A Comparison between the Cytotoxicity Induced by Gossypol in Two Testicular Cell Lines

Neda MahdinezhadGorji [1], SeyedGholamAli Jorsaraei[2], Vida Hojati [3], Ebrahim Zabihi [4], Asieh Khalilpour [4], Zainab Abedian [4], Eisa Tahmasbpour [5]*

Received: 02.08.2014 *Accepted: 28.09.2014*

ABSTRACT

Background: Gossypol is a yellow toxic pigment from the cottonseed that can cause acute or chronic toxicity in humans and animals by affecting the testicular tissues. Nowadays cottonseed is used as food supplement for ruminants specially the sheep. In this study, two different stem cell lines of testicular tissue including GC1-spg (mouse testis) and SFTF-PI43 (sheep testis) cells were used to evaluation of gossypol cytotoxicity.

Methods: The GC-1spg and the SFTF_PI43 cells were cultured in RPMI-1640 supplemented with fetal bovine serum (10%) and antibiotic (penicillin 10^5/ml, streptomycin100µg/ml), and then 5×10^4 cells/well were seeded in 24 well plates. Cultured cells were exposed to four different concentrations of gossypol (1.25, 2.5, 5 and 10µM). After 24 h incubation, cells viability test was performed using Trypan Blue dye exclusion and MTT assay. The Thiobarbituric Acid Reacting Substances (TBARS) and Ferric Reducing Activity Potential (FRAP) assays was performed on media.

Result: In high concentrations (over than 2.5µM), Gossypol showed cytotoxic effects on cells. The IC50 for gossypol (using MTT assays) on SFTF-PI43 and GC-1spg cell lines was 2.2 µM and 3.2 µM, respectively. While the results for FRAP assay did not show any significant differences between the test and control groups, significantly higher lipid peroxidation was observed in SFTF-PI43 cells that were treated with higher doses of gossypol (10µM).

Conclusion: In this research, we found that gossypol has cytotoxic effects on both examined testicular cell lines and increased lipid peroxidation, which is a probable mechanism of its toxicity on cell lines.

Keyword: Cytotoxicity, GC1-Spg, Germ Cell Line, SFTF-PI43, Testis.

IJT 2015; 1188-1195

INTRODUCTION

Nowadays, because of industrialization and change of life style throughout the world, environmental factors that cause infertility are increasing.Harmful substances such as pesticides and chemical fertilizers reduce fertility in human either directly or indirectly [1].In the liver, the majorities of toxins are converted to metabolites and then excreted from the body. However, remained toxins in human body tissue, particularly in human male sex organs,may have negative effects on reproductive function and result in impaired-spermatogenesis, sperm structure and its function [2].Most toxins used as plant pesticides are synthetic, but some others such as vegetable oils, natural essences or volatile oils as well as extracts of some plants that are derived by secondary plant metabolismarenot synthetic.During the primary metabolism, plants create compounds that

1. MSc. Fatemeh Zahra Infertility and Reproductive Health Research Center, Babol University of Medical Sciences, Babol, Iran.
2. Department of Anatomy and Embryology, Fatemeh Zahra Infertility and Reproductive health Research Center, Babol University of Medical Sciences, Babol, Iran.
3. Departmeant of Biology,College of Biological Sciences,Damghan Branch,Islamic Azad Universitiy,Damghan, Iran.
4. Departmeant of Cellular and Molecular Biology, Babol University of Medical Science, Babol, Iran.
5. PhD Student of Clinical Genetics, Member of Young Research Club, Islamic Azad University, Sari Branch, Sari, Iran.
* Corresponding Author: E-mail: alijorsara@yahoo.com

directly contribute to their growth. While during the secondary metabolism, plants produce compounds that serve as defense mechanism against animal attack and combat pests; and are considered as natural toxins [3]. It has been found that these substances or secondary metabolitesenter life cycle of different animals and humans and can cause complications [4]. Gossypol is a natural toxin produced during the plant secondary metabolism. Current studies showed that this component has anticancer, antioxidant attributes, induces oxidative reaction and can cause infertility [5].

Researches on Gossypol have begunin Jiangsu village in Chinabetween 1930 and 1940 because no baby was born during that period. However, before and after that period, villagers had no particular problems related to infertility. The scientists studied the issue and found that villagers, especially men, were experiencing infertility due to overuse of cottonseed oil [6]. Similarly, this problem was also observed in animals. They had reported that higher consumption of cotton products by ruminant and non-ruminant (rodents) animals, can lead to poisoning, shortness of breath, decreased growth rate, appetite and reproduction problems [7].Other studies indicated that Gossypol, a polyphenolic compound with the formula of $C_3H_{24}O_8$,constitutes approximately 0.1 to 100g per kg of cotton plant. It can lead to poisoning if over used by animals and humans [6,7]. However, ruminants are more resistant than rodents (7). This yellow crystalline compound is better solved in organic solvents. Gossypol dissolves in acetic acid and produces Gossypol salt [8]. Geneticists have recently attempted to produce genetically modified Gossypol-free cottonseed, but removing the Gossypol disrupts the immune system of the plant, and thus it is not possible [8]. Recent studies have shown that one of the main effects of gossypol toxicity in ruminant and non-ruminant animals is its negative effects on the reproductive system, impaired spermatogenesis and reduction in sperm count [9,10]. Because of high protein concentration, cottonseed is currently an important source of food and energy for animals, especially rudiments. Therefore, future studies are required to make a better strategy for the treatment of its toxicity and its real mechanisms.

In this study, we aimed to investigate the toxic effects of gossypol acetic acid on two stem cells: $GC1_{-spg}$ (in non-ruminant animals) and SFTF-PI_{43} (in ruminant animals) and its relationship to lipid peroxidation, as a possible mechanism of its cytotoxicity.

MATERIALS AND METHODS

Preparation of Cell Lines

We obtained stem cells from testicular tissue of $GC_{1\text{-}spg}$ (mouse) and SFTF-PI_{43}(sheep) from cell bank of Pastor Institute of Iran and transferred to Babol University of Medical Sciencesunder standard conditions.

Preparation for Cell Culture Conditions

The cells were cultured in Flask 25, FBS 10% (Fetal Bovine Serum), (PAA, Astrulia, A15-104) and penicillin-streptomycin 1% (PAA, Astrulia, A11-504) in medium RPMI-1640 (Astrulia, PAA) and then the cells were grown at 37^0 C, under 5% CO_2 atmosphere and at 90% humidity for 24h.

Preparation of Gossypol Solution

To prepare Gossypol solution (SIGMA–Aldrich, G4382), 0.65 of gossypol was solved in 20 ml of ethanol 96% (Merk Germany).Regarding the dilution of ethanol in 10^{-6}M that makes it ineffective, control group was deleted from culture medium. Then, preparing stock solution, which contained 980µl of a complete culture medium and 20µl of Gossypol solution, dilution was prepared for densities of 10µm, 5µm, 2.5µm, and 1.25µm.

Test Methods

Every day cells were observed under microscope to control growth rate, reproduction as well as possible infection and cell destruction, and the culture media were changed, if necessary.When cells covered approximately 70-80% of the flask surface, we washed them by PBS. Then the cells were detached from the bottom of the flask by Trypsin(0.25%)/EDTA(0.1%) (GBGIBCO Germany);after counting the cells, depending on their quantity, we determined their dilution and passed them in another flask.After the fourth passage, the cells were prepared for toxicity studies. At this time, since there was the

possibility of infection and cell damage, some of them were kept frozen in a tank of liquid nitrogen at 196°C with recorded complete specifications.

The toxicity of Gossypol on Cell Lines

MTT Assay

Gossypol toxic effect on cell lines was evaluated by two methods; MTT (Sigma-Aldrich) and Trypan Blue methods. MTT Powder (Dimethyl Thiazole Diphenyl Bromide) forms by the effect of succinate dehydrogenase enzyme existing in mitochondrial cycles of active cells and breakage of Tetrazolium loop of in soluble formazan crystals (purple). The color is directly related to the number of metabolically active cells. This compound is soluble in acidic isopropanol. Finally, the optical density was measured in a spectrophotometer at two wavelengths of 630 nm and 570 nm.It is noteworthy that the higher absorption rate indicates more live cells.

In total, 5×10^4 cells from GS1-spg and SFTF-PI43 cell lineswere cultured with 1ml of culture medium in 24-cell well-plates and three wells were considered to reduce the error for each concentration. Then cells remained in incubator with control group that lacked any toxic extract, for 24 hours. Gossypol solutions were added tocells and after 24 hours 200µlMTT was added to the plates. Then the plates were incubated for another 4 hours. Finally, optical absorption was measured in a spectrophotometer at two mentioned wavelengths. We used these two wavelengths because the maximum optical densities of formazan crystals are in these limits. Then, differences of ODs in two wavelengths were identified for each well. We obtained percent of live cells using control group in the following formula:

Mean optical absorbance of control cells / Mean optical absorbance of treated cells ×100

Trypanblueassay

This method estimatesthe cells viability based on absorption of Trypanblue by dead cell membranes that have more permeability to this pigment, while the membranes of living cells do not allow this non-electrolyte dye into cells. To determine the vital activity of the cell, 15×10^4 cells of GS1-spg and SFTF-PI_{43}were culturedwith 3 ml of complete medium in 6-cell well plates for24 hours. After that, mentioned concentrations of gossypol were added to each well.Again, after 24 hours,Trypsin was added to them and then washed by PBS. Then, the cells in the wells were counted in volume unit.We prepared Trypan blue solution [0.04% (w/v)] (sigma- Aldrich, Germany) by PBS. Simultaneously, we determined the number of not stained (live) cells and stained (dead) cells with a hemocytometer (neobar).Then, the percentage of cellularvital activity was calculated according following formulas:

Number of alive cells = Average number per square× dilution coefficient × final volume of medium in each well × 10^4

(Viability = number of alive cells /total cells × 100)

Determination of Lipid Peroxidation

The lipid peroxidation of both cell lines, after exposure to gossypol, was measured by thiobarbituric acid reactive substances (TBAR) assay in the medium, previously described by Ohkawa[11]. Briefly,4ml of TBA/TCA and 50µl of BHT were added to all tubes and placed in boiling bath for 15 min. Tubes were centrifuged for15 min at 3000 rpm and the absorption of surfactant was read at 532 nm by spectrophotometer.

Determination of Ferric Reducing Ability of (FRAP) Medium

To measure the total antioxidant power of the medium, the FRAP assay was performed on cell culture medium according to the method of Benzie and Strain [12]. Briefly, the FRAP reagent contained 2.5 ml of a 10mmol/L TPTZ (2,4,6-tripyridyl-s-triazine; Sigma) solution in 40mmol/L HCl plus 2.5ml of20mmol/L FeCl3 and 25ml of 0.3mol/L acetate buffer (pH 3.6). The reagent was freshly prepared and warmed at 37 °C.The working FRAP reagent(1.5 ml) was mixed with 50µl serum or standard in a test tube. After 10 min at 37 °C, the absorbance was determined at 593 nm. $FeSO_4$ at a concentration of 1mmol/L was used as the standard solution. The resultwas expressed as the concentration of antioxidant with a ferric reducing ability

equivalent to that of 1mmol/L FeSO4. The antioxidant power of each medium sample was reported as µM equivalent of FeSO4.

Statistical Analysis

All tests were repeated 3 times. Raw values obtained from the MTT and Trypan blue tests were changed into percentage of each group to the related control group,analyzed by one way ANOVA and t-test and their graphs were provided. Also, $p<0.05$ was considered as statistically significant and logarithm graphs showed cell duplication. According to data obtained from MTT and Trypan blue tests, we drew IC_{50} graph.

RESULTS

Table 1 shows results of GC_1-spg cells vicinity to different concentrations of gossypol by MTT method, which indicates optical absorption of the dilutions with control group in each well. Optical absorption was 1.25µM/ml at the lowest concentrationofGC_1.spg,butat the highest densities (10µM/ml) were 39±6.7 and 85±0.4, respectively. Increasing gossypol dose decreased optimal absorption that indicated decrease in cell reproduction. The results of statistical test showed significant differences between densities of 10.5 and 2.5 with control group, while we did not find significant difference between1.25 and control groups.

Using statistical methods, we concluded that optical absorption decreased in higher doses. Optical density of SFTF-PI43 cells after treatment of gossypolat the lowest and highest concentration were 67±5and 22±1, respectively (Table 1 andFigure 1).

Table2 shows the percentage of cells life cycle after treating with gossypol in Tryptan blue method. The number of non-stained live cells decreasedcompared to control group. Increase in gossypol concentration declined living GCI-SPG and SFTF-PI43 cells gradually. For example, inconcentration of 10µM the percentage ofliving cells was approximately 40%. There was a statistical significance difference between densities of 2.5, 5, and 10µM/ml compared with control group in both cell lines ($p<0.05$); however, there was no significant difference between 1.25 µM/ml concentration and control group.

Gossypol IC_{50} on cell lines was also calculated by MTT assay. These values for GCI-SPG and SFTF-PI43 were 3.2µM/ml, 2.2µM/ml (MTT), respectively. It suggested that about 50% of cell reproduction is aborted in these densities (figure 2).

Malon dialdehyde (MDA) determination in medium (TBARS-assay)

Table 3 illustrates the amount of MDA levels in each group. According to our study, concentrations of MDAincreased with gossypol treatment. The control group in both GC1-SPG and SFTF-PI43 cell lines had the lowest concentrations of MDA, whereas the levels of MDA in 10µM group in SFTF-PI43 was significantly higher than the control group ($p<0.05$). Although the amount of MDA in treated groups was greater than the control group,the difference, particularly in GC1-SPG cell lines, was not significant.

Medium total antioxidant power (FRAP)

Table 4depicts the amount of FRAP in each group. According to our study, the concentration of FRAP did not change significantly with gossypol treatment. There was not a significant difference between groups in both GC1-SPG and SFTF-PI43 cell linesregarding to FRAP.

Table 1. Results of MTT assay in exposure to GCl-spg and SFTF-PI_{43} cell lines with different concentrations of Gossypol. * Significant difference with control group.

Different concentrations of gossypol in RPMI (µM)Cell line	0 (CTRL)	1.25	2.5	5	10
GC1-SPG	100±0/6	85±0/4	56±9/2*	46±3*	39±6/7*
P Value	-	0/17	*0/006	*008/0	3*0/00
SFTF-PI43	100±5	67±5	47±6*	38±4*	22±1*
P Value	-	1420/	3*0/00	0/001*	0/001*

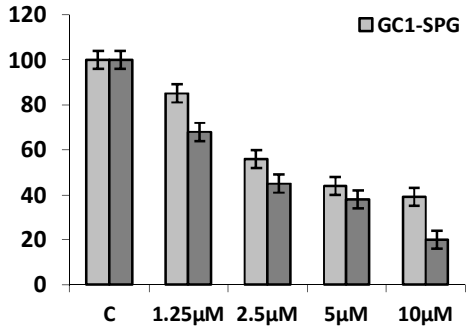

Figure 1. Average percentage of cell lines survival after exposure to gossypol.

Table 2. Trypan blue test in exposure to GCl-spg And SFTF-PI_{43} cell lines with different concentrations of Gossypol.

Different concentrations of gossypol in RPMI (µM)Cell line	0 (CTRL)	1.25	2.5	5	10
GC1-SPG	100%	82%	56%*	48%*	41%*
P Value	-	0.575	0.045*	0.032*	0.009*
SFTF-PI43	100%	80%	51%*	46%*	43%
P Value	-	0.365	0.034*	0.008*	0.005*

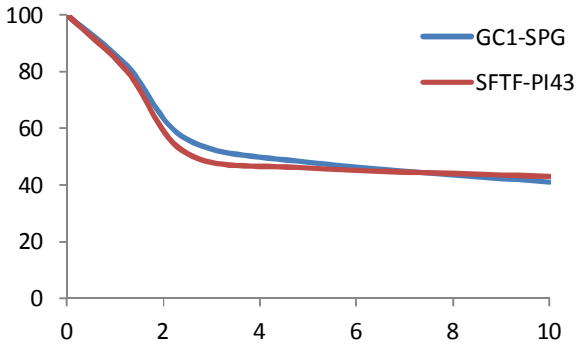

Figure 2. Absolute IC50 curves calculate through MTT assay.

Table 3. Average index of medium MDA.

Different concentrations of gossypol in RPMI(µM)Cell line	0 (CTRL)	1.25	2.5	5	10
GC1-SPG	2.7	2.5	2.8	2.9	3.2
P Value	-	0.425	0.412	0.367	0.162
SFTF-PI43	2.8	3.2	3.4	3.3	4.1
P Value	-	0.375	0.110	0.085	0.042*

* P<0.05 against control

Table 4. Average index of medium FRAP.

Different concentrations of gossypol in RPMI(µM)Cell line	0 (CTRL)	1.25	2.5	5	10
GC1-SPG	55	51	62	60	52
P Value	-	0.425	0.174	0.251	0.194
SFTF-PI43	59	54	59	55	52
P Value	-	0.462	0.890	0.655	0.226

DISCUSSION

Spermatogonia stem cells have unique ability for reproduction and produce mature sperm in the testicles. These cells multiply after puberty and commence spermatogenesis [13]. Exposure to industrial or environmental factors affects the quality of male reproductive function and can cause irreversible infertility or sterility through damage to spermatogonial stem cells [14]. GC1-spg (non-rudiments) and SFTF-PI43 (rudiments) are among the first proven cell lines in testicles that are able to settle on seminiferous tubular basement membrane and through division continue to become spermatozoa [15,16].

Gossypol is a natural toxic pigment of cottonseed that has anti-fertility properties [17]. The results of this study, which was conducted in vitro on cell testicular tissue confirmed that the influence of gossypol varies based on its concentration. In GC1-spg cell line, which is derived from, stem cells of mouse testicles (in concentrations of 10µM/ml, 5µM/ml and 2.5µM/ml), toxicity was observed as compared to the control group. The same effect also occured in cell lines derived from sheep testicles SFTF-PI43.

Mechanisms responsible for the effect of gossypol on male reproductive organs are applied through a variety of routes. In vestigations indicate that effective doses of gossypol in males reduced production and motility and increased abnormalities in the sperm which is because of damage to the mitochondrial membranes in the middle piece of the sperm [6,7].

Content of Dynein that is a protein binding to microtubules is decreased in primary spermatocytes of rats that received oral gossypol acetic acid [18]. Long-term or high doses effects of gossypol on spermatozoid causes severe morphological changes in spermatozoid including damage to sperm acrosome, its mitochondria as well as sperm tail and stunts its growth [19]. One of the functions of gossypol on the epididymisis delaminating epithelial cells of epididymis in both head and tail, while infertility effects of gossypol was created without changes in testosterone levels and androgen hormones [20]. Among other inhibitory activities of gossypol is inhibiting oxidative phosphorylation that results in dysfunction of glycolysis cycle through inhibiting lactate dehydrogenase, which results in the decrease of ATP level and sperm motility. This enzyme is involved in transferring depressant from cytosol to mitochondria and helps sperms to move and produce energy in cytoplasm [21, 22] . Inhibition of type T, calcium channels in the sperm membrane, is also considered one of the gossypol's mechanisms of anti-fertility [23]. Some studies indicated that gossypol has dual property in pro-antioxidant activity. It can play a role as an effective and potent natural antioxidant; and on the other hand, itis a powerful antagonist of iron-induced lipid peroxidation in rat liver microsomes. Laughton et al in their reasrch showed that Gossypol inhibits rat liver microsomal peroxidation, after incubation with ferric/ascorbate (IC50< 0.1µM)[24]. In the present study Gossypol appeared in SFTF-PI43 as pro-oxidant by the FRAP assey.

In recent decades, cell culture systems have provided an important tool to explain the effects of gossypol. Studies on the effect of gossypol on cultured Sertoli cells demonstrated that it could cause infertility through decrease in ATP level of mitochondria [25]. Studies on cultured Sertoli cell showed that gossypol blocks gap junctions of intercellular communications of Sertoli cells with adjacent cells and differentiating generic cells and therefore decreases connexin 43 expression. This gap junction protein relates to regulation of spermatogenesis in adult and removing Cx43 from Sertoli cells causes in delay in the maturation of Sertoli cells [26].

According to this study, gossypol may impair mitochondrial function by apoptotic phenomenon and it can be a possible target for infertility. Gossypol mainly acts on mitochondrial membrane and its ability to join to BCl-2 and BCl-xl (critical regulators of apoptosis) in vitro, inducing the release of cytochrome, and BAK activation are important factors [27,28]. Two models are presented for the performance of gossypol. In the first model, gossypol may directly bind to BCl-2 or BCl-xl, inhibits anti-apoptotic activity, and replaces pro-apoptotic factors, including bax/bak proteins or second BH3. The second BH3 joined to BCl-xl is considered a particular place for interaction with gossypol. In the second possible model, gossypol may directly reacts with pro-apoptotic

BCl-2 family members including bax or bak and forms a complex with apoptotic protease activating factor which subsequently promotes cell death [29,30,31]. The results of our study showed that gossypol reduces viability of GC1-spg and SFTF-PI43 stem cell lines in dose dependent fashion and higher doses of gossypol exert greater inhibitory effects. The results of both toxicity methods match approximately and confirm gossypol toxicity effects on both mentioned cell lines. MTT toxicity and Trypan blue staining tests showed that in vitro conditions, Gossypol sensitivity in both cell lines of the ruminant and non-ruminant is almost identical, while several studies showed resistance in the ruminants. The current study differs from previous ones in some aspects: in previous studies ruminants in their diet consumed gossypol. In the rumen, gossypol joints to free proteins and rumen microbes reduce the toxicity of gossypol and its effect on the tissues by changing the milieu. However, in this study the effect of gossypol on germ cells (stem cells) of the testicles was directly studied. In previous studies, concentration of LD_{50} for gossypol was 500-950 mg/kg in mice and 2400-3340 mg/kg in rats. In this study, the inhibitory concentration of IC50 for gossypol was 3.2µM for GC1-spg and 2.2µM for SFTF-PI_{43}. While Manses reported 7.6µM for IC_{50} of Sertoli cells lines (TM_4), it is possible that the cell lines described in our study are more sensitive than the Sertoli cell line.

CONCLUSION

According to the results, if cottonseed oil is not refined, gossypol will not be removed from it. Therefore, its entry into the body in long time may cause infertility. It should be mentioned that before using cottonseed on livestock, the level of its free gossypol has to be determined by laboratory. In addition, supplements of iron compounds in the diet of animals is recommended to avoid the toxic effects of gossypol.

ACKNOWLEDGMENTS

We are especially thanks to from Babol Medical University.

REFERENCES

1. Fattahi E, Jorsaraei S, Parivar K, Moghaddamnia AA. The effects of a single dosage of Diazinon and Hinosan on the structure of testis tissue and sexual hormones in Mice. Yakhteh Medical Journal. 2010;12(3):405-10.
2. Sarvari A, Naderi MM, Heydari M, Zarnani AH, JEDI TM, Sadeghi MR, et al. Effect of Environmental Risk Factors on Human Fertility. Journal OF Reproduction and Infertility. 2011.
3. Acamovic T, Brooker J. Biochemistry of plant secondary metabolites and their effects in animals. Proceedings of the Nutrition Society. 2005;64(03):403-12.
4. Acamovic T, Stewart CS, Pennycott T. Poisonous plants and related toxins: CABI; 2004.
5. Kovacic P. Mechanism of drug and toxic actions of gossypol: focus on reactive oxygen species and electron transfer. Current medicinal chemistry. 2003;10(24):2711-8.
6. Amini A, Kamkar F. The effects of gossypol on spermatogenesis in NMRI mice. Iranian Journal of Science and Technology. 2005;29(A1):123.
7. Randel R, Chase C, Wyse S. Effects of gossypol and cottonseed products on reproduction of mammals. Journal of Animal Science. 1992;70(5):1628-38.
8. Freedman TB, Cao X, Oliveira RV, Cass QB, Nafie LA. Determination of the absolute configuration and solution conformation of gossypol by vibrational circular dichroism. Chirality. 2003;15(2):196-200.
9. Ganbari F, Jafari Aahangari Y, Ghouchi T, Hasani S. An investigation on effects of cottonseed meal gossypol on some hematological parameters in atabay rams. Journal of Agricultural Sciences.2006.
10. Brocas C, Rivera R, Paula-Lopes F, McDowell L, Calhoun M, Staples C, et al. Deleterious actions of gossypol on bovine spermatozoa, oocytes, and embryos. Biology of reproduction. 1997; 57(4):901-7.
11. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical biochemistry. 1979; 95(2):351-8.
12. Benzie IF, Strain J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Analytical biochemistry. 1996; 239(1):70-6.
13. Singh SR, Burnicka-Turek O, Chauhan C, Hou SX. Spermatogonial stem cells, infertility and testicular cancer. Journal of cellular and molecular medicine. 2011;15(3):468-83.
14. Schmidt JA, Abramowitz LK, Kubota H, Wu X, Niu Z, Avarbock MR, et al. In vivo and in vitro aging is detrimental to mouse spermatogonial stem cell function. Biology of reproduction. 2011;84(4):698-706.

15. Van Pelt AM, Roepers-Gajadien HL, Gademan IS, Creemers LB, de Rooij DG, van Dissel-Emiliani FM. Establishment of cell lines with rat spermatogonial stem cell characteristics. Endocrinology. 2002;143(5):1845-50.
16. Wang Y, Song W, Li S, Guan X, Miao S, Zong S, et al. GC-1 mRHBDD1 knockdown spermatogonia cells lose their spermatogenic capacity in mouse seminiferous tubules. BMC cell biology. 2009;10(1):25-6.
17. Coutinho EM. Gossypol: a contraceptive for men. Contraception. 2002;65(4):259-63.
18. Teng C. Biological activity in the repopulating rat spermatocyte after the withdrawal of gossypol treatment. V. Inhibition and recovery of microtubular dynein. Contraception. 1995;51(5):313-8.
19. Ciereszko A, Dabrowski K. In vitro effect of gossypol acetate on yellow perch (Perca flavescens) spermatozoa. Aquatic toxicology. 2000;49(3):181-7.
20. De Andrade SF, Oliva SU, Klinefelter GR, Kempinas WDG. Epididymis-specific pathologic disorders in rats exposed to gossypol from weaning through puberty. Toxicologic pathology. 2006;34(6):730-7.
21. Stephens D, Critchlow L, Hoskins D. Mechanism of inhibition by gossypol of glycolysis and motility of monkey spermatozoa in vitro. Journal of reproduction and fertility. 1983;69(2):447-52.
22. Leblanc ML, Russo J, Kudelka AP, Smith JA. An *In Vitro* Study Of Inhibitory Activity Of Gossypol, A Cottonseed Extract, In Human Carcinoma Cell Lines. Pharmacological research. 2002;46(6):551-5.
23. Shi YL, Bai J-P, Wang W-P. Ion-channels in human sperm membrane and contraceptive mechanisms of male antifertility compounds derived from Chinese traditional medicine. Acta pharmacologica Sinica. 2003;24(1):22-30.
24. Laughton MJ, Halliwell B, Evans PJ, Robin J, Hoult S. Antioxidant and pro-oxidant actions of the plant phenolics quercetin, gossypol and myricetin: effects on lipid peroxidation, hydroxyl radical generation and bleomycin-dependent damage to DNA. Biochemical pharmacology. 1989;38(17):2859-65.
25. Monsees TK, Winterstein U, Schill W-b, Miska W. Influence of gossypol on the secretory function of cultured rat sertoli cells. Toxicon. 1998;36(5):813-6.
26. Zhou D-R, Zhou Y-C, Cui G-H, Guo X, Qin J, Gui Y-T, et al. Gossypol repressed the gap junctional intercellular communication between Sertoli cells by decreasing the expression of Connexin43. Toxicology in vitro. 2008;22(7):1719-25.
27. Oliver CL, Miranda MB, Shangary S, Land S, Wang S, Johnson DE. (−)-Gossypol acts directly on the mitochondria to overcome Bcl-2-and Bcl-XL-mediated apoptosis resistance. Molecular cancer therapeutics. 2005;4(1):23-31.
28. Lei X, Chen Y, Du G, Yu W, Wang X, Qu H, et al. Gossypol induces Bax/Bak-independent activation of apoptosis and cytochrome c release via a conformational change in Bcl-2. The FASEB journal. 2006;20(12):2147-9.
29. Zhang M, Liu H, Guo R, Ling Y, Wu X, Li B, et al. Molecular mechanism of gossypol-induced cell growth inhibition and cell death of HT-29 human colon carcinoma cells. Biochemical pharmacology. 2003;66(1):93-103.
30. Parone PA, Martinou J-C. Mitochondrial fission and apoptosis: an ongoing trial. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research. 2006;1763(5):522-30.
31. Balakrishnan K, Wierda WG, Keating MJ, Gandhi V. Gossypol, a BH3 mimetic, induces apoptosis in chronic lymphocytic leukemia cells. Blood. 2008;112(5):1971-80.