

A possible Ameliorating Effects of *Tribulus terrestris* on Testicular Dysfunction Induced by Xenoestrogens Exposure in Adult Rats.

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ABSTRACT

Human exposure to xenoestrogen as Soybeans or Bisphenol- A were claimed to cause testicular alterations. Recently investigations on *Tribulus terrestris* in ameliorating the putative toxic effect of xenoestrogen have been reported. Ninety adult male rats of Wister origin was used in this experiment. The groups were divided into nine groups; two groups were lifted as control receiving water and corn oil respectively. The other seven groups were treated with Soya isoflavon (SIF43mg/Kg), Bisphenol-A(BPA3mg / kg) and *Tribulus terrestris* (TT 100mg / kg) ,SIF+BPA, SIF+TT, BPA+TT and finally SIF+BPA+TT for 58 successive days. By the end of the experiment ,body weight, relative sex organs weight, Biochemical estimation for estradiol and testosterone hormones, Aspartate aminotransferase AST, alanine aminotransferase, Alkaline phosphatase ALP and acid phosphatase ACP in testicular tissue were done. Also histological studies, morphometric measurements and immune histopathological studies on testicular tissues were done .SIF and BPA treated groups showed significant increase in circulating estradiol levels, AST and ALT which was normalized by TT administration, while SIF+BPA group reduced estradiol. Insignificant change in estradiol level was observed in TT treated group. Reduction in testosterone levels, ALP and ACP activity were observed in SIF, BPA and SIF+BPA groups, co administration of TT to these groups normalize estradiol, testosterone levels and their activity of these enzymes. Histological observation revealed testicular damage in the form of spermatogenic arrest ,edema together with reduction in leydig cells counts in SIF,BPA alone or when given together .the incidence and severity of these lesions were significantly diminished in TT+SIF and TT+ BPA groups. TT administration significantly augmented the putative deleterious effect of SIF combined with BPA on testicular tissues.

Key words: Xenoestrogen, *Tribulus terrestris* - infertility.

Introduction

There are many factors that can affect the infertility as hormonal disruption, which can be reduced as follicle stimulating hormone (FSH), luteinizing hormone (LH),and testosterone levels in case of abnormal semen parameters. Also infertility can result from disorders of the testicles themselves or an abnormality affecting other hormonal system including the hypothalamus, pituitary, thyroid and adrenal gland .Low testosterone (male hypogonadism) and other hormonal problems have a number of possible underlying causes (Loughlin, 2012).

The other factors that can affect the infertility is overexposure to certain environmental chemicals compound such as xenoestrogens that can reduce sperm production or sperm function. Xenoestrogen is a man-made chemical compounds which mimics the effect of estrogen in the human body. It can disrupt the hormonal system in both men and women and cause infertility (Swerdloff, 2012). Xenoestrogens are either phytoestrogens or synthetic estrogens, which are able to bind to estrogen receptors, and to mimic estrogenic activities in a cell and tissue specific manner (Patel and Sigman, 2008). Phytoestrogens are naturally occurring in plants as soy beans and other legumes containing isoflavone notably genistein which binds to estrogen receptors in the body and thus tone dawn effects of excessive quantities of estrogen by rendering the body relatively insensitive to synthesized estrogen. It has been previously indicated that soy containing diet may delay male reproductive development with manifestation detected in adulthood in rats (Knight and Eden, 1996). The recorded studies focused on male fertility showed that soy lowered testosterone level (Lund *et al.*, 2004 ; Dillingham *et al.*, 2005 and Goodin *et al.*, 2007), and lowered sperm concentration (West *et al.*, 2005).

The relation between chemical pollution and reproductive health has always been a public health concern. It was reported that BPA was one of the important chemicals used principally as a monomer in the manufacture of plastics and other products and have been detected in food and water consumes by animals and

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peoples (Krishnan *et al.*, 1993 ; Brotons *et al.*, 1995). BPA is a well-known endocrine disruptor that imperfectly effects physiologic estrogen via membrane-bound estrogen receptors (mER α , mER β).

BPA can act as an anti-androgen by blocking of dihydrotestosterone human androgen receptors (Krishnan *et al.*, 1993). Steinmetz *et al.*, (1998) found that treatment with single high dose of BPA induced inhibition of testicular steroidogenesis. Also plasma free testosterone levels were dramatically decreased by treatment of mice with BPA. These studies have shown that BPA has adverse effects on the reproductive system. Takahashi and Oishi,(2003) also showed that BPA can cause a weight decrease in the testis epididymis, prostate, seminal vesicles and a decrease in sperm production. Moreover, it has been shown (in-vivo) that BPA has a stimulatory effect on sertoli cells in rats (Wistuba *et al.*, 2003).

Tribulus terrestris (TT) is a plant of tropical regions, traditionally used as aphrodisiac, analgesic and diuretic agent(Elahi *et al.*, 2013).TT is a completely natural non-hormonal herbal product extracted from the herb TT in the family *Zygophyllaceae*. TT has been shown to enhance sexual behavior in an animal model (Gauthaman *et al.*, 2002). TT contains biologically active substances such as steroids, saponins, flavonoids, alkaloids, unsaturated fatty acids, vitamins, tannins (Adaikan *et al.*, 2000). Gauthaman and Adaikan,(2005) study, showed that TT has caused an increase in testosterone and 71%improvement in the recovery of libido and sexual reflexes, when there is a complete absence of libido before treatment. Animal studies in rats and rabbits have demonstrated that administration of TT can produce statistically significant increases in levels of testosterone, dihydrotestosterone and dehydroepiandrosterone, (Gauthaman and Ganesan, 2008).

The aim of the present work was to evaluate the possible testicular disruptive effects of xenoestrogens (soybean or bisphenol -A) when given orally, and to explore the possible potential protective effects of *Tribulus terrestris* on testicular dysfunction induced by these compounds.

Materials and Methods

- 1- SoyIsoflavones mixture (SIF) tablet (50 mg) contains (genistein, daidzin, and saponin, extracted from soybean) was purchased from Mepaco Medi food Ltd. (Sharkeia , Egypt).
- 2- Bisphenol-A (BPA) 4,4 isopropylidenediphenol-1-2, 2-bis- (4-hydroxy.phenyl) –propane C₁₅H₁₆O₂ purchased from Sigma- Aldrich chemie-Gmb.
- 3-*Tribulus Terrestris* (TT) leaves extract manufactured by Nerhadou International and Nutraceuticals, capsule contains TT extract 250 mg.

Animals

Adult male albino rats weighed (200 to 300) g. were obtained from the animal house of the National Organization for Drug Control and Research (NODCAR).They were housed under controlled laboratory conditions, two weeks prior to experiment for acclimatization and received standard diet and water *ad libitum* during the study period. All animal procedures and the experimental protocols were approved by the Institutional Ethics Committee at NODCAR and were carried out in accordance with the Guide for the Care and use of laboratory Animals.

Experimental groups

Ninety rats were randomly assigned to the following nine groups:

- 1- Control water group (CW): animals received Tween 80(1%) in distilled water (5ml /kg; orally).
- 2- Control oil group (CO): animals received corn oil (5ml / kg; orally).
- 3- SIF-group: animals received orally soyisoflavone (43 mg / kg) hydro suspension by Tween 80 (1%) according to Khan and Sultana,(2011) for 58 days.
- 4- BPA -group : animals received orally Bisphenol- BPA dissolved in corn oil at 3mg/kg/day according to Sakaue *et al.*, (2001) for 58 successive days.
- 5-TT-group: animals orally administered TT at 100mg / kg according to Sharma *et al.*, (2013) for the same period.
- 6-SIF+BPA- group: animals received combined treatment of (SIF + BPA) for the same period.
- 7-SIF+TT group: animals supplemented with SIF+ TT for the same period.
- 8-BPA+TT group: animals received BPA + TT for the same period.
- 9-SIF+BPA+TT group: animals received triple regimen (SIF+BPA+TT) for the same period.

Biochemical Estimation

Collection of serum and testicular sample

At the end of the experimental period, the final body weights of the animals were recorded. Blood samples were collected from the retro orbital plexus in plastic centrifuge tubes, left to clot at 4°C for 30 minutes and serum was obtained by centrifugation at 3000 rpm for 20 minutes. Then, rats were anaesthetized and dissected testes, prostate gland, epididymis and seminal vesicles were immediately removed where cleared of adhering tissues, rinsed in ice-cold saline and weighed. The relative weight of organs (%) was calculated as g/100 g body weight.

Hormonal assays in serum

Serum estradiol (E) and testosterone (T) were measured by the method of Tsang *et al.*, (1980) & Tietz (1995) using commercial ELISA kits from Nova Tec. Immunodiagnostica (Dietzenbach, Germany) and Bio Check (Foster city, U.S.A.) respectively.

Determination of Testicular Markers

Frozen testicular tissues were homogenized in ice cold medium as 10% w/v. Homogenate was mixed with 0.1M Tris buffer (pH 8.1) and centrifuged for 40 min at 4°C and 15,000 rpm. The resulting cytosolic fraction was used for the determination of the activities of Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) were determined by using kits according to the method of (Reitman and Frankel, 1957). Alkaline phosphatase (ALP) and Acid phosphatase (ACP) using commercially available kits (Kind and King, 1954).

Histopathological studies

Testes were collected, fixed in 10% buffered formalin, for paraffin blocks, serial sections were prepared at 4µ, then, stained with haematoxylin and eosin (H&E) according to the method of Bancroft *et al.*, (1996). The sections were viewed and photographed using Olympus light microscope.

Morphometrical Measurements

Measuring Diameter and heights of seminiferous tubules

The tubular diameter and height of the seminiferous tubules epithelium were measured at 100X magnification using Image analysis system (Image Prop Plus version 5). At least 20 tubular profiles that were round or nearly round were chosen randomly and measured for each animal. Epithelium height was measured in 50 seminiferous tubules per rat using (40X) power at two points on the same axis of each seminiferous tubule, the mean of these two readings was taken as the epithelium height according to Kalisnik, (1981).

Leydig Cells count

Ten random fields of testis tissue per animal were examined in H&E stained slides using (100X) power and counted the number of Leydig cells according to (Otoom *et al.*, 2004).

Immunohistochemical studies

For immunohistochemical techniques, testes tissues were taken from animals of both control and treated groups, immediately after dissection, tissues were fixed in 10% neutral formalin and then 5 micrometer thickness sections were prepared. Sections were stained immunohistochemically to visualize B-cell lymphoma 2 (BCL-2) positive cells, using anti-Bcl-2 (Hsu *et al.*, 1981).

Statistical Analysis

The data obtained from the biochemical analysis of different groups are presented as Mean ± Standard Error (mean ± SE). The significance of the difference between the groups was calculated by one-way analysis of

variance (ANOVA) followed by Duncan and (Dunnnett t (2-sided) procedure for multiple comparisons at $P < 0.05$ using the SPSS-PC computer software package version 18.

Results

The impacts of SIF, BPA and TT on final body weight gain are shown in Table (1). There was no significant difference in the body weight gain among the SIF, TT, SIF +TT, BPA+ TT and SIF+BPA +TT groups when compared to control groups. However, the body weights gain in the BPA and BPA +SIF groups were significantly less than other groups. In SIF group, there were no significant changes neither in the body weight nor the relative prostate weight and relative epididymis weight, meanwhile a statistically significant decrease in testes relative weight and relative seminal vesicles weight were indicated between SIF group and other groups comparing to control. Data recorded in BPA group revealed significantly lower values in body weight, relative testes weight and relative seminal vesicles weight at $p < 0.05$ in comparison with control groups coupled with significant increase in relative epididymis weight and in significant in relative prostate weight. The recorded results from TT treated group demonstrated significant increase in both seminal vesicles and prostates relative weights when compared to controls, meanwhile, data revealed insignificant difference between the control groups and TT group in body weight, relative testis and epididymis relative weights.

Effect of SIF, BPA and TT on serum estradiol and testosterone hormone are shown in table (2). The data revealed that SIF or BPA alone or combined with TT, significantly increase serum estradiol hormonal level more than the two control groups. The recorded data of (BPA+SIF) groups displayed significant decrease in comparison to control, or SIF, BPA, TT groups respectively. Also, the animals groups treated with SIF or BPA or their combination showed significant lower values in serum testosterone hormone when compared with control groups, meanwhile, TT group showed significant increase in serum testosterone hormone. The changes of the activities of AST, ALT, ALP, and ACP in testis homogenate after oral administration of treated rats with SIF, BPA, or TT for 8 weeks are shown in histogram (1, 2, 3 and 4). The administration of SIF, BPA or their combination exerts significant increase in AST and ALT enzymatic activity as comparing to controls, Figs (1, 2). The results also proved that, ALP and ACP display significant decrease in SIF, BPA or their combination in comparison to control (fig. 3,4)

Histological testes results

SIF: The histological studies for testis of this group revealed mild toxicity appeared in the form of irregularity in shape of many seminiferous tubules together with spermatogenic arrest. Some other seminiferous tubules with spermatogenic lineage were detached from their basement membrane. Focal areas of capsular thickening, and dilated congested subcapsular blood vessels. Interstitial edemas were detected in many areas Fig (5, 6).

BPA: The toxic impact of this group was mild to moderate, where many seminiferous tubules showed reduction in size and spermatogenic arrest, detachment and degenerative changes in many spermatogenic lineage especially spermatides. Focal areas of capsular thickening, beside dilated congested subcapsular blood vessels. Interstitial inflammatory aggregates were also observed Fig (7, 8).

TT: Mild pathological alteration due to TT, where testicular tissues display detachment of spermatogenic lineage in few seminiferous tubules with spermatogenic arrest, together with intraepithelial vacuoles. Other seminiferous tubules showed desquamated cells in their lumen Fig (9).

SIF+ BPA: Moderate to severe toxic impact has been detected in this combined treatment; where the incidence and severity of spermatogenic arrest were highly significant compare to the mono treatment, in addition to sever intraepithelial vacuoles. Many interstitial spaces with edema, and dilated congested vasculature were also showed Fig (10, 11).

SIF+ TT: Mild toxicity was observed due to TT treatment where mild degrees of seminiferous tubules damage were noted, beside intraepithelial vacuoles, few seminiferous tubules reflecting spermatogenic arrest, in addition to few irregular in shape and atrophy. Focal capsular thickening together with giant cell were occasionally seen Fig (12).

BPA+TT: Male rats orally given combined with BPA + TT, displayed mild toxic impact on testicular tissues, where intraepithelial vacuoles were diffused in most of the seminiferous tubules. Few seminiferous tubules display spermatogenic arrest, while in many seminiferous tubules basement membranes separating from the underlying layers were observed. Focal capsular thickening with congested dilated underlying vessels could be seen in Fig (14).

SIF+BPA+TT: Testicular tissues of animals exposed to the three regimens, display mild pathological alterations in form of spermatogenic arrest in many of seminiferous tubules, together with interstitial edema with congested dilated vasculature Fig (15, 16).

The Bcl-2 expression

In testicular tissue of control animals, the majority of interstitial Leydig cells showed Bcl-2 positive staining Fig (17). Meanwhile, in testicular tissues of animals received SIF or BPA or both display very few cells positive for Bcl-2 immune stain Fig (18, 19). TT treatment reflects high Bcl-2 expression in leydig cells Fig (20). Testes of SIF+BPA treated rats showing weak expression of Bcl-2 in Leydig cells as shown in Fig (21). Administration of TT combined with either BPA or SIF showed up regulation in the expression of Bcl-2 in Leydig cells (Figs 22& 23). Leydige cells in the group of animals received triple treatment (TT, SIF and BPA) showed high expression of Bcl-2 immunostain reflecting the ameliorating effect of TT as seen in Fig (24).

Table 1: Effects of SIF, BPA and TT on body weight and relative sex organs weight of adult male rats when administered for 8 weeks.

Parameter Groups	% B.WT. change	% Relative testis	% Relative epididymis	% Relative seminal	%Relative prostate
CW	46.88 ^{cd} ±2.73	1.05 ^c ±0.02	0.17 ^{abc} ±0.01	0.26±0.01	0.16 ^{ab} ±0.01
CO	46.78 ^{cd} ±4.09	0.94 ^{dc} ±0.05	0.18 ^{bc} ±0.01	0.36 [*] ±0.03	0.22 ^{*d} ±0.02
SIF	51.18 ^{de} ±0.88	0.64 ^{*b} ±0.03	0.16 ^{ab} ±0.01	0.17 [*] ±0.01	0.16 ^{ab} ±0.01
BPA	35.71 ^{*ab} ±0.95	0.49 ^{*a} ±0.03	0.20 ^c ±0.01	0.16 [*] ±0.01	0.18 ^{bc} ±0.01
TT	54.30 ^c ±2.90	0.92 ^{dc} ±0.02	0.17 ^{cd} ±0.01	0.38 [*] ±0.02	0.26 ^{*c} ±0.01
SIF+BPA	38.29 ^b ±1.76	0.79 ^c ±0.05	0.15 ^a ±0.01	0.27±0.01	0.14 ^{*a} ±0.01
SIF+TT	49.97 ^{de} ±0.65	0.93 ^{*d} ±0.05	0.17 ^b ±0.01	0.26±0.01	0.23 ^d ±0.02
BPA+TT	42.29 ^{bc} ±1.26	0.89 ^{*cd} ±0.03	0.18 ^{cd} ±0.01	0.32 [*] ±0.02	0.19 ^{cd} ±0.01
SIF+BPA+TT	31.30 ^{*a} ±1.99	1.01 ^{de} ±0.06	0.18 ^{cd} ±0.04	0.34 [*] ±0.01	0.16 ^{ab} ±0.01

Results were expressed as mean ± SE for each 10 rats.

* Significant difference against control at P<0.05 (Dunnnett t (2-sided)).

Groups have the same letter mean non- significant at P < 0.05(Duncan).

Groups have different letters means significant at P < 0.05(Duncan).

Table 2: Hormonal profile of adult male rats exposed to SIF, BPA and TT after 8 weeks of administration.

Parameter Groups	Estradiol (pg/ml)	Testosterone (ng/ml)
C W	31.74 ^b ±1.96	6.35 ^b ±1.90
C O	33.03 ^b ±0.53	6.87 ^b ±0.18
SIF	38.95 ^{*c} ±0.62	3.72 ^{*a} ±0.14
BPA	42.26 ^{*cd} ±0.97	2.98 ^{*a} ±0.04
TT	31.76 ^b ±1.51	11.67 ^{*c} ±0.38
SIF+BPA	23.41 ^{*a} ±0.91	3.33 ^{*a} ±0.54
SIF+TT	39.69 ^{*cd} ±1.62	8.99 ^{*c} ±0.39
BPA+TT	36.74 ^{*c} ±0.79	6.95 ^b ±0.19
SIF+BPA+TT	30.57 ^b ±1.33	6.78 ^b ±0.60

Results were expressed as mean ± SE for each 10 rats.

* Significant difference against control at P<0.05 (Dunnnett t (2-sided)).

Groups have the same letter mean non- significant at P < 0.05(Duncan).

Groups have different letters means significant at P < 0.05(Duncan).

Table 3: Morphometrical analysis (height, diameter of seminiferous tubules and Leydig cell count) for testes of adult male rat exposed to IF, BPA and TT and their combination after 8 weeks of administration.

Parameter Groups	Height /μ	Diameter/μ	Leydig cell count
C W	9.08 ^c ±0.21	36.0 ^c ±0.93	49.2 ^c ±3.39
C O	8.29 ^c ±0.04	34.9 ^c ±0.60	49.2 ^c ±3.39
SIF	8.83 ^c ±0.49	29.5 ^{*a} ±0.98	27.3 ^{*c} ±2.98
BPA	4.13 ^{*b} ±0.11	31.0 ^{*b} ±.82	25.1 ^{*ab} ±3.09
TT	8.93 ^c ±0.22	31.0 ^{*b} ±1.03	48.8 ^c ±5.24
SIF+BPA	2.81 ^{*a} ±0.08	30.6 ^{*b} ±0.76	24.4 ^{*a} ±1.73
SIF+TT	4.83 ^{*b} ±0.19	29.8 ^{*b} ±0.78	36.7 ^{*d} ±4.40
BPA+TT	4.48 ^{*b} ±0.17	35.6 ^c ±1.86	27.0 ^{*bc} ±2.13
SIF+BPA+TT	6.06 ^{*c} ±0.21	35.2 ^c ±1.40	36.9 ^{*d} ±2.38

Results were expressed as mean ± SE for each 10 rats.

* Significant difference against control at P<0.05 (Dunnnett t (2-sided)).

Groups have the same letter mean non- significant at P < 0.05(Duncan).

Groups have different letters means significant at P < 0.05(Duncan).

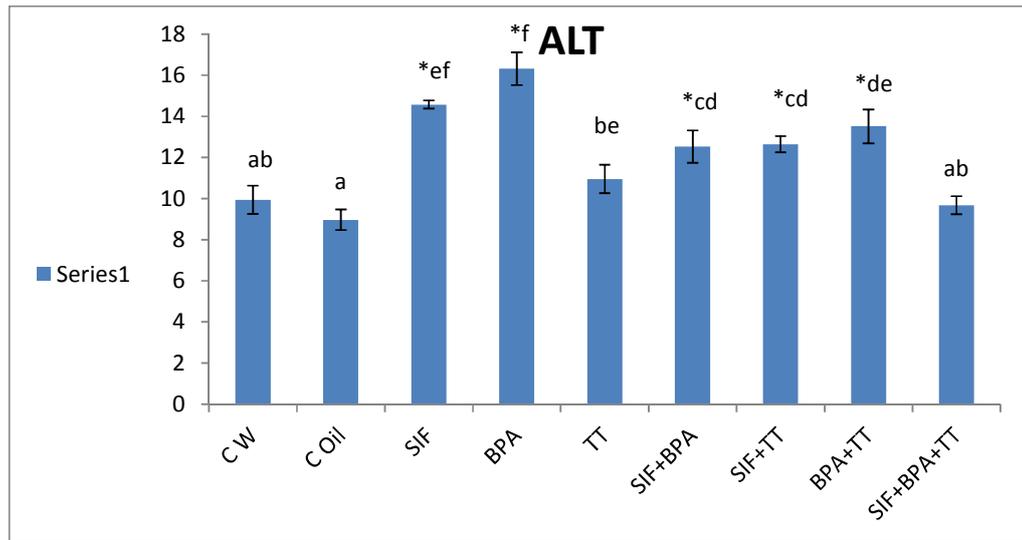


Fig. 1: Effects of SIF, BPA and TT on ALT activity of adult male rats after 8 weeks of administration. Results were expressed as mean \pm SE for each 10 rats.

* Significant difference against control at $P < 0.05$ (Dunnett t (2-sided)).
 Groups have the same letter mean non- significant at $P < 0.05$ (Duncan).
 Groups have different letters means significant at $P < 0.05$ (Duncan).

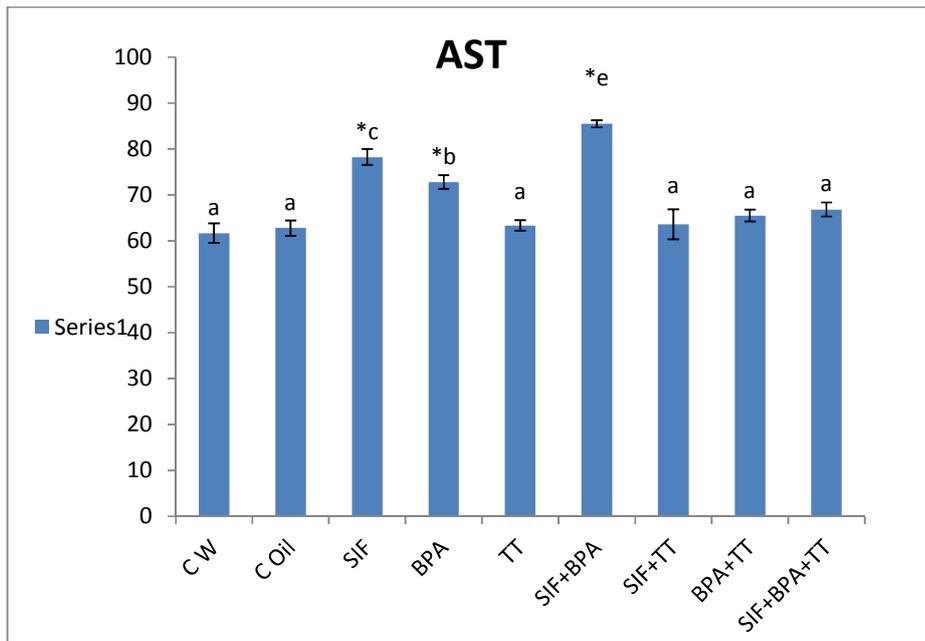


Fig. 2: Effects of SIF, BPA and TT on AST activity of adult male rats after 8 weeks of administration. Results were expressed as mean \pm SE for each 10 rats.

* Significant difference against control at $P < 0.05$ (Dunnett t (2-sided)).
 Groups have the same letter mean non- significant at $P < 0.05$ (Duncan).
 Groups have different letters means significant at $P < 0.05$ (Duncan).

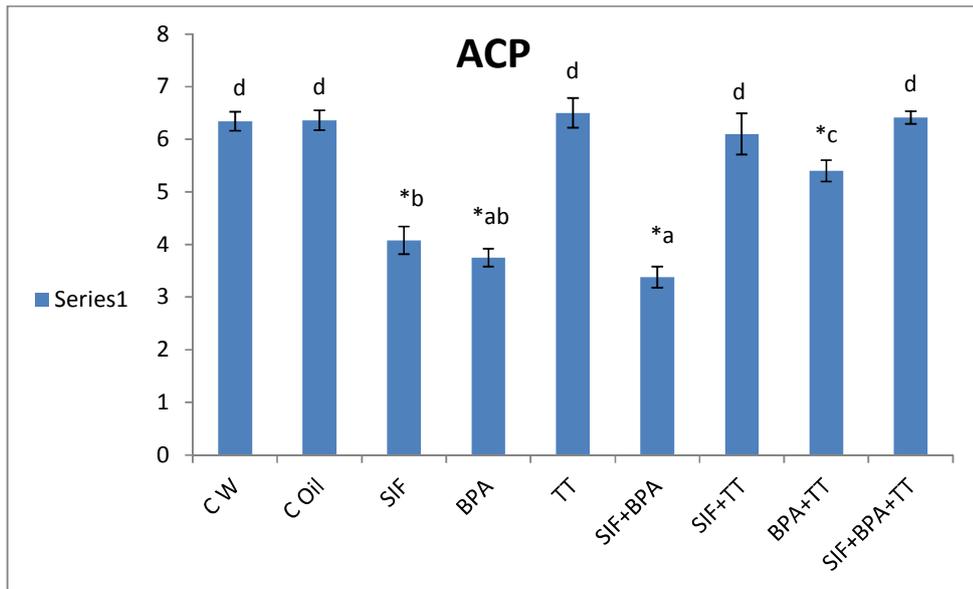


Fig. 3: Effects of SIF, BPA and TT on ACP activity of adult male rats after 8 weeks of administration. Results were expressed as mean \pm SE for each 10 rats.
 * Significant difference against control at $P < 0.05$ (Dunnett t (2-sided)).
 Groups have the same letter mean non- significant at $P < 0.05$ (Duncan).
 Groups have different letters means significant at $P < 0.05$ (Duncan).

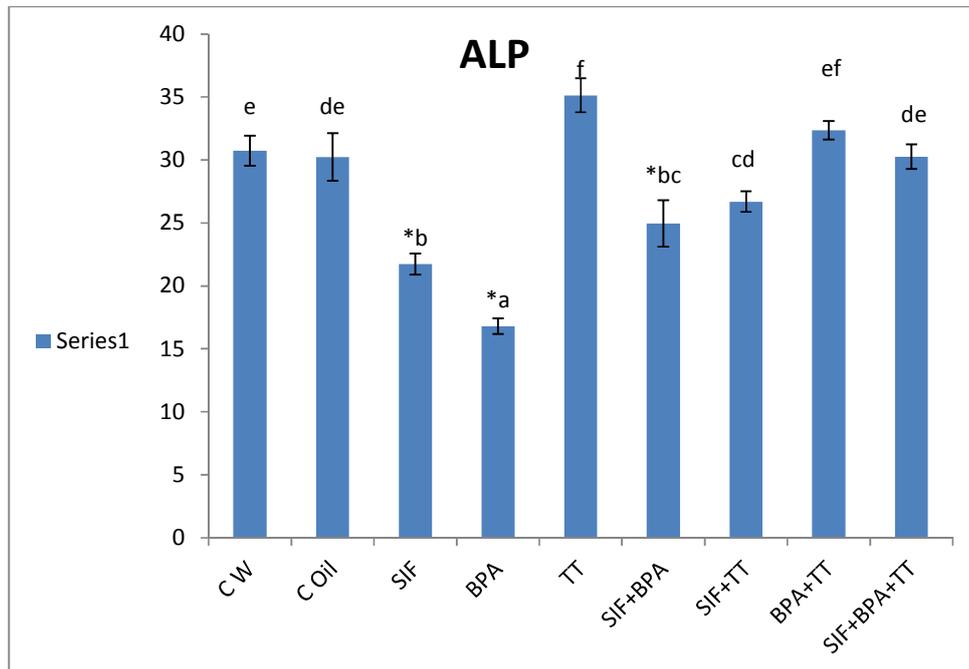
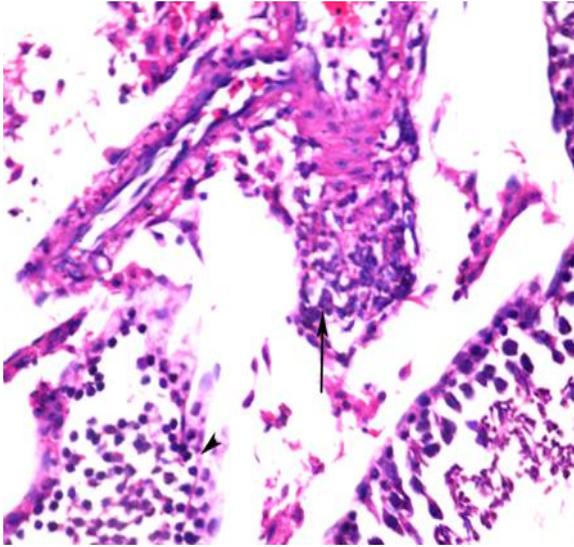
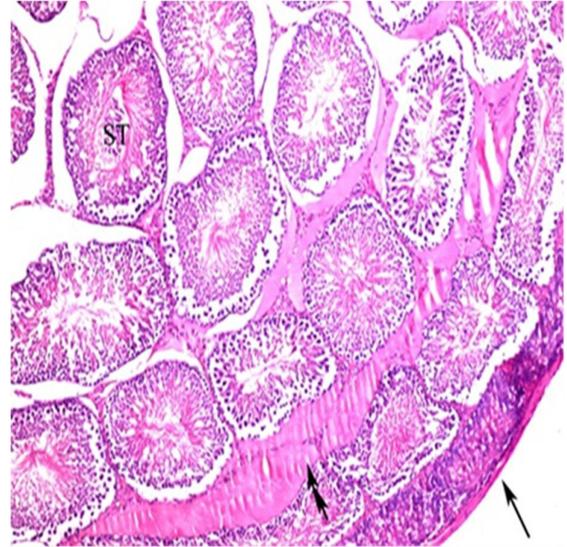


Fig. 4: Effects of SIF, BPA and TT on ALP activity of adult male rats after 8 weeks of administration. Results were expressed as mean \pm SE for each 10 rats.
 * Significant difference against control at $P < 0.05$ (Dunnett t (2-sided)).
 Groups have the same letter mean non- significant at $P < 0.05$ (Duncan).

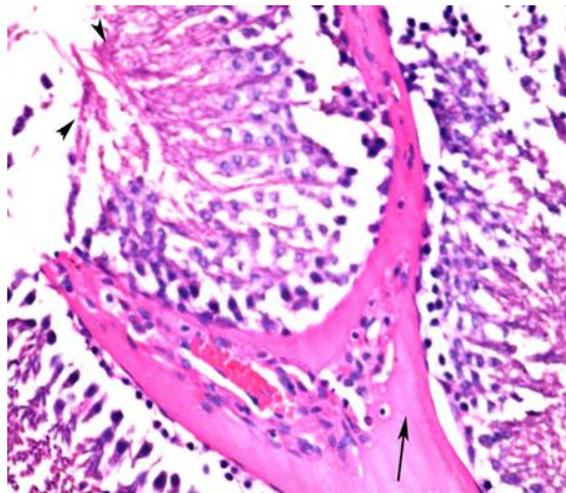
Groups have different letters means significant at $P < 0.05$ (Duncan).



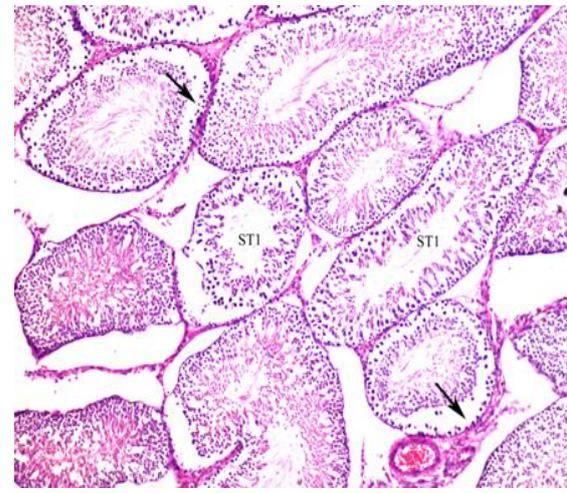
5-Photomicrograph of testicular tissues of SIF- treated group showing interstitial inflammation (arrow) (H&E,X: 400.)



6- Photomicrograph of testicular tissues of SIF-treated group demonstrating capsular thickening , congested dilated testicular blood vessel(arrow),seminiferous tubules (ST) (H&E,X: 200).



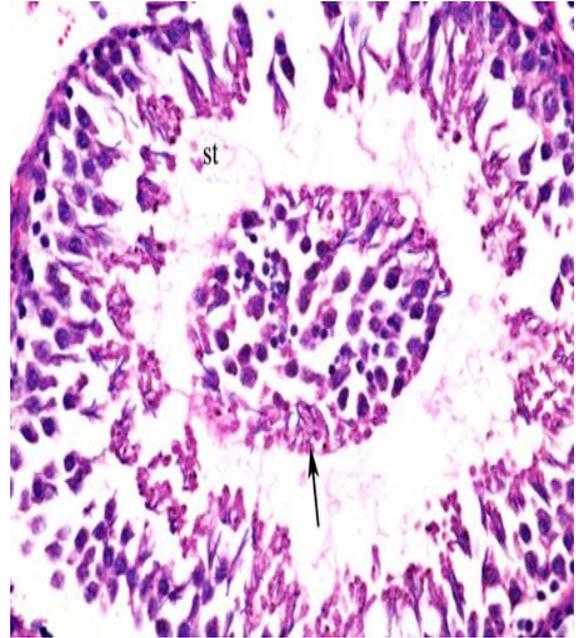
7- Photomicrograph of testicular tissues of BPA-treated group showing edema in interstitial space (arrow) degenerated spermatides (arrow head) (H&E, X:400).



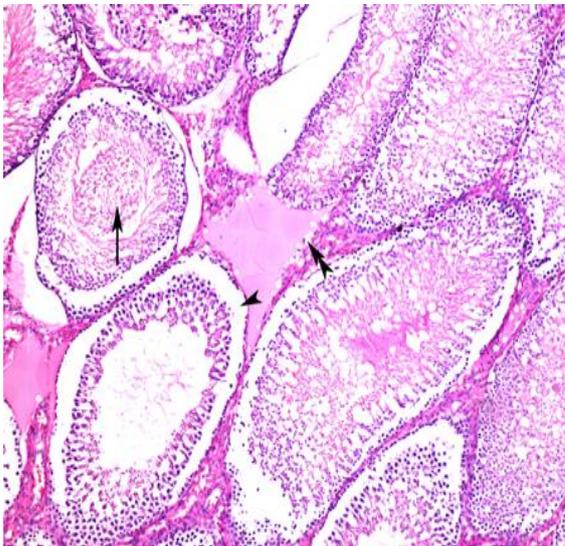
8-Photomicrograph of testicular tissues of BPA-treated group showing spermatogenic arrest in seminiferous tubule (ST1) basement membrane separating from the underlying epithelial cell layer in some seminiferous tubule(ST1) (arrow) (H&E,X:200).



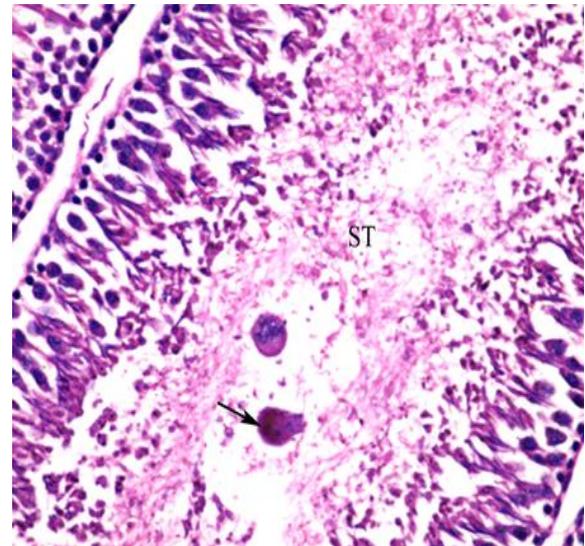
9- Photomicrograph of testicular tissues TT- treated group focusing on vacuoles in intraepithelial layer (Arrow), (H&E, X: 200).



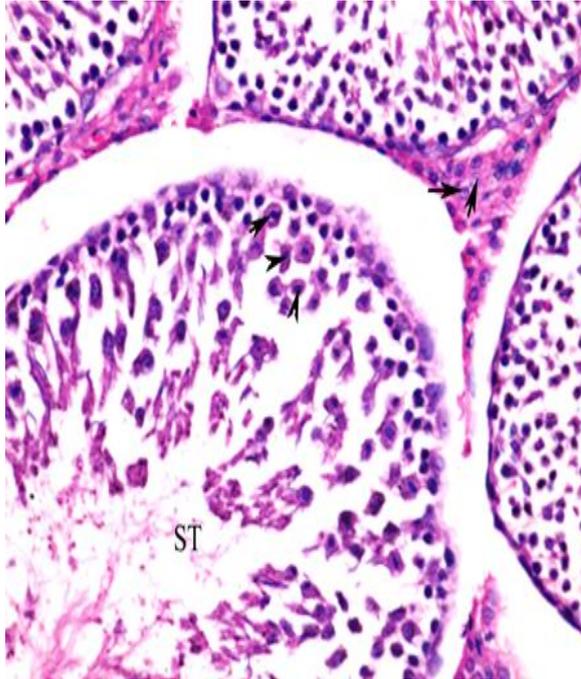
10- Photomicrograph of testicular tissues SIF+BP treated group, demonstrating desquamated cells (Arrow) in lumen of seminiferous tubules (ST), H&E.X:400).



11- Photomicrograph of testicular tissues SIF+BPA treated group showing separating basement) membrane from underlying epithelial cells (arrow head), desquamated cells in lumen of seminiferous tubules (arrow), edema in interstitial spaces (double arrow) (H&E, 200).



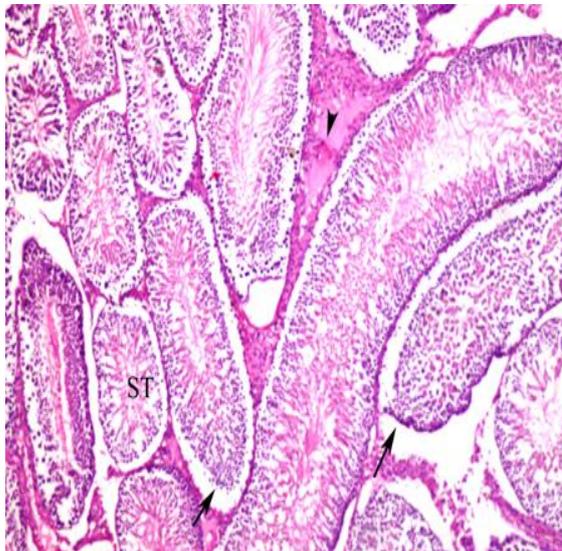
12- Photomicrograph of testicular tissues of SIF+TT treated group, showing giant cells (Arrow) in lumen of seminiferous tubules, H&E, X: 400.



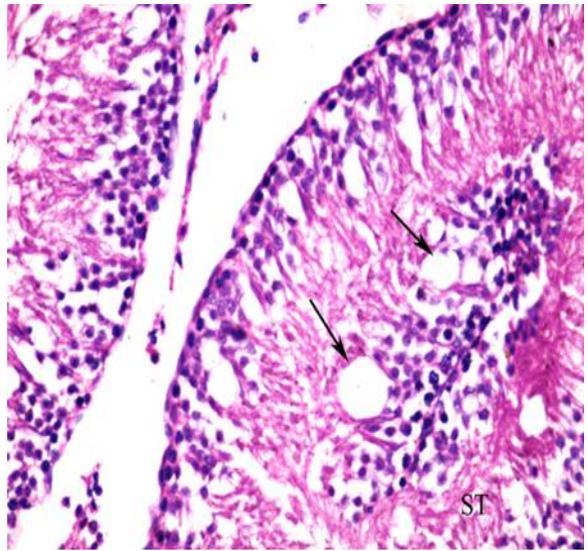
13- Photomicrograph of testicular tissues of SIF+TT treated group, showing Leydig cells in interstitial space (Arrow), degenerated spermatocytes (Arrow head), H&E, X: 400.



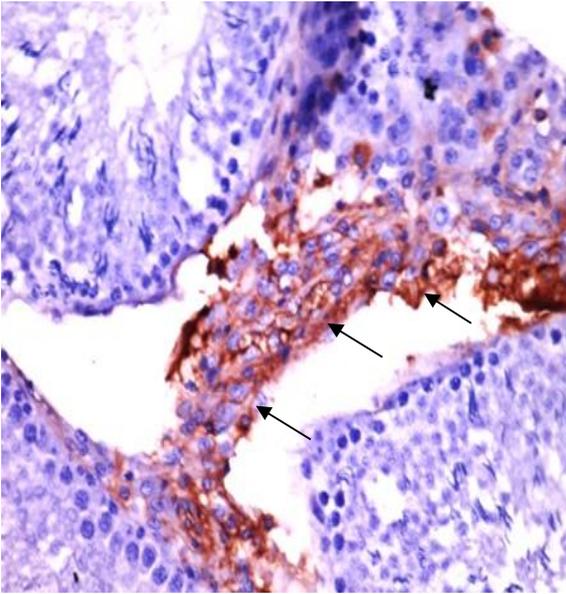
14- Photomicrograph of testicular tissues of BPA+TT treated group, showing testicular tissues replaced by adipose tissues (AT), H&E, X: 200.



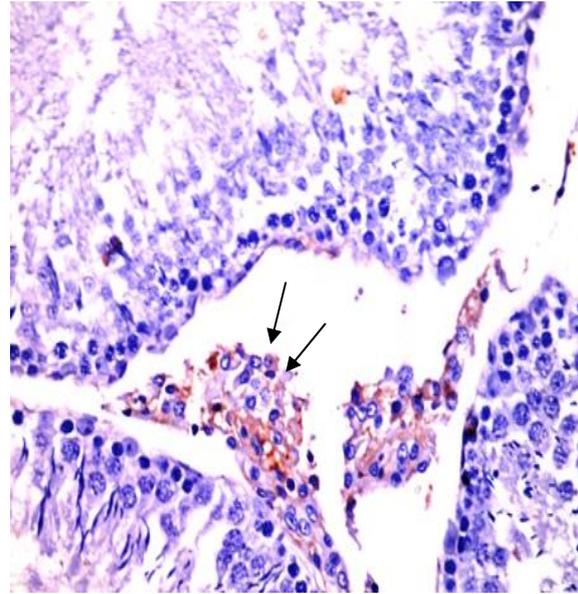
15- Photomicrograph of testicular tissues of SIF+BPA+TT- treated group, showing many intact seminiferous tubules (ST), separating basement membrane from underlying epithelial cells (Arrow), eosinophilic material deposited in interstitial spaces. H&E, X: 200.



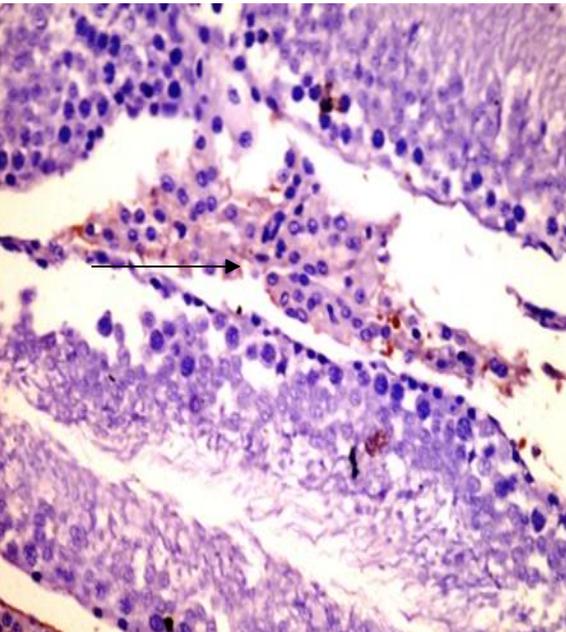
16- Photomicrograph of testicular tissues of SIF+BPA+TT- treated group, demonstrating interstitial epithelial vacuoles in between spermatogenic cells. H&E, X: 400.



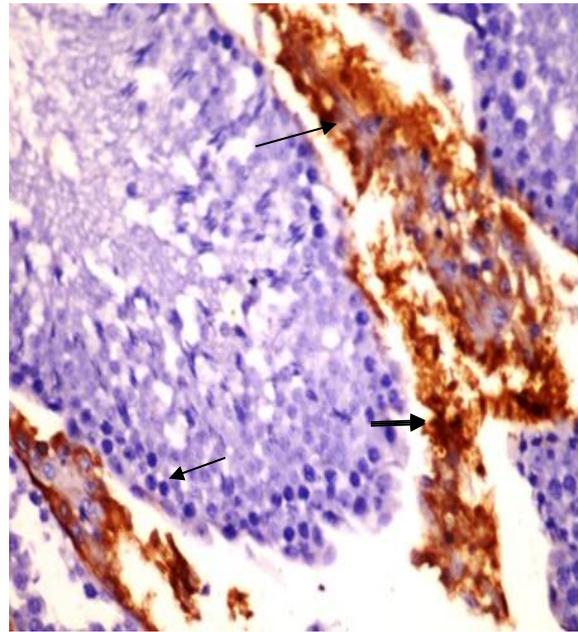
17- A photomicrograph obtained from testis of a control rat showing marked expression of Bcl-2 in Leydig cells (arrows), Bcl-2 immunestain, counter stained with hematoxylin, X 400



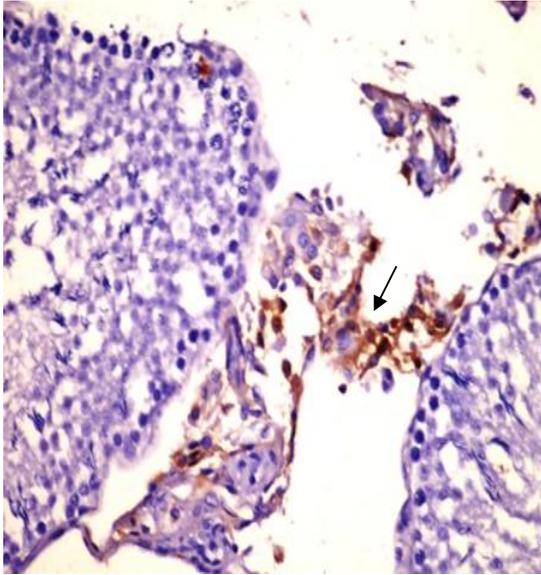
18- A photomicrograph obtained from testis of SIF-treated rat showing very few stained Leydig cells for Bcl-2 (arrows), (Bcl-2 immune stain, counter stained with hematoxylin, X 400).



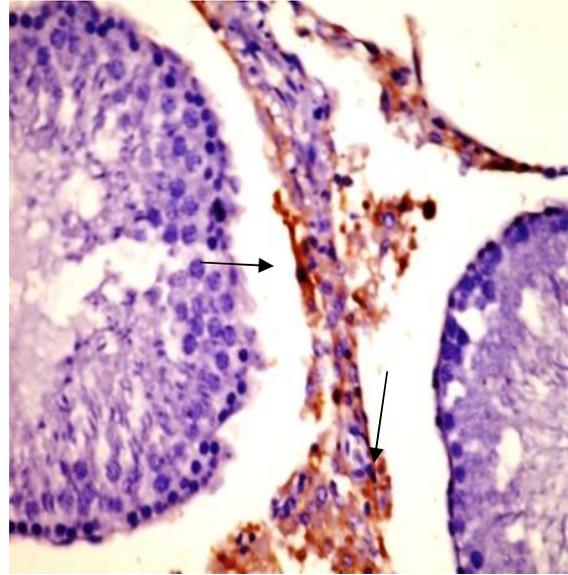
19-A photomicrograph obtained from testis of a rat treated with BPA showing few stained Leydig cells for Bcl-2 (arrow), (Bcl-2 immune stain, counter stained with hematoxylin, X 400).



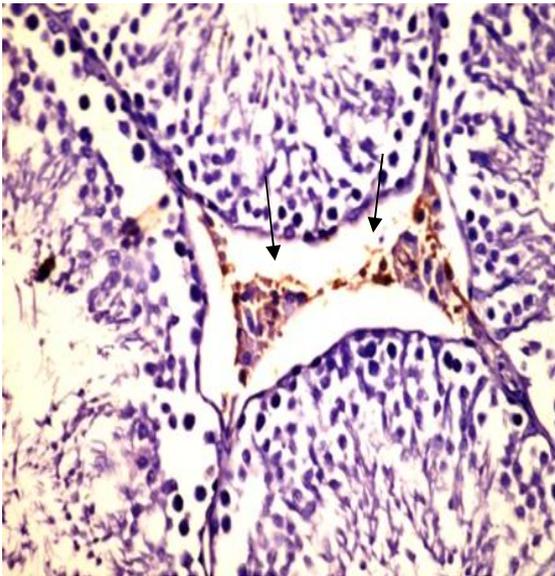
20-A photomicrograph obtained from testis of a rat treated with TT showing nearly normal expression in leydig cells for Bcl-2 (arrow), (Bcl-2 immune stain, counter stained with hematoxylin, X 400).



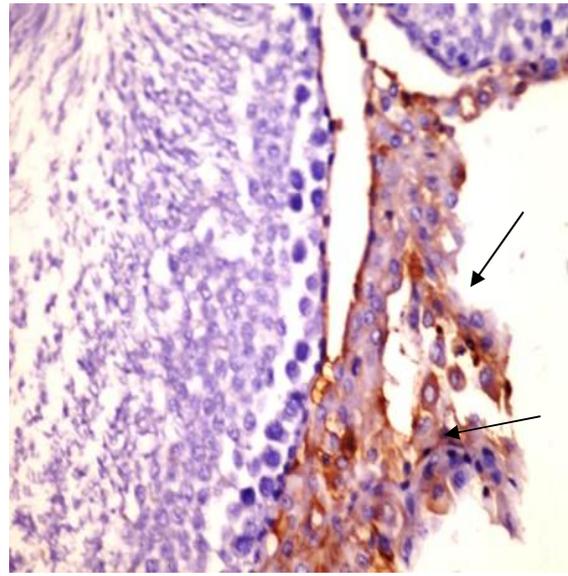
21-A photomicrograph obtained from testis of SIF+BPA treated rat showing weak expression of Bcl-2 in Leydig cells (arrow), (Bcl-2 immune stain, counter stained with hematoxylin, X 400



22-A photomicrograph obtained from testis of SIF+TT treated rat showing moderate expression of Bcl-2 in Leydig cells (arrows), (Bcl-2 immune stain, counter stained with hematoxylin, X 400



23-A photomicrograph obtained from testis of BPA+TT treated rat showing moderate expression of Bcl-2 in Leydig cells (arrows), (Bcl-2 immune stain, counter stained with hematoxylin, X 400



24-A photomicrograph obtained from testis of SIF+BPA+TT treated rat showing high expression of Bcl-2 in Leydig cells (arrows), (Bcl-2 immune stain, counter stained with hematoxylin, X 400

Discussion

The results of the present study showed that apart from the treated groups BPA and combination of BPA+ SIF + TT have a significant decrease in body weight when compared to control. In addition to the relative weights of testes and seminal vesicles were significantly decreased in BPA and/or SIF-treated groups. These alterations in body and organs weight are classical indices for development of toxicity, so reduction in body weight or organ weight are potential sign of toxicity of BPA (Yuan *et al.*, 2012; Zhang and Sen, 2009).

These results run parallel with the histopathological results. The possible cause of the decrease in testis weight may be due to the depletion of spermatogenic elements and spermatozoa (Mishra *et al.*, 2009). TT administration with either BPA or SIF produced significant increase in body weight and testis weight compare to BPA or SIF alone, this data reflect the effect of TT in gaining weights, this can be attributed to the fact that oral administration of this extract to laboratory animals resulted in the stimulation of spermatogenesis and the proliferation of the spermatogonia, which involved cell divisions of the spermatocytes and spermatids (Elahi *et al.*, 2013).

It is well known that estradiol E and testosterone T are the major hormones in male animals, which play important roles in male reproduction. In male animals E and T, are produced by testis. In the present study, the effect of BPA, SIF on reproductive hormones could cause varying degrees of pathological alterations. BPA, SIF and SIF + TT cause significantly increase in concentration of serum estradiol hormone.

In SIF treated group serum estradiol was elevated this is due to the estrogen contents composed of endogenous estrogen plus the weak phytoestrogen from soybean. Also soybean increase SHBP to bind excess of estrogen present (Serag El Din *et al.*, 2011). Also SIF treated group caused significant decrease in serum testosterone level may be due to the ability of soyisoflavone to inhibit the enzymes involved in steroid hormone synthesis. In fact various types of cell culture studies have demonstrated the ability of isoflavones to inhibit 3 β -HSD an enzyme involved in the conversions of pregnenolon to progesterone, DHEA to androstenedione and 5-androstenediol to testosterone (Whitehead and Lacey, 2000; Ohno *et al.*, 2002; Ohno *et al.*, 2003 and Ohno *et al.*, 2004).

Also inhibit the enzyme 17 β -HSD involved in the conversion of DHEA to 5-androstenediol and androstenedione to testosterone (Kayisli *et al.*, 2002). It is generally believed that soyisoflavone affect the estradiol feedback regulation in the hypothalamus–pituitary axis, and alter endogenous testosterone level (Yuan *et al.*, 2012). BPA binds to estrogen receptor (ERs) in the pituitary gland, resulting in direct suppression of FSH secretion (Hanaoka *et al.*, 2002) which ERs in the pituitary gland and that estradiol directly inhibits gonadotropin secretion at the pituitary level in men. BPA is considered by some researchers to be one of the most potent reproductive toxicants (Maffini *et al.*, 2006).

Akingbemi *et al.*, (2004) described an inhibitory effect of BPA on testicular steroidogenesis at low exposure levels in pubertal rats, which they ascribed to an ER-mediated effect. BPA might also act as an androgen receptor antagonist, preventing endogenous androgens from regulating androgen-dependent transcription. The disruption of the androgen receptor–androgen interaction has been speculated to be significant in eliciting adverse effects on the male reproductive system, including sexual dysfunctions (Li *et al.*, 2009). Alternatively, there could be differential effects of BPA on the metabolism of testosterone and estrogen.

BPA also significantly decreased the activity of enzymes involved in the hydroxylation of testosterone, including the cytochrome P450 isoforms for testosterone 2 α -hydroxylase and testosterone 6 β -hydroxylase, CYP2C11/6 and CYP3A2/1, respectively, in isolated rat livers both of which could lead to a net increase in circulating testosterone (Heringa *et al.*, ; 2004 Zhou *et al.*, 2008). Our results are in agreement with a study released by (Dobrzynska and Radzikowska, 2013) which demonstrated that BPA, in weak concentrations, is sufficient to produce a negative reaction on the human testicle. To evaluate the effect of BPA and SIF on testes biochemical metabolism, levels of ACP, ALP, AST and ALT were detected in testis of adult male rats. Data in our study indicated that BPA- and/or SIF- treated groups caused significant increase in AST and ALT in testicular tissue. AST and ALT are important aminotransferase in testis tissue which are widely distributed in mitochondria, and are associated with reduction in the integrity of spermatozoa membrane and the frequency of intact acrosome spermatozoa.

The activities of AST and ALT increased when the membrane of spermatozoa decreased (Yang *et al.*, 2010) The present study showed that the activities of AST and ALT were obviously decreased insignificantly in TT, TT+BPA and TT+SIF groups respectively, compare to individual BPA or SIF treated group, which returned to the normal values. The present results indicated that TT is important factor that improves the ability of anti-stress in testis cells including spermatogenic cells, and protected spermatozoa.

In testes, ALP is associated with the division of spermatogenic cells and the transportation of glucose to spermatogenic cells (Yan *et al.*, 2010). ACP marker of testicular toxicity and a reduction in its activity reflects decreased testicular steroidogenesis and/or reduced secretion of gonadotropin (El-koshary, 2009). ACP located in lysosome of leydig cells and involved in the protein synthesis by abduction of sex hormones (Zhang

and Sen, 2009 ; Samarth and Samarth, 2009). Yang *et al.*,(2010) revealed that, ACP is one of the markers of dyszoospermia that associated with the denaturation of seminiferous epithelium and phagocytosis of sertoli cells.

The present study found that the decreased activities of ACP and ALP were notable manifestations of BPA or SIF induced testicular damage that was ameliorated by treatment with TT. The results indicated that TT may be important in spermatogenesis by improving the lipid and energy metabolism by increasing the spermatogenic cells division in testis(Yang *et al.*, 2010). Changes in the activity of ALP and ACP may be used as an indicator of spermatogenesis function. When significantly decreased ALP, ACP were reflect testicular degeneration, and consequently suppressed testosterone secretion.

In the present work, histological, immunohistochemical and morphometrical tools were performed to assess in evaluating the effect of either PBA or SIF on testis tissues and the role of TT in ameliorating these deleterious impact. Histological examination of the testicular tissues of SIF-group, revealed mild toxic impact where spermatogenic arrest, spermatogenic lineage detached from their basement membrane. Capsular thickening together with edema were observed. This is came along with Ekaluo *et al.*, (2013), they reported that soybean in different doses caused significant dose- dependent toxicity effects ranging from mild degeneration of sperms in testicular tubules to excessive necrosis and hemorrhage. Ekaluo *et al.*, (2013) revealed that Soya bean may have disrupted the synergy between testosterone and follicle stimulating hormone during the process of spermatogenesis (Gelain *et al.*, 2005)and (Ikpeme *et al.*, 2010).

Concerning morphometrical analysis reveal significant decrease in leydig cell count, reduction in seminiferous tubules height and diameter, the fact that, the diameter and height of seminiferous tubules reflect quantity of germ cells may help in confirmed the toxic impact of SIF on spermatogenesis.

In the present work, decrease in seminiferous tubules height and diameter were in consonance with the finding of (Akinola *et al.*, 2007; Assinder *et al.*, 2007), and these finding are suggestive of a reduction in the quantity of germ cells that constitute the wall of seminiferous tubules .Data In the current work, revealed significant reduction in Leydig cells number, associated with reduction in testosterone level , these findings were accordance with (Bennetau-Pelissero *et al.*, 2001).

In BPA- treated rat, testis revealed spermatogenic arrest in many seminiferous tubules together with degenerative changes in spermatogenic lineage especially spermatides, these observation were confirmed by (Karumari and Balasubramanian, 2014)study where, testis revealed cellular changes like edema between the seminiferous tubules., wide empty lumen and scanty cellular components.

Parameter such as tubular diameter, Seminiferous epithelium height can also give information about testicular damage degree(Vendramini *et al.*, 2010), therefore the morphometrical data include seminiferous diameter and height and leydig cells count was recorded, and display highly significant reduction in all these items. Similar results were detected by other investigators with different BPA doses and routes in rats and mice, the possible explanation in that BPA decrease the level of circulating testosterone, resulting in atrophy of seminiferous tubules (means reduction in diameter), degeneration of germ cells and complete absence of spermatogenesis (Norazit *et al.*,2012).Also previous studies reported that administration of BPA, significantly decreased the weights of testis and epididymis , which may be due to the inhibition of spermatogenesis, decreased elongated spermatids and steroidogenic enzyme activity (Takahashi and Oishi, 2003) also it may cause severe parenchymal atrophy in the seminiferous tubules cause reduction in testis and epididymis weights. BPA affects testicular functions in terms of leydig cells and sertoli cell functions (Tohei *et al.*, 2001)In the current study, co-administration of TT to BPA or SIF, produce protective effect against SIF or BPA induce toxicity.

This protective effects, was seen in either testis histological profile or in the normalized figures of seminiferous height ,diameter and leydig cells count, this is may attributes to the effect of TT on testis tissues, where it can stimulate spermatogenesis and increase the activity of sertoli cells (Elahi *et al.*, 2013).Wealth of evidence suggested the ameliorating role of TT, where it claimed to increase the body weight natural testosterone levels, stimulate spermatogenesis and increase the activity of sertoli cells (Gauthaman *et al.*, 2002 ; Sangeeta *et al.*, 1994 and Jagadeesan *et al.*, 2005).

The present histological and immunohistocheal data, on either TT, or co-administration with SIF or BPA revealed significant ameliorated effects concerning Bcl-2 expression or leydig cell count and seminiferous tubular diameter or height.

In previous study by (Zhang *et al.*, 2013), they stated that expression of BCL-2 in control mice group , located in spermatogenous cells , while in BPA group it was mainly expressed in leydig cells and sertoli, and this expression was decreased in BPA and SIF groups, also BPA can decrease the quality of leydig cells (Carlsen *et al.*, 1992; Chitra *et al.*, 2003; Wu and Zhang, 2005). Bcl-2 in BPA treated group showed weak expression, this is came along with (Wang *et al.*, 2010).

It could be concluded that both BPA and SIF induced disruption in testicular enzymes, tissues and hormonal levels which can effect on fertility of male rats. The administration of TT ameliorating these deleterious impacts.

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