Journal of Animal and Veterinary Advances 11 (22): 4102-4107, 2012
ISSN: 1680-5593
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In vitro Assessment of Gossypol Induced Toxicity in Xinjiang Fine-Wool Sheep Leydig Cell

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Abstract: In this research, researchers assessed the gossypol induced toxicity on Xinjiang Fine-Wool sheep Leydig cell to get a better understand of the reproduction toxicity of gossypol on the male ruminant. The Leydig cells with high purity were separated successfully from a testicle of 3 months old Fine-Wool sheep through a method of Percoll Density Gradient Centrifugation. Cytotoxicity assays indicated mitochondrial is sensitive to gossypol induced toxicity. Hoechst staining and DNA-FCM with PI staining revealed gossypol mainly causes cell apoptosis of the Leydig cell. Cell cycle analysis by FCM indicated cell cycle arrest on G0/G1 phase. Leydig cell is the main site of testosterone biosynthesis and testosterone is the critical steroid hormone which determines the initiation and maintenance of spermatogenesis as well as expression of the male phenotype. Thus, cytotoxicity effects of gossypol on the Leydig cell revealed by this research indicated that the reduction of spermatogenesis induced by gossypol in male ruminant at least partially associated with the cytotoxicity effects of gossypol on the Leydig cell.

Key words: Xingjiang Fine-Wool sheep, Leydig cell, gossypol, toxicity, steroid hormone

INTRODUCTION

Whole Cottonseed (WCS), a by-product from the cotton (genus *Gossypium*) ginning process is considered a good feed stuff for ruminants due to its high content of digestible energy and protein and moderate fiber. However, WCS contains a polyphenolic compound, gossypol which can cause toxicity if consumed at high doses over several weeks (Risco and Chase, 1997). Although, most Free Gossypol (FG) combines soluble proteins in the rumen and discharges with the feaces, the absorbed FG is difficult to be metabolized and accumulates in the body to exert its negative effects (Willard *et al*., 1995). In male ruminants, gossypol toxicity can manifest as reproductive problems such as increased sperm abnormalities, reduced sperm motility, reduced scrotal circumference and suppressed testosterone concentrations in cattle (Smalley and Bicknell, 1982; Risco *et al*., 1993; Chenoweth *et al*., 1994), goats (Solaiman *et al*., 2009) and deer (Brown, 2001; Gizejewski *et al*., 2008; Bullock *et al*., 2010).

Leydig cell is known to the main site of testosterone biosynthesis. Testosterone is the critical steroid hormone which determines the initiation and maintenance of spermatogenesis as well as expression of the male phenotype (Sharpe, 1987).

Xinjiang is the major cotton production area in China. The WCS is a potential feed supplement for Xinjiang Fine-Wool sheep. In this research, researchers separated the interstitial Leydig cells from the testis of Xingjiang Fine-Wool sheep and assessed the toxicity of gossypol on the cells *in vitro* to further investigate the negative effects of gossypol on this important economical ruminant in Xinjiang area.

MATERIALS AND METHODS

Cell separation and identification: The leydig cells were separated from a testicular of 3 months old Fine-Wool sheep through a method of Percoll density gradient centrifugation as earlier described (Fumarola *et al*., 1982). In brief, the testicular was subjet to

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mechanicaldispersion and trypsinization. Single cell suspensions were added to Percoll solutions (Sigma) in four densities (solutions containing 60, 34, 26 and 21% Percoll). The gradient was centrifuged at 3000 r min^{-1} for 30 min obtaining four layers and the cells at the interface of the third layer were gently aspirated and washed twice in Phosphate Buffer (PBS).Then the cells were cultured in Dulbecco Minimum Essential Medium (DMEM) with 10% fetal calf serum.

The purity of Leydig cells was examined by histochemical staining for 3β-hydroxysteroid dehydrogenase (Sigma) according to the histochemical method of Mendelson *et al.* (1975) with some modifications. Briefly, Leydig cells were incubated in a 24 well plate with 0.4 mL $well^{-1}$ staining solution containing 0.15 mg mL^{-1} nitro-bluetetrazolium, 0.36 mg mL^{-1} nicotinamide adenine dinucleotide and 0.5 mM dehydroepiandrosterone in 0.05 M PBS, pH 7.4 for 2 h at 37°C. The positive cells were stained a dark blue color.

Treatment of Leydig cells with gossipol: Rcemic (±) gossypol (sigma) was dissolved in Dimethyl Sulphoxide (DMSO) at a concentration of 512 mg mL^{-1} and stored at -20°C as a stock solution. The stock was diluted to the required concentration with serum free medium immediately before use. The final DMSO concentration did not exceed 0.1% DMSO throughout the study. At ~80% confluence in the appropriate microtitre plates 5×10^4 cells mL^{-1} per well, 0, 2, 4, 8, 16, 32, 64, 128, 256 and 512 μg mL^{-1} of freshed prepared gossypol solution was added to Leydig cells to measure pertinent dose-effect relationship of the gossypol to the cell.

MTT assay: Cell viability was assessed by measuring mitochondrial succinate dehydrogenase activity using a modified MTT assay (Mosmann, 1983). Briefly, following incubation with the range of gossypol doses (0-512 μg mL^{-1}) for 24 and 48 h, the gossypol containing medium was removed and replaced with sterilized MTT solution. This MTT solution was freshly prepared in DMEM containing no phenol red and FBS. The plates with added MTT solution were then wrapped in a aluminum foil and placed in the 5% CO_2 incubator for 1 h at 37°C. The MTT solution was removed and 100 μL of DMSO was added to each well to dissolve the blueformazancrystals. The optical density was measured at 570 nm wave lengths using the ELISA spectrophotometer (Thermo-maxmicroplatereader, Molecular Devices Corp. MenloPark, California, USA).

Lactate Dehydrogenase (LDH) leakage assay: Cell membrane integrity was assessed by Lactate Dehydrogenase (LDH) leakage into the culture medium.

Following exposure to the range of gossypol doses (0-512 μg mL^{-1}) for 24 and 48 h, the culture medium was aspirated and centrifuged at 3000 rpm for 5 min in order to obtain a cell free supernatant. The activity of LDH in the medium was determined using a commercially available kit from Sigma Diagnostics (LD-L50). The assay is based on the conversion of lactate to pyruvate in the presence of LDH with parallel reduction of NAD. The formation of NADH from the above reaction results in a change in absorbance at 340 nm. Aliquots of media and warm reagent were mixed in a 96 well plate (Fisher) and absorbance was recorded using a micro plate spectrophotometer system (Spectra max190-Molecular Devices).

Hoechst staining: The Leydig cells were stained with DNA-specific Hoechst 33342 dye to distinguish normal cells from cells with apoptotic nuclear morphology. About 10^5-10^6 cells cultured in 1 mL medium with the range of gossypol doses (0-512 μg mL^{-1}) for 48 h at 37°C in a 10% CO_2 humidified atmosphere. Cells were then detached from the plates by treatment with trypsin-EDTA, washed extensively and resuspended in a 4% paraformaldehyde fixative solution. Then cells were placed onto silicate slides, air-dried and stained with 10 μg mL^{-1} Hoechst 33342 dye (Sigma-Aldrich) for 10 min. Slide was then washed with phosphate buffered saline solution. Fluorescence microscopy (at an emission wavelength of 460 nm) was used to visulize chromatin condensation and nuclear fragment.

DNA extraction and electrophoresis: The Leydig cells were harvested by centrifugation at 200 g for 10 min and lysed with 0.2 mL ice-cold lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.2% Triton X-100). Proteinase K (50 μg mL^{-1}) was added and the cells were incubated for 1 h at 37°C. The DNA was sequentially extracted with equal volumes of phenol/chloroform/isoamyl alcohol (25:24:1). The DNA recovered from the aqueous phase was precipitated by adding 2 volumes of 100% ethanol and maintained over night at -20°C. The DNA was then collected by centrifugation, air dried and resuspended in 15 μL of Tris 10 mM/EDTA 1mM buffer, pH 8.0. The samples were mixed with 5 μL of gel loading buffer (0.33% bromophenol blue and 40% (w/v) sucrose) and loaded onto a 0.8% agrose gel (runing buffer 1×90 mM Tris/90 mM Boric acid/2 mM Boric acid/2 mM EDTA). Ethidium bromide was included to allow visualization of the DNA under UV light.

Flow cytometric analysis: To determine the effect of gossypol on the cell cycle, cells were treated with the range of gossypol doses for 48 h washed and fixed with

70% ethanol. After incubation overnight at -20°C, cells were washed with PBS and then suspended in staining buffer (propidium iodide, 10 mg mL^{-1}; Tween-20, 0.5% Rnase, 0.1% in PBS). The cells were analyzed using a FACS Vantage flow cytometer that used Cell Quest acquisition and analysis programs (BectonDickinson, SanJose, CA). Gating was set to exclude cell debris, cell doublets and cell clumps.

RESULTS AND DISCUSSION

Cell separation and identification: The Percoll gradient centrifugation yielded cells of three major populations. The top layer consisted of small round cells and cell debris. Layer-2 was composed of spermatozoa and a small fraction of Leydig cells. The cells in the third layer consisted of >90% Leydig cells as they were positively stained by the 3β-HSD Method (Fig. 1b). According to the 3β-HSD staining, the laye r-3 cells were confirmed as Leydig cells. After 72 h incubation, most Leydig cells glowed adherently and stretched in forms of polygon, triangle or oval (Fig. 1a).

Cytotoxicity assays: Cell viability was assayed in Leydig cell cultures exposed to gossypol under different concentrations (0-512 μg mL^{-1}) for 24 and 48 h. cytotoxicity was determined with MTT assay and LDH leakage assay. In MTT assay, gossypol showed dose and time dependent inhibitory effects on the growth of Leydig cells and statistically significant decreases in cell viability were shown in the does range of 64-512 μg mL^{-1} for 48 h

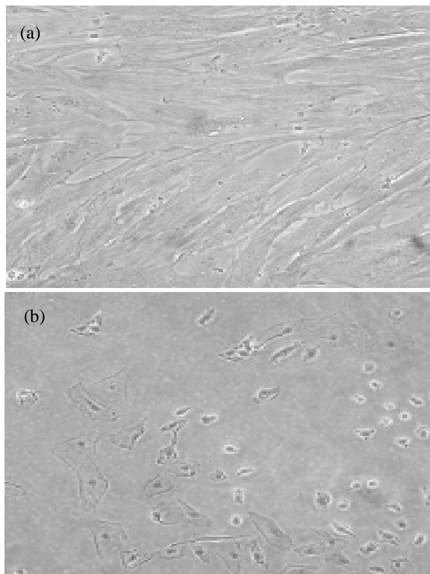

Fig. 1: a) The seperation culture and b) identification of leyding cell in Xingjiang Fine Wool sheep

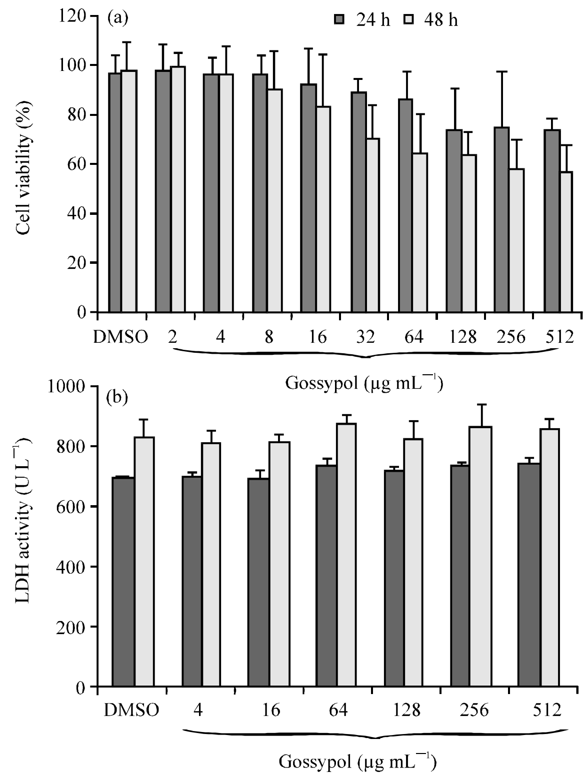

Fig. 2: a) Gossypol mediate cytoxicity in Fine Wool sheep Leydig cells as revealed by MTT assay and b) LDH assay *Indicated p<0.05 in comparison to the vehicle control (DMSO) by ANOVA test

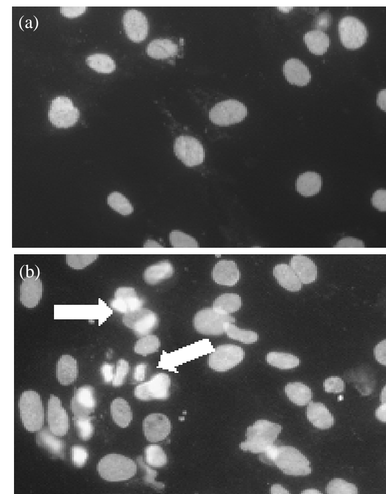

Fig. 3: Gossypol induces apoptosis in Fine Wool sheep Leydig cells as revealed by Hoechst staining. Arrows indicate cells show chromatin condensation and nuclear fragmentation a) DMSO; b) Gossypol (64 μg mL^{-1})

Fig. 2. However, The LDH leakage results indicated that gossypol had no effect on the cell membrane (Fig. 2b).

Hoechst staining: Hoechst staining of gossypol treated Leydig cells revealed chromatin condensation and nuclear fragmentation, both of which are characteristic features of apoptosis (Fig. 3).

DNA extraction and electrophoresis: As shown in Fig. 4, the typical DNA ladders which are characteristic features of apoptosis was not observed by the treatment of the gossypol with the rang of does (0-128 μg mL^{-1}).

Flow cytometric analysis: In the case of apoptosis, DNA-FCM with Propidiumiodide (PI) showed a sub-population, designated A0 cells with reduced DNA stainability. The peak is below the normal G0/G1 region, (Nicoletti *et al*., 1991). It is believed that the reduced DNA stain ability is the consequence of progressive loss of DNA from the cells, due to activation of endogenous endonuclease and subsequent leakage of the low molecular weight DNA products prior to measurement (Arends *et al*., 1990; Ormerod *et al*., 1993). Thus, the percentage of apoptotic cells could be counted through screening and calculating the of the FCM peaks. As shown in Fig. 3, the increasing areas of the A0 cells and decreasing G0/G1 regions revealed an increasing apoptotic cells percentage in the Leydig cells after the treatment of the range of gossypol doses for 48 h.

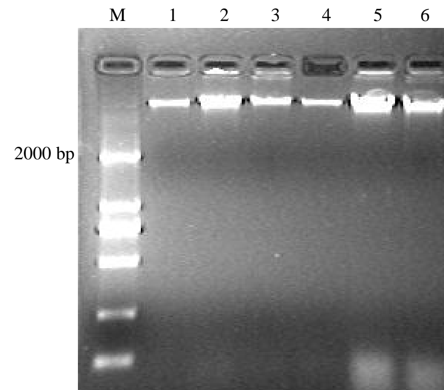

Fig. 4: Internucleosomal DNA fragmentation in Fine Wool sheep Leydig cells after exposure to gossypol for 48 h. Line M: the molecular mark of 2 kb DNA ladder; Line 1, DNA from cells treated by DMSO; Line 2-6, DNA from cells treated by 0, 16, 32, 64 and 128 μg mL^{-1} gossypol, respectively

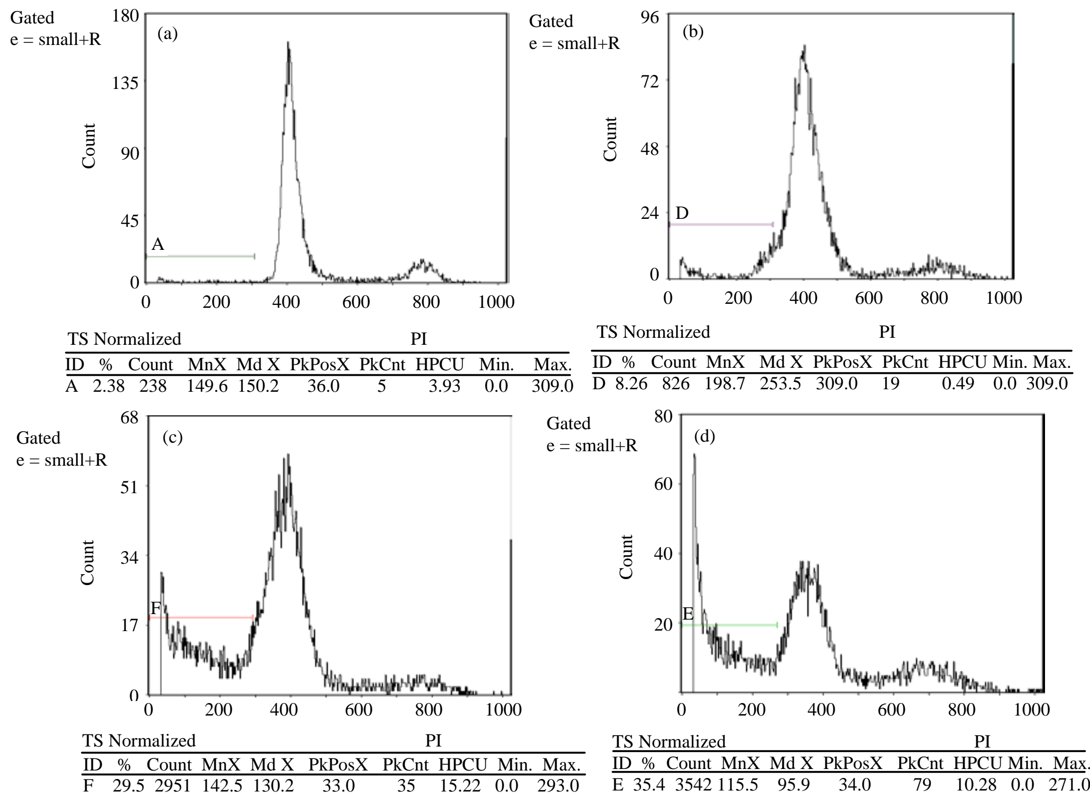

Fig. 5: FCM of cellular DNA content of fine Wool Sheep Leydig cells treated with gossypol for 48 h. a-d) 0, 64, 256 and 512 mg L^{-1} respectively

Cell cycle analysis by FCM indicated exposure with 48 h of (64 μg mL^{-1}) gossypol treatment showed a progressive accumulation of cells in the G1 phase (83.5%, compared with baseline of 76.5%), accompanied by a decreasing number of cells in the S phase (7.28% compared with baseline of 10.8%) or in the G2-M phase (9.25% compared with baseline of 12.9%) (Fig. 5 and 6).

A primary concern with feeding large amounts of cottonseed is the possibility of gossypol toxicity and a potential depression in fertility across a variety of mammalian species (National Coordinating Group on Male Antifertility Agents, 1979; Qian and Wang, 1984; Randel *et al*., 1992) including sheep and male ruminants (Lindsey *et al*., 1980; Arieli, 1998). Some studies indicated gossypol appears to exert unique and selective effects on the male reproductive system and causes degeneration of seminiferous tubules in the parenchyma of the testicles. However, the toxicity of gossypol on the Leydig cell which is important to fertility was seldom assessed. The male primary steroid hormone, Testosterone (T) is produced almost exclusively by Leydig cells in the testis. T promotes acquisition of the male phenotype and maintains fertility in adulthood (Sharpe, 1987). Some studies showed that the impairment of Leydig cells had adverse consequences for male fertility.

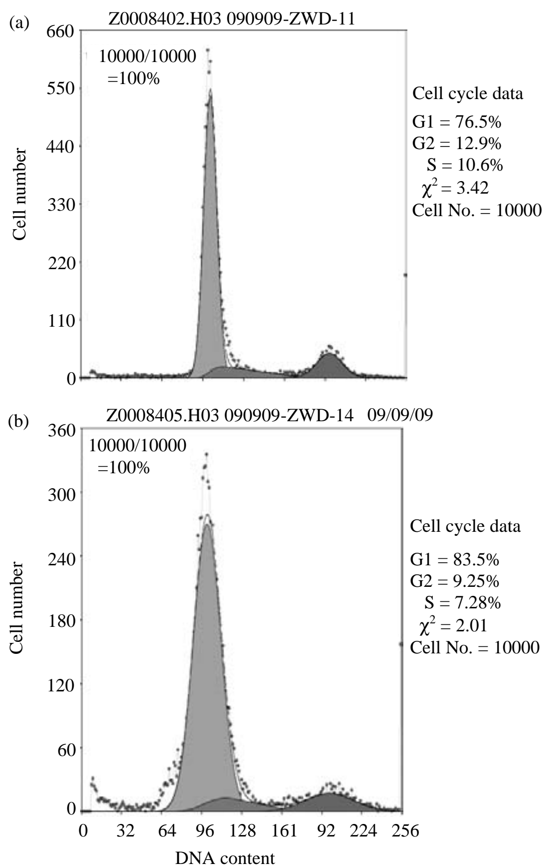

Fig. 6: Cell cycle analysis of fine-wool sheep Leydig cells treated by gossypol for 48 h by FCM. Percentages of cell populations at each phase of the cell cycle are indicated in each histogram. a) 0 μg mL^{-1}, b) 64 μg mL^{-1}

In this research, cytotoxicity assays demonstrated that in Leydig cells the MTT assay revealed toxicity before any effect with the LDH leakage assay which can be attributed to the fact that gossypol may have an effect on mitochondria. Mitochondria is one of the most important organelles in the cells which plays a crucial role in the mitochondrial apoptosis signal transduction pathway. Earlier reports have indicated that gossypol initiated mitochondrial apoptotic pathway through inhibiting the functions of Bcl-2/Bcl-xL/Mcl-1 proteins (Mohammad *et al*., 2005a, b; Meng *et al*., 2008; Etxebarria *et al*., 2008), the anti-apoptotic Bcl-2 family members.

Some researches have reported DNA breaks being secondary to cytotoxic induced by gossypol in some cell lines. DNA breaks were observed when cell viability was reduced to <70% (Quintana *et al*., 2000). In this research, chromatin condensation and immediate reduction in DNA stainability were observed by hoechst staining and DNA-FCM with PI staining. However, the typical DNA ladder caused by apoptosis was not seen in DNA electrophoresis. Accordingly, gossypol induced toxicity on the Leydig cells was in a pattern associated with early apoptosis and may be not primarily genotoxic in the cells. Results of cell cycle effect of gossypol indicated cell apoptosis induced by gossypol was associated with induction of G1 arrest and a reduction in the number of cells in S phase.

CONCLUSION

This research revealed that in Fine-Wool sheep Leydig cells, mitochondrial is sensitive to gossypol induced toxicity. Cell apoptosis induced by gossypol may through mitochondrial apoptotic pathway and associated with the cell cycle arrest on G0/G1 phase of the Leydig cells. This information helps to get a better understand of the reproduction toxicity of gossypol on the male ruminant. Reduction of spermatogenesis, one of the gossypol effects upon the male reproductive system was mostly reported in ruminant. Spermatogenesis is driven by testosterone which is produced exclusively by Leydig cell. Cytotoxic effects of gossypol on the Leydig cell revealed by this research indicated that the reduction of spermatogenesis induced by gossypol at least partially associated with the cytotoxic effects of gossypol on the Leydig cell.

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