in group B significant ($P < 0.05$) when compared with the control group A, but extremely significant decrease was observed in groups C, D and E ($P < 0.01$). Notably, the decreases of SOD and GSH-Px activities were correlated with the concentration of gossypol.

DNA damage of gossypol on piglet sertoli cells

Comet tail length formed in comet assay is an important parameter in evaluating the DNA damage. After UV excitation, sertoli cell DNA from gossypol treated groups showed an orange-red color. DNA fragments migration were observed in gossypol treated groups (Figure 2a, b), suggesting that, gossypol induces DNA damage in sertoli cells. Table 3 showed that, 90.43% control cell were categorized at grade 0, no cells were categorized at grade 3 and 4. The increased gossypol concentration caused more severe DNA damage. The rate of DNA tail was used to show the degree of DNA damage. An increased DNA tailing rate was observed along with the

Table 2. Effects of gossypol on malondialdehyde (MDA) content, superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities of piglet sertoli cells.

Group	MDA (nmol/mgprot)	SOD (U/mgprot)	GSH-Px (U/mgprot)
Control (A)	2.67 ± 0.26	210.37 ± 24.63	18.87 ± 3.43
B	3.13 ± 0.47*	198.58 ± 21.32*	16.68 ± 3.26*
C	5.19 ± 1.60**	178.86 ± 22.64**	13.96 ± 2.97**
D	5.46 ± 0.88**	137.57 ± 21.57**	12.05 ± 2.18**
E	11.24 ± 1.98**	105.13 ± 19.48**	9.63 ± 2.27**

Compared to the control group, "*" indicates significant difference ($p < 0.05$), "**" indicates extremely significant difference ($p < 0.01$).

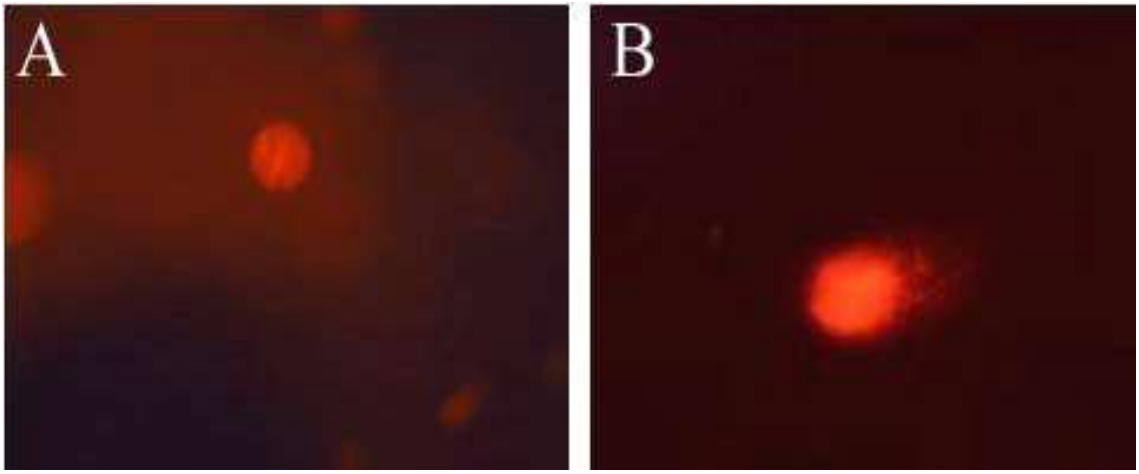

Figure 2. The single-cell gel electrophoresis (comet assay) showed the DNA damage of gossypol on piglet sertoli cells: (A) DNA image of control group; (B) comet assay revealed that damaged DNA of gossypol-treated group B contained strand breaks migrates farther in the gel than intact DNA, creating an image resembling a celestial comet. Magnification: 400×.

Table 3. Effect of gossypol on DNA damage of piglet sertoli cells.

Group	Total cell number	Cell number of different degree					Rate of DNA tail (%)
		Grade 0	Grade 1	Grade 2	Grade 3	Grade 4	
Control (A)	100	90.43 ± 4.34	5.17 ± 1.02	4.40 ± 1.23	0.00 ± 0.00	0.00 ± 0.00	9.57 ± 1.25
B	100	86.33 ± 4.51	6.29 ± 2.03	4.61 ± 1.62	1.65 ± 1.14	1.12 ± 0.53	13.67 ± 1.46
C	100	68.94 ± 5.73**	18.28 ± 3.79**	5.39 ± 0.97*	4.82 ± 1.13*	2.57 ± 0.46*	31.06 ± 3.13**
D	100	60. 52 ± 3-48**	20.05 ± 2.68**	8.76 ± 1.55**	6.13 ± 1.35**	4.41 ± 1.3**	39.35 ± 2.57**
E	100	40.12 ± 2.53**	24.06 ± 2.85**	12.24 ± 2.32**	13.56 ± 1.75**	10.02 ± 2.41**	59.88 ± 4.31**

Compared to the control group, "

increased gossypol concentration. These data indicates that, the overall level of DNA damage gradually increased in an obvious dose-response to gossypol (Table 3).

DISCUSSION

Breeding domestic livestock and aquatic animals with cottonseed cake has significant economic benefits. However, gossypol has been found toxicity in male reproductive system as a feedstuff additive. This study showed that, gossypol inhibited the growth of piglet sertoli cells in a dose-dependent manner. Thus, the current may shed insight into male reproductive toxicity of gossypol in clinic. We used 10% fetal calf serum (FCS) for the growth and survival of piglet sertoli cells. Recently, we demonstrated

that 10% FCS promotes proliferation of piglet sertoli cells (Zhang et al. 2010). The control group and gossypol-treated groups were cultured with FCS and thus fetal calf serum has no effect for us to investigate the toxicity of gossypol on piglet sertoli cells.

Previous research of gossypol on male reproductive toxicity is mainly focused on the structure of testis, epididymis and spermatogenic cells. Gossypol causes sparse array of testis seminiferous tubule, ambiguous or self-dissolving nucleus of epitheliums and reduces the amount of sperm, unclear sperm structure and swelling mitochondrial. Gossypol inhibits testicular lactate dehydrogenase activity. Thereby it undermines the animal spermatogenic epithelium, results in abnormality and death of sperm, or even no sperm. Finally, it causes sire infertility, reduces mitochondrial function and results in infertility (Monsees et al., 1998). Many researches suggests that, apoptosis plays an important role in testis regression (Furuta et al., 1994). Apoptotic spermatogenic cells can be found at all levels, especially at spermatocytes and sperm cells. The apoptotic cells occasionally aggregate chain-like or piles. Andrade et al. (2006), utilized hemicastration and electron microscopy to confirm that the epididymis is a definitive target of gossypol (de Andrade et al., 2006). Sertoli cell junctional protein maybe the early target of toxins such as gossypol (Fiorini et al., 2004). The effects of gossypol on sertoli cells are still unclear.

Oxidative stress can cause potential harm to animal organism. When oxidative stress is over generated or the enzymatic and non-enzymatic antioxidant defense systems are inefficient, it can stimulate some chain reactions causing cytotoxic effect. Superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) are the two major part of oxidase defense system. Superoxide dismutase is closely related to the cellular oxidative metabolism which widely exists *in vivo*. It is a natural scavenger of reactive oxygen species (ROS) and superoxide anion radicals. In the antioxidant enzymes, SOD is the first enzyme to combine with active oxygen free radicals. SOD specifically combines with superoxide anions and acts synergistically with GSH-Px in preventing cell membrane lipid peroxidation and damaging metabolites' formation. They directly capture and eliminate free radicals (Alul et al., 2003). Through hydrogen atom donors' effect of blocking or terminating radical consecutive reaction chain, superoxide dismutase prevents the action of free radicals, suppress cell membrane lipid peroxidation during pathological lesion and protect biological macromolecules and membranes from damage. Glutathione peroxidase converts hydrogen peroxide (H_2O_2) into H_2O. GSH-Px has a strong ability of scavenging lipid peroxide and hydrogen peroxide which were induced by active oxygen species and hydroxyl radicals. Thus, SOD and GSH-Px can protect biological macromolecules and membranes from damages. The values of SOD, GSH-Px and malondialdehyde (MDA) content of oxidative metabolites

Malondialdehyde is often considered as the major hallmark of lipid peroxidation damage, which causes secondary damage to cell functions, genotoxicity and carcinogenesis (Korchazhkina et al., 2003). DNA damage is the major effect of gossypol in piglet sertoli cells. The tail ratio in gossypol treated groups from 5 µg/ml was remarkably different from the control, as detected by single cell gel electrophoresis. This result suggests that, gossypol can cause DNA damage in piglet sertoli cells at high concentration. Activities of superoxide dismutase, glutathione peroxidase and malondialdehyde content in gossypol treated groups B to E were significantly different from the control, whereas DNA damage was observed in groups exposed to gossypol at concentrations above 5 µg/ml. The DNA damage induced by gossypol could be as a result of its stimulation of hydroxyl radicals production (El-Sharaky et al., 2009); gossypol also causes DNA-breaking (Li et al., 2000). Thus, gossypol has a marked effect on the superoxide dismutase, glutathione peroxidase activities, malondialdehyde content and DNA damage of sertoli cells.

Conclusion

The present revealed that gossypol has obvious toxic effects on piglet sertoli cells, as evidenced by the inhibition of sertoli cell proliferation and DNA damage, increased malondialdehyde level, as well as reduced superoxide dismutase and glutathione peroxidase activities of piglet sertoli cells, which may lead to decrease in reproduction of male pigs and as well decreased their reproductive capacity.

ACKNOWLEDGEMENT

This work was supported by the Department of Education of Hunan Province, China (Grant No. 03A020).

REFERENCES

Alul RH, Wood M, Longo J, Marcotte AL, Campione AL, Moore MK, Lynch SM (2003). Vitamin C protects low-density lipoprotein from homocysteine-mediated oxidation. Free Radic. Biol. Med. 34: 881-891.

Cerelli JS, Johnson L (1999). Potential daily sperm production, Sertoli cell number, and seminiferous tubule characteristics in beef bulls fed conventional or gossypol-enriched diets. J. Androl. 20: 519-528.

Coutinho EM (2002). Gossypol: a contraceptive for men. Contraception, 65: 259-263.

de Andrade SF, Oliva SU, Klinefelter GR, De Grava Kempinas W (2006). Epididymis-specific pathologic disorders in rats exposed to gossypol from weaning through puberty. Toxicol. Pathol. 34: 730-737.

Dirami G, Ravindranath N, Pursel V, Dym M (1999). Effects of stem cell factor and granulocyte macrophage-colony stimulating factor on survival of porcine type A spermatogonia cultured in KSOM. Biol. Reprod. 61: 225-230.

Dodou K, Anderson RJ, Small DA, Groundwater PW (2005). Investigations on gossypol: past and present developments. Expert Opin. Invest. Drugs, 14: 1419-1434.

Dym M, Jia MC, Dirami G, Price JM, Rabin SJ, Mocchetti I, and Ravindranath N (1995). Expression of c-kit receptor and its autophosphorylation in immature rat type A spermatogonia. Biol. Reprod. 52: 8-19.

El-Sharaky AS, Wahby MM, Bader El-Dein MM, Fawzy RA, El-Shahawy IN (2009). Mutual anti-oxidative effect of gossypol acetic acid and gossypol-iron complex on hepatic lipid peroxidation in male rats. Food Chem. Toxicol. 47: 2735-2741.

Fiorini C, Tilloy-Ellul A, Chevalier S, Charuel C, Pointis G (2004). Sertoli cell junctional proteins as early targets for different classes of reproductive toxicants. Reprod. Toxicol. 18: 413-421.

Flohe L, Gunzler WA (1984). Assays of glutathione peroxidase. Methods Enzymol. 105: 114-121.

Furuta I, Porkka-Heiskanen T, Scarbrough K, Tapanainen J, Turek FW, Hsueh AJ (1994). Photoperiod regulates testis cell apoptosis in Djungarian hamsters. Biol. Reprod. 51: 1315-1321.

He Z, Jiang J, Hofmann MC, Dym M (2007). Gfra1 silencing in mouse spermatogonial stem cells results in their differentiation via the inactivation of RET tyrosine kinase. Biol. Reprod. 77: 723-733.

He Z, Kokkinaki M, Jiang J, Dobrinski I, Dym M (2007) Isolation, characterization, and culture of human spermatogonia. Biol. Reprod. 82: 363-372.

Heath RL, Packer L (1968). Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch. Biochem. Biophys. 125: 189-198.

Korchazhkina O, Exley C, Andrew Spencer S (2003). Measurement by reversed-phase high-performance liquid chromatography of malondialdehyde in normal human urine following derivatisation with 2,4-dinitrophenylhydrazine. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 794: 353-362.

Li AS, Bandy B, Tsang SS, Davison AJ (2000). DNA-breaking versus DNA-protecting activity of four phenolic compounds *in vitro*. Free Radic. Res. 33: 551-566.

McCord JM, Fridovich I (1969). Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J. Biol. Chem. 244: 6049-6055.

Monsees TK, Winterstein U, Schill WB, Miska W (1998). Influence of gossypol on the secretory function of cultured rat sertoli cells. Toxicon. 36: 813-816.

Mosmann T (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods, 65: 55-63.

Ojha P, Dhar JD, Dwivedi AK, Singh RL, Gupta G (2006). Effect of antispermatogenic agents on cell marker enzymes of rat Sertoli cells *in vitro*. Contraception, 73: 102-106.

Romualdo GS, Klinefelter GR, de K (2002). Postweaning exposure to gossypol results in epididymis-specific effects throughout puberty and adulthood in rats. J. Androl. 23: 220-228.

Sharma S, Kumar M, Goyal RB, Manivannan B, Lohiya NK (1999). Reversible antispermatogenic effect of gossypol in langur monkeys (Presbytis entellus entellus). Adv. Contracept. 15: 15-27.

Singh NP, McCoy MT, Tice RR, Schneider EL (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res. 175: 184-191.

Ye W, Chang HL, Wang LS, Huang YW, Shu S, Sugimoto Y, Dowd MK, Wan PJ, Lin YC (2010). Induction of apoptosis by (-)-gossypol-enriched cottonseed oil in human breast cancer cells. Int. J. Mol. Med. 26: 113-119.

Zhang M, He Z, Wen L, Wu J, Yuan L, Lu Y, Guo C, Zhu L, Deng S, Yuan H (2010). Cadmium suppresses the proliferation of piglet *Sertoli cells* and causes their DNA damage, cell apoptosis and aberrant ultrastructure. Reprod. Biol. Endocrinol. 8: p. 97.

Zhou DR, Zhou YC, Cui GH, Guo X, Qin J, Gui YT, Cai ZM (2008). Gossypol repressed the gap junctional intercellular communication between *Sertoli cells* by decreasing the expression of Connexin43. Toxicol. *In Vitro*. 22: 1719-1725.