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RESEARCH ARTICLE

Sodium Thiosulfate (Hydrogen Sulfide Donor): Ameliorates the Pituitary-testicular Axis Dysfunction Induced by Cyclophosphamide and/or Ionizing Gamma Radiation in Albino Rats

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ABSTRACT

Background: Hypothalamic-pituitary-testicular axis represents a complex biological structure organ regulating reproductive function. The present study aimed to investigate the potential protective effect of sodium thiosulfate (STS) as a hydrogen sulfide donor on the pathophysiological effects of cyclophosphamide (CYP) and/or ionizing gamma radiation (IR) on pituitary-gonadal axis through the improvement of pathological, biochemical, and hormonal alteration in male rats. **Materials and Methods:** Rats of experimental groups were received STS (400 mg/kg b.w. for 14 days, i.p.) before treatment with CYP and/or IR, except normal control rats received saline only. Then, all groups received STS 3 times per week for 3 successive weeks during CYP and/or IR. The other groups were then treated with fractionated doses of CYP (50 mg/kg/week by i.p. for 3 weeks) and/or IR (3 Gy/week for 3 weeks, up to a total cumulative dose of 9 Gy). **Results:** STS had significantly ameliorated the CPA and/or IR-induced oxidative stress in rats by reducing the malondialdehyde level and elevating the superoxide dismutase, catalase, glutathione (GSH) peroxidase, and reduced GSH activities in both pituitary gland and testicular tissues. In addition, STS elevated the reduced luteinizing hormone, follicle-stimulating hormone, and the male sex hormone, testosterone. Furthermore, STS also reduced pathological alterations changes in the pituitary and testicular glands, as well as decrease caspase-3 and tumor necrosis factor-α ($P < 0.001$). **Conclusions:** This study shows that STS has protective effects, as evidenced by reduced oxidative stress and apoptosis, as well as improved histopathological changes in both pituitary gland and testicular tissues.

Keywords: Sodium thiosulfate, Ionizing gamma radiation, Cyclophosphamide, Oxidative stress, Pituitary, Testis, Pathophysiology and immunohistochemistry

INTRODUCTION

Management of many cancers has been achieved by aggressive chemotherapy and radiotherapy.[1] The use of anticancer drugs affects reproductive function, including fertility, in both human and experimental animals.[2]

The hypothalamic-pituitary-testicular axis represents a complex neuroendocrine feedback loop regulating reproductive function. Gonadotropin-releasing hormone (GnRH), a hormone produced by specific neurons in the hypothalamus, forms the final common pathway regulating reproductive function.[3] GnRH induces the conflation and stashing of the luteinizing hormone (LH) and the follicle-stimulating hormone (FSH). FSH is responsible for the activation of the seminiferous tubules and production of sperm. Testicular function

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is controlled by the central nervous system through FSH and LH. Testosterone is positively linked with sexual intimacy in men and plays a significant role in spermatogenesis in adult males.[4] The male reproductive system is highly susceptible to chemotherapy and radiotherapy.[5]

Chemotherapy is currently one of the most important tools in the fight against cancer; many of its side effects are well known. However, similar as cognitive impairments are still being studied.[6] Chemotherapeutic drugs for cancer treatment reveal that no drug has been advocated and as widely used as cyclophosphamide (CYP) for the past 60 years.[7] CYP is a bifunctional alkylating agent and has been reported to give have a better therapeutic index than other alkylating agents in animals.[8]

CYP causes low brain weight, severe hemorrhagic lesions, and scant parenchymal cells in the pituitary gland. Additionally, that was linked to a potential reduction in the overall number of cells that constitute of pituitary gland which causes decrease the levels of LH and FSH.[9]

CYP causes reproductive toxicity, including azoospermia, oligospermia, histological changes in the epididymis and testis, decreased weight of reproductive organs, and impaired fertility and growth in humans and experimental animals. Because of the high frequency of cellular division in the cells of the seminiferous epithelium, the testis is extremely sensitive to chemotherapeutic drugs.[10]

Radiotherapy (ionizing gamma radiation [IR]) is used to cure cancers. The effects of ionizing radiation at the organism level depend on the response of the cells. The effects of ionizing radiation on the organism are determined by cell response. When radiation hits a cell, it might damage any cellular organelles and macromolecules. Unrepairable damage leads to cell death, while mis-repaired alterations leave mutations in surviving cells. Mis-repaired alterations cause mutations in surviving cells, while unrepairable damage leads to cell death.[11]

IR often causes delayed and progressive cognitive impairments, such as deficits in memory, attention, and executive function.[12] Radiation convinced endocrinopathies, influenced mental and physical development as well as lipid peroxidation that damaged the hypothalamus and pituitary gland which leads to hypopituitarism.[13]

Testicular tissue, a radiosensitive organ, has a variety of cells that differ in their degree of radiosensitivity. The spermatogonia are very radiosensitive and kill at doses <3 Gy in the differentiation period. The infertility following irradiation is caused due to the apoptosis of spermatogonia rather of getting genetic differences.[14]

Sodium thiosulfate (STS) is a major oxidation product of hydrogen sulfide (H_2S), an endogenous signaling molecule and the third member of the gas transmitter family. STS is currently used in the clinical treatment of CYP and ionizing radiation toxicities in cancer therapy. Expanding evidence shows that STS has antioxidant and anti-inflammatory properties, making it a potential therapeutic candidate molecule that can target multiple molecular pathways in various diseases and drug-induced toxicities.[15]

STS can guard endocrine organs and acclimate hormone secretion through an antioxidative effect and ion channel regulation. H_2S performs a function with inside the endocrine system to guard the secretion of pituitary hormones and testis function.[16] Sodium thiosulfate appears to be a promising agent to be used concomitantly with many anticancer agents to reduce their adverse effects through several mechanisms including strong antioxidant ability, anti-inflammatory, and anti-apoptotic effects.[17]

In this study, we aimed to minimize side effects of CYP and/or IR by investigating the protective effect of STS against pathophysiological alterations induced by CYP and/or IR.

MATERIALS AND METHODS

Ethical approval

All procedures described were studied and approved by the research ethics committee for experimental studies (human and animals subjects) at National Center for Radiation Research and Technology (NCRRT) of the Egyptian Atomic Energy Authority (EAEA) (EAEA, Cairo, Egypt) (registration number vet cu 2305 2022451).

Chemicals and reagents

STS (Chem Center 5580 La Jolla Blvd., San Diego, CA), CYP injection (CYP, Endoxan-N 1 g), and all

other chemicals were of analytical grade and were obtained from Sigma Aldrich Chemical Co. (St. Louis, Missouri, United States of America [USA]).

Radiation facility

Whole-body gamma irradiation was carried out using the facilities provided by the NCRRT. Acesium-137 irradiation unit (Gamma cell-40) biological irradiator is manufactured by Canada Ltd., Ottawa, Ontario, Canada. The rate of cesium-137 was 0.41 Gy/min at the time of the experiment.

Experimental animals

Sixty-four male Wister albino rats were purchased from the Holding Company for Biological Products and Vaccines, VACSERA, Cairo, Egypt. The experiment was conducted at the Animal Research Facility, Faculty of Veterinary Medicine, Cairo University. The animals had 7 days of acclimatization period before experimentation, and they were maintained in a 12 h of light-dark cycle with the regulatory temperature at 21–23°C with free access to food and drink.

Experimental design

Rats were randomly allocated into eight groups (eight rats per group) as follows:

- Group 1 (normal control negative): Rats received only saline intraperitoneally
- Group 2 (STS): Rats received STS (400 mg/kg/day) for 14 days intraperitoneally then 3 times per week for 3 successive weeks
- Group3 (CYP): Rats received a single dose of CYP (50 mg/kg) per week for 3 successive weeks, intraperitoneally
- Group 4 (R): Rats exposed to IR (3 Gy/week up to a cumulative dose of 9 Gy after 3 weeks)
- Group 5 (CYP + R): Rats exposed to IR, then CYP was injected (50 mg/kg/week), intraperitoneally, 24 h after exposure to IR
- Group 6 (STS + CYP): Rats treated with STS (400 mg/kg/day) i.p. for 14 days before i.p. doses of CYP then 3 times per week for 3 successive weeks during CYP treatment
- Group 7 (STS + R): Rats received STS (400 mg/kg/day) i.p. for 14 days before IR then 3 times per week for 3 successive weeks during IR treatment
- Group 8 (STS + CYP + R): Rats received STS (400 mg/kg/day) i.p. for 14 days before and 3 times per week during CYP and IR treatment.

Samples' collection

Rats were fasted overnight 48 h after CYP injection and anesthetized with an intraperitoneal injection of sodium thiopental (20 mg/kg). Blood samples were collected in serum vacuum tubes from rats under anesthesia by heart puncture following normal laboratory procedures. The blood was allowed to coagulate and then centrifuged at 4000 rpm for 10 min.[17] A portion of testis was homogenized in PBS buffer (0.1 M, pH 7.4) to prepare 10% (w/v) tissue homogenate for biochemical analysis. In addition, the other portions of the pituitary gland and testis were preserved in 10% neutral buffered formalin for histopathological and immunohistochemical examination.

Assessment of oxidative stress

Malondialdehyde (MDA), a lipid peroxidation marker, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and reduced glutathione (GSH) activities were also assessed in the pituitary gland and testis tissue homogenates of all animal groups using commercial kits (Bio Diagnostic Company, Egypt).[18]

Biochemical analyses of hormones

LH, FSH, and testosterone were quantified in serum according to the manufacturer's instruction by enzyme-linked immunosorbent assay kits (Hitachi company).[19]

Histopathological examination

Tissue samples from the pituitary gland and testis were cut and fixed in 10% neutral-buffered formalin. The tissues were routinely processed before embedding in paraffin wax. The paraffin-

embedded blocks were cut into 5 μm thick sections and stained with hematoxylin and eosin (H&E) for histopathological examination Masson's trichrome in the pituitary gland to differentiate between acidophilic and basophilic cells.[20]

In addition to, testicular injury was also semi-quantitatively assessed in five stained sections by PAS high microscopic fields (40×), using different pathological parameters such as degenerate round spermatids and spermatocytes, according to the method of, O'Donnell *et al*.[21] using a grading system 1 = just detectable above normal levels and 2 = consistently present in a high proportion (>50%) of Stage VII/VIII tubules and tubular degeneration and/or atrophy, according to the method of, Lanning *et al*.[22] using a grading system 1 ≤ 10% of tubules affected in seminiferous tubules, 2 = 11–25% tubules affected in seminiferous tubules, 3 = 25–50% tubules affected in seminiferous tubules, 4 = 51–75% tubules affected in seminiferous tubules, and 5 = 76–100% tubules affected in seminiferous tubules.

Immunohistochemical analysis

Caspase 3 in pituitary gland and testicular tissue, as well as tumor necrosis factor-α (TNF-α) in testis were measured histochemical using the method described in Saleh *et al*.[23] Briefly, the sections were deparaffinized in xylene, rehydrated in descending grades of alcohol, and incubated in 3% hydrogen peroxide (H_2O_2). The sections were then incubated with as primary antibodies. Caspase-3(sc-373730; Santa Cruz Biotechnology, USA), an apoptotic marker, and TNF-α (sc-52746; Santa Cruz Biotechnology, USA), an inflammatory marker. Diaminobenzidine (Sigma, USA) was added to demonstrate the immune response. The immunohistochemical staining for caspase 3 and TNF-α semi-quantitatively assessed in 10 high microscopic power fields (40×) as described by Hegazy.[24] Two main criteria, the color intensity and the percentage (%) of positively stained cells, were basically used for this assessment. For color intensity, a grading system scaled from 0 to 3 was used, in which Grade 0 = no staining, Grade 1 = weak staining, Grade 2 = moderate staining, and Grade 3 = strong staining. Similarly, a grading system scaled from 0 to 3 was used for the assessment of the percentage (%) of positively stained cells in high-power fields, in which Grade 0 denotes 0%, Grade 1 denotes <30%, Grade 2 denotes 30–70%, and Grade 3 denotes >70%. The total immunoreactivity score of each stained section is the sum of these two criteria.

Statistical analyses

The obtained data were statistically analyzed using Statistical Package for the Social Sciences for Windows software (Statistical Package for the Social Sciences [SPSS] version 20.0, SPSS Inc., Chicago, USA). Descriptive statistical values are presented as the mean ± standard error ($n = 8$ per group). The one-way analysis of variance (ANOVA) followed by a *post hoc*, Tukey test was used to assess the differences between groups for parametric tests, while the statistical analysis of histopathological scores and IHC staining scores non-parametric tests was performed by Kruskal–Wallis test followed by Dunn's test. $P \leq 0.05$ was considered a statistically significant.

RESULTS

STS alleviates the pituitary and testicular oxidative stress biomarkers in CYP and/or IR-treated rats

Elevated pituitary and testicular MDA levels have been recorded in the CYP, R, and CYP + R groups compared to the normal controls, as shown in Tables 1 and 2. Furthermore, testicular SOD, CAT, GP_X, and GR levels were significantly reduced ($P < 0.0001$) in CYP, R, and CYP+R groups compared to the normal controls. Pre-treatment with STS significantly reduced pituitary and testicular MDA levels and elevated the levels of SOD, CAT, as well as GP_X and GR activities, compared to the untreated groups. In addition, there was almost non-significant difference ($P > 0.05$) between the STS group and the control group.

STS alleviates serum pituitary and testicular hormonal profiles in CYP and/or R-treated rats

In CYP and/or IR-treated rats (CYP, R, and CYP + R groups), a significant decrease in

Table 1: Ameliorative effect of STS on the pituitary antioxidant biomarker in CYP and/or irradiated rats

	Pituitary gland				
Groups	**MDA (nmol/gm tissue)**	**SOD (U/gm tissue)**	**Catalase (U/gm tissue)**	**GPx (U/gm tissue)**	**GSH (mmol/gm tissue)**
Control	16.00±2.31^{b}	7.84±0.56^{bc}	46.37±2.85^{bc}	103.1±6.86^{bc}	15.36±0.45^{bc}
STS	13.91±1.46^{bc}	8.32±0.43^{bc}	46.05±2.76^{bc}	102.1±4.86^{bc}	15.5±1.13^{bc}
CYP	27.76±1.58^{a}	2.49±0.16^{a}	15.22±1.01^{a}	34.52±3.36^{a}	7.77±0.67^{a}
R	41.19±2.21^{ac}	1.97±0.36^{a}	15.62±0.27^{a}	45.09±4.11^{a}	7.400±0.15^{a}
CYP+R	46.71±1.88^{ac}	1.57±0.2^{a}	12.38±1.25^{a}	25.88±2.96^{a}	6.56±0.22^{a}
STS+CYP	13.29±0.79^{bc}	4.71±0.45^{abc}	35.12±0.74^{abc}	64.36±4.68^{ac}	10.61±0.43^{abc}
STS+R	22.13±1.67^{b}	5.45±0.1^{abc}	23.97±0.92^{abc}	74.09±3.55^{abc}	12.79±0.09^{bc}
STS+CYP+R	29.82±3.6^{abd}	4.52±0.26^{abcd}	34.28±1.5^{abcd}	63.22±4.86^{acd}	10.49±0.32^{abd}

Data were expressed as (Mean±SEM). Values are statistically significant when $P \leq 0.05$. p^{a}: Significant compared to the control group, p^{b}: Significant compared to the radiation group, p^{c}: Significant compared to CYP group, and p^{d}: Significant compared to CYP+R group (one-way ANOVA, *post hoc* test, Tukey test), STS: Sodium thiosulfate, CYP: Cyclophosphamide, MDA: Malondialdehyde, SOD: Superoxide dismutase, GSH: Glutathione

Table 2: Testicular oxidative stress biomarkers in CYP and/or R-treated rats

	Testes				
Groups	**MDA (nmol/gm tissue)**	**SOD (U/gm tissue)**	**Catalase (U/gm tissue)**	**GPx (U/gm tissue)**	**GSH (mmol/gm tissue)**
Control	44.44±4.57^{bc}	31.07±0.59^{bc}	132.3±5.93^{bc}	56.59±4.18^{bc}	19±0.6^{bc}
STS	45.94±1.07^{bc}	31.68±0.43^{bc}	153.8±7.25^{bc}	59.78±2.89^{bc}	19.98±1.08^{bc}
CYP	109.3±5.09^{a}	15.16±1.12^{a}	51.27±5.27^{a}	18.96±1.43^{a}	11.69±0.31^{a}
R	92.17±5.59^{a}	17.87±1.13^{a}	44.50±1.75^{a}	27.06±3.73^{a}	13.76±0.65^{a}
CYP+R	154.5±8.76^{abc}	10.78±0.59^{ab}	21.93±2.64^{a}	12.98±1.67^{a}	10.45±0.17^{ab}
STS+CYP	57.26±1.72^{bc}	29.66±1.29^{bc}	91.63±8.78^{abc}	36.70±4.19^{ac}	13.45±0.43^{a}
STS+R	55.56±156^{bc}	31.48±0.46^{bc}	81.46±9.46^{abc}	45.21±5.53^{bc}	18.86±0.31^{bc}
STS+CYP+R	64.42±3.99^{abcd}	32.43±0.31^{bcd}	61.32±2.81^{ad}	23.72±2.24^{a}	14.28±1.11^{ad}

Data were expressed as Mean±SEM. Values are statistically significant when $P \leq 0.05$. p^{a}: Significant compared to the control group, p^{b}: Significant compared to the radiation group, p^{c}: Significant compared to CYP group, and p^{d}: Significant compared to CYP+R group (one-way ANOVA, *Post hoc* test, Tukey test). STS: Sodium thiosulfate, CYP: Cyclophosphamide, MDA: Malondialdehyde, SOD: Superoxide dismutase, GSH: Glutathione

testosterone, FSH, and LH hormone levels was found ($P < 0.001$) compared to the control group. After STS treatment (STS + CYP, STS + R, and STS + CYP + R), there was a significant increase in the levels of testosterone, FSH, and LH hormone ($P < 0.001$) when compared to the untreated groups [Figure 1].

STS restores pituitary and testicular histopathological alterations incorporated with CYP and/or IR

Pituitary gland alterations

The micromorphology of the anterior pituitary in the control and STS-treated groups showed primarily three types of cells: Acidophils, basophils, and chromophobes. The acidophils were identified using eosin stain, while the basophils were identified using hematoxylin and chromophobes with very little staining.

The outline of the cells in the pituitary of control and STS-treated rats showed a normal outline of the cells [Figure 2a and b], as well as an abundance of basophiles as indicated by the Mason trichrome stain [Figure 3a and b].

However, in the CYP-treated group, some cells appeared shrunken, in addition to the pyknotic nuclei seen in basophiles and acidophils [Figure 2c]. Pituitary glands of the R group showed basophile and acidophil degeneration and apoptosis, as well as severe congestion of hypophyseal sinusoids [Figure 2d] and a reduction in basophilic cell numbers stained blue by MTC [Figure 3c and d].

However, in the CYP-treated group, some cells appeared shrunken, in addition to the pyknotic nuclei seen in basophiles and acidophils [Figure 2c]. Pituitary gland of R group showed basophile and acidophil degeneration and apoptosis, as well as severe congestion of hypophyseal sinusoids

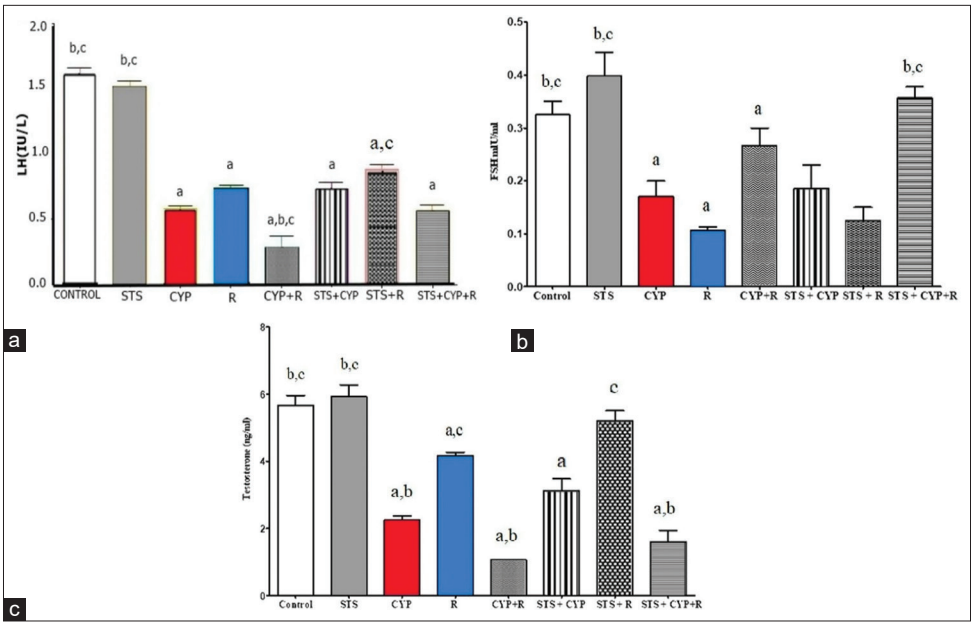

Figure 1: Effect of STS on the level of (a) LH, (b) FSH, and (c) testosterone in the serum of male albino rats in all experimental groups. Data were expressed as Mean ± SEM. P[a]: Significant compared to control group, P[b]: Significant compared to R group, P[c]: Significant compared to CYP, and P[d]: Significant compared to CYP + R group (one-way ANOVA, *post hoc* test, Tukey test). FSH: Follicular-stimulating hormone, LH: Luteinizing hormone

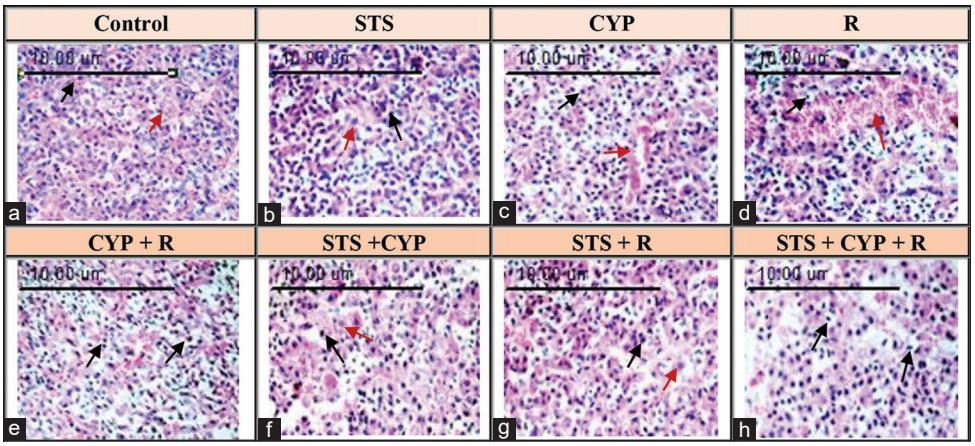

Figure 2: Photomicrographs of the pituitary gland showing: (a and b) Normal anterior pituitary, three types of cells acidophils, basophils, and chromophobes (arrows), (c) shrunken cells and apoptotic nucleus (arrows), (d) severe congestion and apoptosis (arrows), (e) irregular orientation of adenohypophysis cells and extensive apoptosis in basophiles and acidophils arrows, (f and g) restoration of basophilic and acidophilic cells arrow, and (h) degeneration and apoptosis of basophilic and acidophilic cells in a small number of anterior pituitary arrows (stain: H&E; scale = 10 μ)

[Figure 2d] and a reduction in basophilic cell numbers stained blue by MTC [Figure 3c and d].

In contrast, more pronounced alterations were displayed in the pituitary gland of the CYP and R-treatedgroups.Thesealterationswerecharacterized by irregular orientation of adenohypophysis cells and extensive apoptosis of basophiles, acidophils, and decline of chromophores [Figure 2e]; absence of blue stained cells by Mason's trichrome stained section [Figure 3e].

STS pre-treatment in the CYP and R groups resulted in significant improvement of histological lesions and an apparent regular arrangement of its cells [Figure 2f and g]. Mild alleviation was recorded in the STS + CYP + R group, which showed few numbers of apoptotic bodies [Figure 2h] as well as the presence of a large number of blue stained cells in both STS + CYP and STS + R groups by MTC [Figure 3f, g, and h].

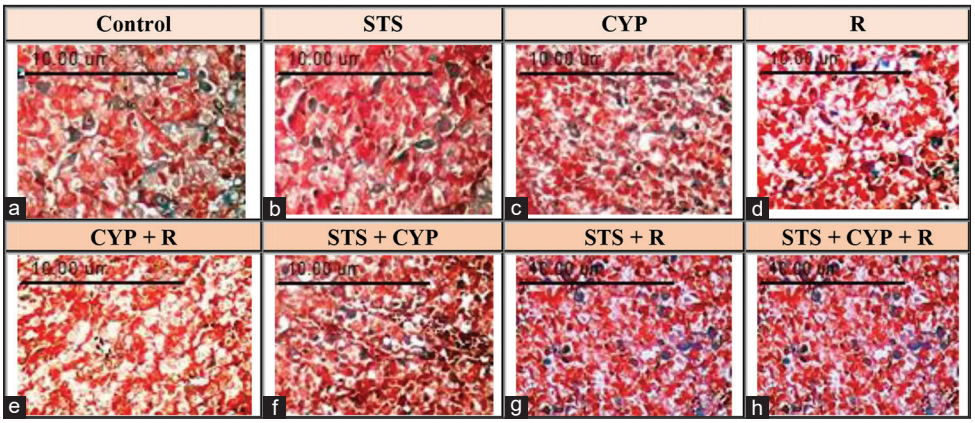

Figure 3: Photomicrographs of the pituitary gland showing: (a and b) Normally arranged anterior pituitary cells with a large number of blue stained cells, (c and d) few numbers of basophilic cells, (e) absence of basophilic cells, and (f-h) restoration of blue stained cells (basophilic cells) (MTC stain; scale = 10 μ)

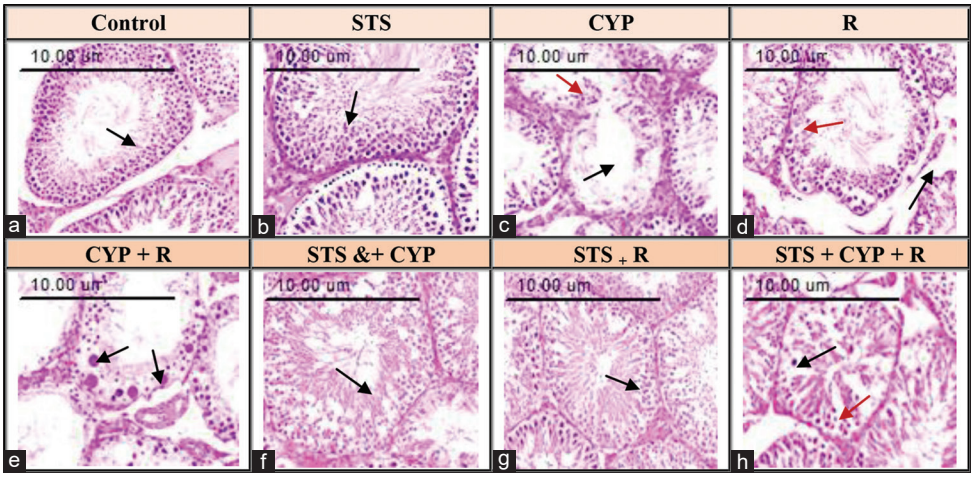

Figure 4: Photomicrographs of testis showing: (a and b) Normally arranged seminiferous tubules with a synchronized population of maturing germ cells arrow, (c) apoptosis of germ cells and extensive vacuolation of Sertoli cells arrow, (d) atrophy of seminiferous tubules with reduction of germ cells arrow, (e) massive deterioration of seminiferous tubules that are completely devoid of germ cells with numerous multinucleated round spermatids arrow, (f) restoration of seminiferous tubules, (g) a few number of degenerated spermatogenic cell layer arrow, and (h) degeneration and apoptosis of germ cells in few seminiferous tubules, arrow (stain: H&E; scale = 10 μ)

Testicular tissue alterations

Testes of normal control and STS-treated rats showed normal histological structure, with compactly arranged seminiferous tubules. The seminiferous tubules revealed a synchronized population of maturing germ cells [Figure 4a and b]; on the contrary, severe histopathological alterations were demonstrated in the CYP and R groups. Diffuse reduction of germ cell numbers with sparse remaining apoptotic germ cells and extensive vacuolation of Sertoli cells in addition to proliferation of interstitial Leydig cells were demonstrated in the testes of the CYP group [Figure 4c]. Massive degeneration and/or apoptosis of germ cells with intensely eosinophilic spermatogonia and spermatids were noticed. In addition, shrinkage of Leydig cells with a decrease in their number and size was demonstrated in the testes of the R group [Figure 4d].

More deleterious alterations were demonstrated in the CYP + R group, including distortion of seminiferous tubules, which are completely devoid of germ cells, with numerous numbers of multinucleated round spermatid giant cells and Sertoli cells [Figure 4e].

Pre-treatment with STS revealed significant amelioration of the previously mentioned pathological lesions, particularly in the STS + CYP and STS + R groups. The number of degenerated seminiferous tubules was significantly reduced in the STS + CYP group, which revealed restoration of most of seminiferous tubules with

individual degeneration and/or apoptosis of germ cells [Figure 4f]. Similarly, few degenerated seminiferous tubules with degeneration and/or apoptosis of their germ cells were demonstrated in the STS + R group [Figure 4g]. On the other hand, little amelioration was recorded in the STS + CYP + R group, which revealed degeneration and/or apoptosis of germ cells in most seminiferous tubules [Figure 4h]. Pathological lesions score of testicular injury was shown in illustration [Figure 5].

STS attenuates caspase-3 and TNF-α expressions

The total immune reactivity score (TIS) of caspase-3 recorded in pituitary gland and testes of different animal groups. In addition, TNF-α in testicular tissue is illustrated.

Caspase-3 expression

Absence of caspase-3 expression was demonstrated in the pituitary gland of normal control and STS group [Figure 6a]. Increased caspase-3 expression level was recorded in basophilic and acidophilic cells of CYP group [Figure 6c]. Strong positive stained cells were recorded in the R group, in pituitary gland [Figure 6d].

A significant increase of caspase-3 positively stained cells was recorded in the CYP + R group, in pituitary tissue [Figure 6e]. Conversely, pre-treatment with STS revealed a significant reduction of caspase-3 expression with a significant decrease of (TIS), particularly in STS + CYP and STS + R in affected pituitary glands [Figure 6f and g]. In addition, decreased caspase-3 positively stained cells were

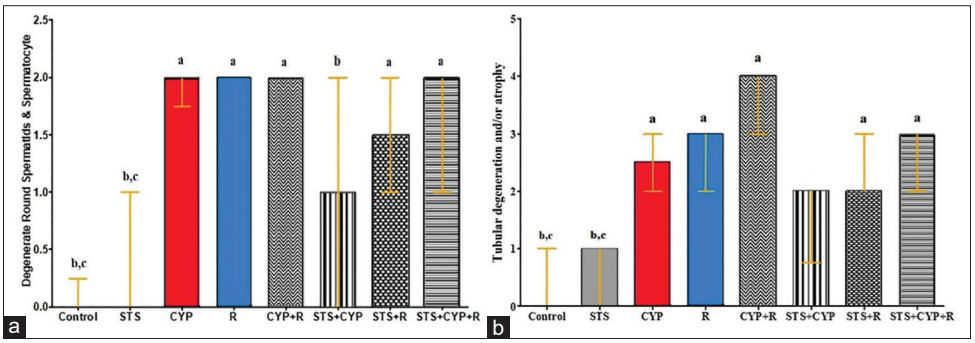

Figure 5: The pathologic lesion score of testicular injury. Effect of STS on testicular score in CYP and/or IR-induced testicular toxicity; (a) degenerated round spermatid and spermatocyte. (b) Tubular degeneration and/or atrophy. All results were presented as median with interquartile range (IQ; $n = 8$), by Kruskal–Wallis test. p^a: Significant compared to control group, p^b: Significant compared to R group, P^c: Significant compared to CYP, and P^d: Significant compared to CYP + R

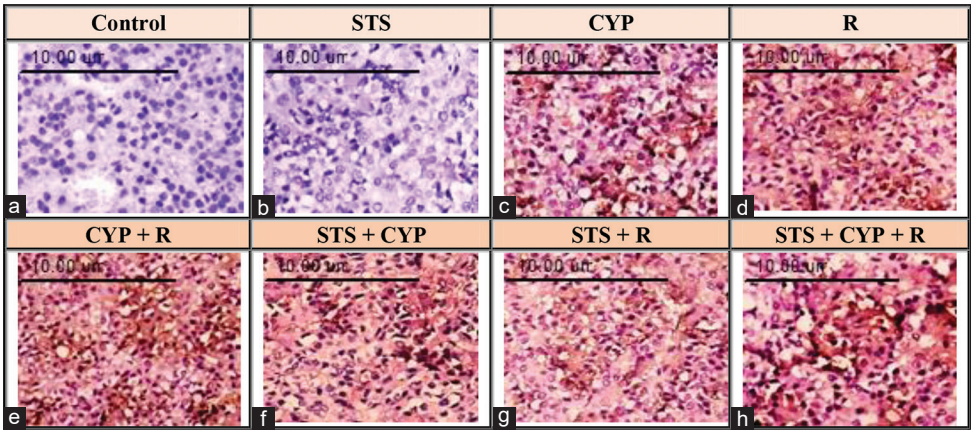

Figure 6: Immunohistochemical analysis of caspase-3 expression in pituitary gland showing: (a and b) Absence of caspase-3 expression in adenohypophysial tissue, (c) strong brown staining of the basophilic and acidophilic cells, (d) positive caspase-3 expression in some cells, (e) positively stained cells with strong brown cytoplasm and/or nuclear staining in all cells, (f and g) few positively stained cells, and (h) scattered basophilic and acidophilic cells with brown nuclear staining (scale = 10 μ)

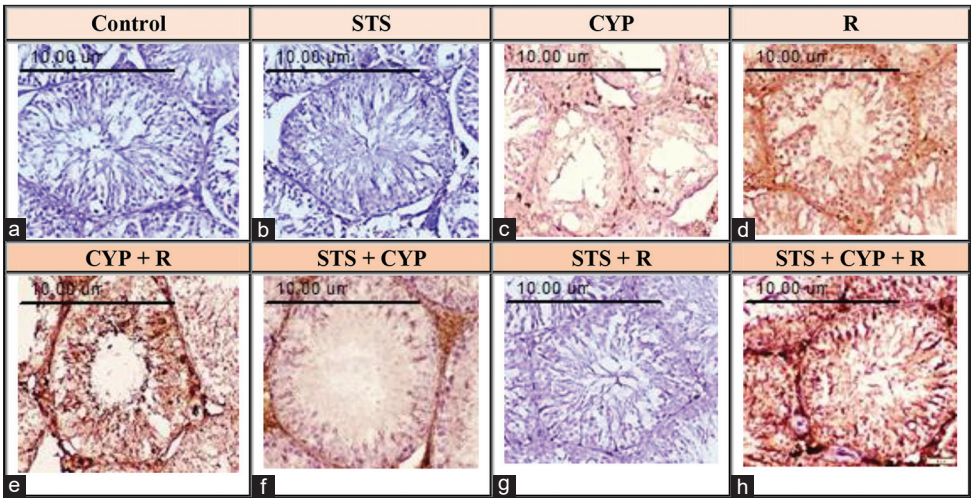

Figure 7: Immunohistochemical analysis of caspase-3 expression in testicular tissue showing: (a and b) Absence of caspase-3 expression in testes, (c) strong caspase-3 brown staining of the germ cells, (d) positive caspase-3 expression demonstrated in all seminiferous tubules, (e) positively stained cells with strong brown cytoplasm and/or nuclear staining in all germ cells of seminiferous tubules, (f and g) few positively stained cells in the seminiferous tubules, and (h) sparse spermatogonia and interstitial cells with brown nuclear staining (scale = 10 μ)

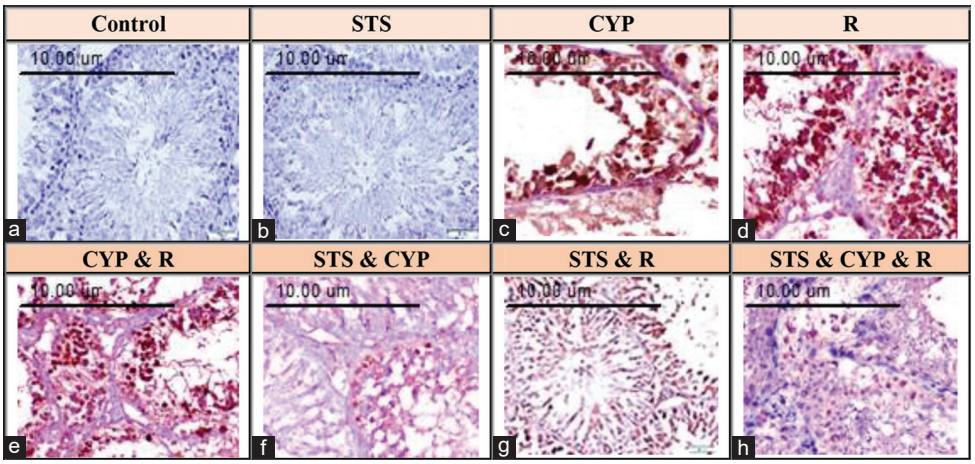

Figure 8: Immunohistochemical analysis of TNF-α expression in testicular tissue showing: (a and b) No expression of TNF-α in testes, (c) brown stained cells in the testicular germ cells and interstitial cells, (d) strong brown stained cells demonstrated in all seminiferous tubules, (e) positively stained cells with strong brown cytoplasm and/or nuclear staining in all germ cells and the interstitial cells, (f) weak cytoplasmic staining in the seminiferous tubules, (g) few positively stained cells in the seminiferous tubules, and (h) abundant positively cells in testes (scale = 10 μ)

demonstrated in the pituitary of the STS + CYP + R group [Figure 6h].

None significant changes of caspase-3 expression level were demonstrated in the testes of normal controls and the STS group [Figure 7a and b]. In contrast, increased caspase-3 expression, with a significant increase in TIS, was recorded in the testes of the CYP group [Figure 7c]. Strong positive stained cells were observed in the R group [Figure 7d].

A significant increase of TIS, along with a significant increase in caspase-3 positively stained cells, was recorded in the CYP+R group of testicular tissue [Figure 7e].

Conversely, pre-treatment with STS revealed a significant reduction of caspase-3 expression with a significant decrease of TIS particularly in the STS + CYP and STS + R groups in affected testicular tissue [Figure 7f and g]. In addition, decreased percentages of caspase-3 positively stained cells with weak cytoplasmic and/or nuclear staining were demonstrated in pituitary and testis of the STS + CYP + R group [Figure 7h].

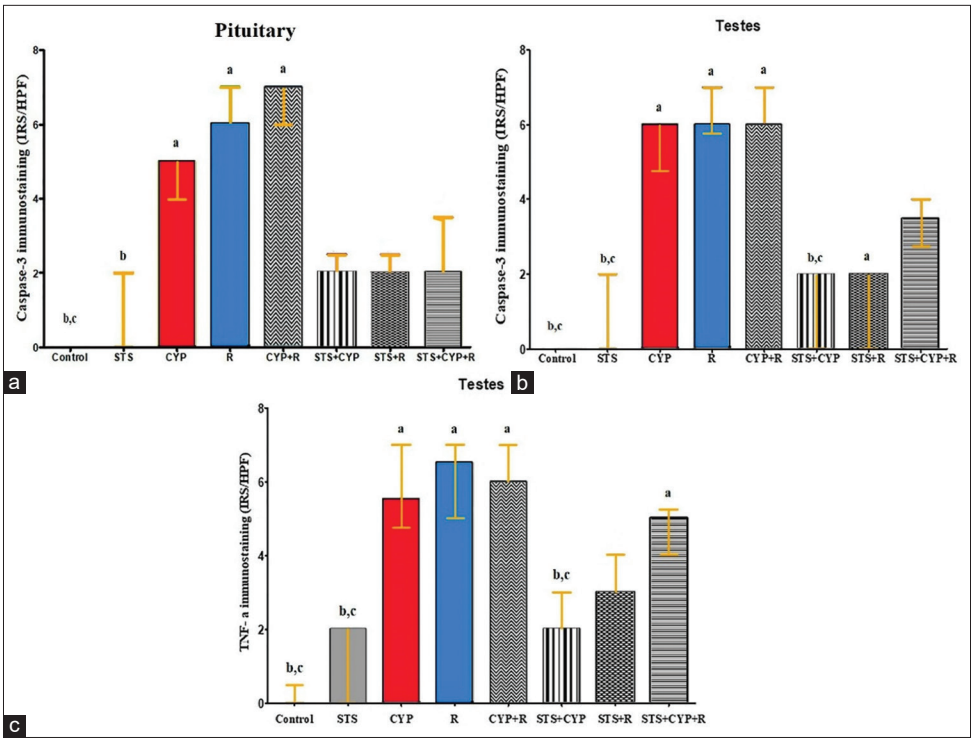

Figure 9: (a) Pituitary glandcaspase-3 immunostaining, (b) testis caspase-3 immunostaining, and (c) testis TNF-α immunostaining. All results were presented as median with interquartile range (IQ; *n* = 8), by Kruskal–Wallis test. p^a: Significant compared to control group, p^b: Significant compared to R group, P^c: Significant compared to CYP, and P^d: Significant compared to CYP + R

TNF-α expression

TNF-α expression was not detected in normal or STS-treated testicular tissue [Figure 8a and b]. Meanwhile, in the testes of CYP groups, there was a remarkable increase in TNF-α expression, as well as a significant increase in TIS [Figure 8c]. Furthermore, numerous strong browns, positively stained cells were frequently demonstrated in seminiferous tubules of the R group [Figure 8d].

In the CYP + R group, germ cells and interstitial cells showed a more noticeable increase in TNF-α positive stained cells with strong brown staining [Figure 8e]. Pre-treatment with STS significantly reduced the elevated TNF-α expression in all pretreated groups [Figure 8f and g], but there was a little improvement in the STS + CYP + R group, which had a high number of positively stained cells [Figure 8h]. STS attenuates caspase-3 and TNF-α expressions, total immune reactivity score (TIS) of caspase-3 recorded in pituitary gland and testis of different animal groups. In addition, TNF-α in testicular tissue is illustrated in Figure 9.

DISCUSSION

The present study evaluates the potential protective effectofSTSagainstpathophysiologicaldisturbances, oxidative stress, and immunohistochemical changes caused by CYP and/or IR inside the pituitary gland and testes of rats' model. CYP is a cytotoxic chemotherapeutic alkylating agent used to deal with several types of tumors and organ transplant rejection in addition to autoimmune diseases.[9] IR is a massive environmental risk factor that causes oxidative damage, organ dysfunction, and metabolic disturbances in biological systems through way of means of generating diverse kinds of reactive oxygen species (ROS).[11]

In the present study, injection of CYP and/or exposure to IR induced antioxidant disturbances in the pituitary and testis of rats. The disturbance was demonstrated by considerable depression in the SOD, CAT, and GPx activities in the pituitary and testis as well as significant reduction in GSH and increase in MDA level. The histopathological examination revealed sever degeneration and apoptosis of pituitary gland[13] and severe arrest of

spermatic tissue and necrosis of cells involved in spermatogenesis and testosterone production.[25] CYP and/or IR induce abroad spectrum of toxicities and tissue dysfunctions.[13,25]

The most recent research demonstrates that CYP and IR cause testicular toxicity as shown by an increase in MDA levels in these tissues as well as a depletion of SOD, CAT, and GSH-Px activities. These results demonstrated that the enzymatic antioxidant molecules were insufficient to neutralize the free radicals produced by CYP and/or IR.[26] MDA is a cellular polyunsaturated fatty acid marker of oxidative stress that is produced.[27] SOD is the main protective antioxidant enzyme that converts superoxide radicals (O_{-2}) and singlet oxygen ($^1O_2$) to H_2O_2.[28] As a result, lipid peroxidation is suppressed by H_2O_2 being removed via CAT.[29] H_2O_2 and lipid peroxides are reduced by GSH-Px and GSH.[30] The test is' antioxidant defense mechanisms are inhibited by the reduction of those antioxidant enzymes when combined with the unchecked buildup of H_2O_2.[30]

Pre-treatment with STS improved the antioxidant protection machine in testicular tissue. STS will elevate the antioxidant activity through a lot of mechanisms, consisting of suppression ROS directly, GSH cell degree modulation, and increasing antioxidant expression.[31]

In the present study, the significantly lowered plasma FSH level that was associated with CYP and/or IR can be attributed to two factors: First, the deleterious alteration of the pituitary histoarchitecture with a consequent decrease in pituitary FSH secretion, and second, a possible decreased responsiveness of the Sertoli cells to the available plasma FSH. It is, however, not unlikely that a possible proliferation of the spermatogonia through its active binding with FSH was altered by the decreased plasma level of testosterone (possibly secondary to the reduced plasma LH level) that was associated with CYP administration and/or exposure to IR.[32] A present study established that CYP and/or ionizing gamma-radiation induce their deleterious effects in biological systems principally by inducing oxidative stress through the generation of ROS.[11]

In the present study, the pituitary gland of an exposed group of rats showed areas of congestion and apoptosis in basophilic and acidophilic cells. Also, after CYP administration and/or exposure to ionizing gamma radiation.[9,33] Furthermore, after exposure to ionizing gamma radiation, we observed apoptosis of the anterior pituitary gland due to increased lipid peroxidation, which damaged the hypothalamus and pituitary gland, resulting in hypopituitarism accompanied by a decrease in FSH and LH levels.[33]

In addition, we showed sever degeneration and abundant apoptotic cells as well as the absence of chromophobe cells and necrosis in the CYP + R group. This agreement with Ekeleme-Egedigwe *et al*.,[25] Zhou *et al*.[33] suggested that CYP and/or IR induce endocrine disorders that could be triggered by the generation of ROS and lipid peroxidation.

Regarding steroidogenesis, testosterone and FSH levels were significantly reduced following treatment with CYP and gamma radiation. Our findings are in accordance with Ebokaiwe *et al*.[34] Testosterone and FSH are important androgens, which play a pivotal role in the development and maturation of male sexual organs and also have a critical role in spermatogenesis and the development of sperm.[32] Moreover, several studies reported that experimental radiation exposures had significantly decreased serum testosterone in males, confirming the direct effects of radiation on Leydig cell steroidogenesis, which is the main source of circulating testosterone.[32] About 75% of the total testosterone is generated by the interstitial Leydig cells to maintain spermatogenesis.[35]

Testicular toxicity induced by CYP and gamma radiation in the present study is characterized by degeneration and/or apoptosis of germ cells, particularly spermatogonia and spermatids. In contrast to Sertoli or Leydig cells, spermatogonia are rapidly dividing germ cells that respond to cytotoxic agents[36] Moreover, spermatogonia are reported to be the most sensitive cells to cytotoxic agents, followed by stem cells, maturing spermatocytes, and spermatids, and finally spermatozoa. Equivalent to the other germ cell types, spermatocytes are vulnerable to the diversity of agents that trigger apoptosis such as androgen deficiency and cytotoxic agents.[37]

The present study showed that not only spermatogonia and spermatocytes are the most sensitive cells to radiation and cytotoxic agents but

also Leydig cells are affected. Being outside of the protective blood-testis barrier, Leydig cell function may be vulnerable to indirect effects impacting gonadotropin release in addition to direct toxic effects on steroidogenesis.[38] Decreases in Leydig cells' number and size were demonstrated in the irradiated group, which led to a dramatic reduction in steroidogenesis.[38]

Consequently, Leydig cell atrophy is associated with decreased spermatogenesis, which is manifested by depletion of the elongating spermatid and Stage V11/ V111 germ cell degeneration.[21,39,40] On the other hand, Leydig cell hyperplasia, which is demonstrated in CYP group, occurred as a compensatory response to decreased spermatogenesis. The multinucleated round spermatid giant cell, demonstrated in the current study, is one of the most characteristic indicators of tubular degeneration, which occurred as a result of spermatid retention or spermatocyte degeneration with subsequent expansion of the intercellular bridges as previously reported by Özatik *et al*.[41]

In addition, the misshaping of seminiferous tubules and contracted tubular lumen is attributed to the decreased production of seminiferous tubule fluid by Sertoli cell, which is an androgen-dependent function and modulated by the presence of elongating spermatids.[38]

Romano *et al*.[42] attributed the significant reduction in Leydig cell LH receptors brought on by radiation as the cause of the steroidogenic lesions seen in experimental studies following radiation exposure. LH is the primary hormonal factor that regulates Leydig cell function through its specific receptor, which is coupled to both the adenylate cyclase and phospholipase-C pathways.[43,44] The decrease in LH receptors is explained by Somosy.[45] They demonstrated that radiation has the ability to enhance the quantity and activity of lysosomes in various cells, which, in turn, increases the lysosomal degradation of LH receptor.[46]

Our results of immunohistochemistry revealed that CYP and/or IR induce apoptosis evidenced by increased the pituitary gland and testicular expression of caspase 3.[47] It has been reported that CYP and/or IR-induced apoptosis are triggered by ROS, which upregulate apoptosis receptors and stimulate the regulation of pro-apoptosis caspase proteins such as Caspase-8 and Caspase-9.

On the other hand, pre-treatment with STS decreased the pituitary gland and testicular caspase-3 expression with decreased the percentage of caspase-3-positive cells. STS was seemed to inhibit apoptosis through inhibition of p38 and caspase 3 as previously confirmed by Rinaldi *et al*.[48]

Treatment with CYP and IR, in addition to oxidative stress, may cause the pro-inflammatory response by boosting the expression of pro-inflammatory cytokines like TNF-a in testicular tissue.[47,49] In addition, CYP has been identified as an activator of the transcription of inflammatory cytokines (TNF-α), interleukins, and other mediators of apoptosis.[50] In addition, IR can cause inflammation and reduction/oxidation reactions by activating the TNF-protein kinase c-dependent pathway.[51]

Pre-treatment with STS, in contrast, reduced testicular TNF-α expression. STS has an antioxidant impact by stimulating multiple antioxidants such as nuclear factor erythroid 2 and also modulating several pro- inflammatory signal systems such as nuclear factor-kappa and suppressed TNF-α. Therefore, STS can be ameliorated the pathological alterations induced by cyclophosphamide and /or gamma radiation.[52,53]

CONCLUSIONS

The present study shows that STS ameliorates the side effects of CYP and/or ionizing gamma radiation on Pituitary and testis through inhibition of antioxidative stress and regulation of hormones secretion. These findings suggest that STS could be useful in protection of pituitary gland and testis from chemo and radiotherapy in cancer patient.

CONFLICTS OF INTEREST

The authors state that they have no conflicts of interest.

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