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GENOTOXIC AND HEMOCHROMATOSIS EFFECT OF IRGASAN TREATMENT AND THE MITIGATING EFFECT OF VITAMIN E IN MALE ALBINO MICE

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INTRODUCTION

Several antimicrobial agents and preservatives (soaps, shampoos, detergents, disinfectants, cosmetics and pharmaceutical products) are commonly used in the personal care products. Different parts of our body like blood, milk, and various organs and tissues are the target of those chemical accumulations due to their continuous use with detectable concentrations. Irgasan (IGSN) is one of those antimicrobial agents that widely used as preservative in toothpastes, soaps, shampoos, and cosmetics and reported to be a highly toxic chemical for aquatic flora and fauna and thus has been included in the probable list of endocrine disruptors because of its resemblance with known non-steroidal estrogens or its mimetic. Furthermore, heat and ultraviolet irradiation convert IGSN and its chlorinated derivatives to various chlorinateddibenzo-p-dioxins that might be harmful for different biological systems. The mode of action of IGSN as an Endocrine-disrupting chemical (EDC) is controversial, in which IGSN exposure in Japanese medakafry (Oryziaslatipes) for fourteen days showed a weak androgenic effect. Another study reported that IGSN metabolite may be a weak estrogenic compound with the potential to induce vitellogenin while decreasing the hatchability, as well as delaying the hatching in females.IGSN has also been reported to inhibit testosterone-induced transcriptional activity as it functions as an anti-androgen agent. Exposure of IGSN to the human may be a consequence of its presence in the cosmetics and other human use products that could be absorbed mainly across the skin or through the gastrointestinal tract.

Environmental contaminants distribute the pro-oxidant/antioxidant balance of testicular cells

that in turn affects their functions, accompanied by downstream pathways such as apoptosis. In turn this increases reactive oxygen species (ROS) levels and apoptosis and affects the normal functioning of the testis tissue. Therefore, there is a great demand to use a good antioxidant to reduce this deleterious effect.

The present study was aimed to evaluate the histopathological, DNA damage and testicular hormonal evaluations induced by IGSN treatment. Moreover, the study was aimed to assess the protector role of vitamin E pre-treatment in testes of albino male mice.

MATERIALS AND METHODS

Animals: Twenty-five albino male mice (Mus musculus; 8-10 weeks old; 26-30 g body weight) were obtained from the animal house of the King Fahad Center for Medical Research, King Abdul-Aziz University in Jeddah. We have followed the European Community Directive (86/609/EEC) and National Rules on Animal Care.

Tested drugs

1. Irgasan (IGSN) was dissolved in corn oil and injected intraperitoneally (i.p.) daily at 15 mg/kg for 2 consecutive days.
2. Vitamin E (Vit E) (α-tocopherol acetate, Sigma) was dissolved in corn oil and orally was administrated daily for 2 consecutive days at dose level of 50 mg/kg.[17]

Treatment Schedule: Group 1: Negative control group, untreated mice. Group 2: Vehicle group, mice were injected i.p. with 50 µL corn oil. Group 3: Vitamin E (Vit E) group, mice were administrated orally with 50 µL Vit E in corn oil (50 mg/kg). Group 4: Irgasan (IGSN) group, mice were injected i.p. with 50 µL IGSN (15 mg/kg). Group 5: Vitamin E+Irgasan (Vit E+IGSN), mice were orally administrated with 50 µL Vit E in corn oil followed by i.p. injection of 50 µL IGSN (15 mg/kg+50 mg/kg).

Animals were killed by cervical dislocation and testicular tissues were used for further assays.

Molecular Evaluation

Comet assay: Frosted microscopic slides were dipped into 1.0% hot normal melting point agarose with the remove of excess agarose from the underside of the slide. Testes tissues were homogenized in cold Hank`s Balanced Salt Solutions and only 10 µL of homogenate was mixed with 65 µL of low melting point agarose (0.5%) at 37°C, and was spread on the

frosted slides. Then the slides were left in lysis solution (2.5 M NaCl, 100 mM Na2EDTA, 10 mMTris, NaOH to pH 10.0, 1% Triton-100 and 10% DMSO) for 2 hours at 4°C. The slides were left in electrophoresis buffer (NaOH, TE buffer) for 20 mins and then electrophoresed at a constant current of 300 mA, for 35 mins. After complete electrophoresis, the slides were neutralized using Tris-HCl buffer (5 mins for three washes) at pH 7.5, followed by cold ethyl alcohol for 10 mins and then left to dry overnight. The slides were stained by using 80 µL ethidium bromide (20 µg/ mL) for 20 mins.[18] The slides were covered and viewed under an epifluorescence microscope (Zeiss epifluoresent) with an attached CCD camera. Images were saved as electronic files and for each sample, 50 isolated comets were selected randomly and measured for comet tail length, %DNA in tail and tail moment using COMETSCORE software based on the definition.

Histopathological Evaluation

Testes were removed and fixed in 10% neutral buffer formalin, washed with tap water, dehydrated in a series of ethyl alcohols, then cleared in xylene, and finally embedded in 60°C paraffin wax to obtain block. Blocks were cut at 5 microns using a microtome. Testis sections were stained using Hematoxylin and Eosin dyes[20,21] for the investigation of general histological changes under light microscope at magnification 400X.

Tissue Hormonal Evaluation

Preparation of tissue homogenates

Testes were removed, washed in 0.9% saline and then dried on filter paper. 100 mg tissue was homogenized in 1 ml of 1X PBS and stored overnight at -20°C. After two freeze-thaw cycles were performed to break the cell membranes, the homogenates were centrifuged for 5 min at 5000 x g, 2-8°C. The supernatant was removed and assayed immediately.

Sex hormones assay

Luteinizing hormone (LH) and follicle stimulating hormone (FSH) in MIU/mg and testosterone (T) in ng/mg were measured using enzyme linked immunosorbant assay (ELISA) kits per manufacture structure. LH levels in the testes homogenate were determined using Mouse luteinizing hormone ELISA Kit (Catalog No. CSB-E12770m), FSH levels in the testes homogenate were determined using Mouse follicle stimulating hormone ELISA Kit (Catalog No. CSB-E06871m) purchased from Cusabio Biotech Co. (CUSABIO) (Baltimore, USA). Testosterone hormone levels in the testes homogenate were determined using Mouse Testosterone ELISA Kit (Cat. No. KT-52637) purchased from Kamiya Biomedical Co.

Statistical analysis

Comparisons among groups were analyzed for statistical significance by using student t-test. Data were represented as mean ± standard error (SE). Differences were considered significant at $p<0.05$. Statistics were carried out using statistical analysis systems (SAS) program for comet and hormonal evaluations. Graphs and statistical analysis were executed using Sigma Plot 12 statistics software (Systat Software, Inc., San Jose, CA).

RESULTS

The genotoxic potential of Irgasan was evaluated by using comet assay, in which Figure 1a and 1b shows typical nuclei of undamaged cells for negative control group; while Figure 1c and 1d is a representative photomicrograph for IGSN group showing DNA damage observed as comets.

Basic-Clinical-Pharmacy-Photomicrograph

The results show a significant increase in tail length, %DNA in tail and tail moment for IGSN group in comparison with the negative control group as shown in Figure 2. However, Vit E preoral administration to IGSN group shows remarkable decrease in tail length but still significant in comparison to negative control group. In addition, a VitE+IGSN group shows a significant reduction in %DNA in tail and tail moment in comparison to IGSN group. However, for the tail moment results still significant in comparison to negative control group.

Basic-Clinical-Pharmacy-Irgasan

Figure 2: A histogram showing the effect of Irgasan (IGSN) on the DNA (DNA damage was represented by Comet assay) in mice testicular cells. Significant difference ($P<0.05$) using Student's t-test, in which: *statistically compared with negative control group; #statistically compared Vit E+IGSN group with IGSN group.

Basic-Clinical-Pharmacy-Negative-Control

The testicular histopathological results of the IGSN treated group in the current study was supported by sex hormonal dysfunction evident by significant decrease in Testosterone, FSH, LH hormones when compared with the negative control group. However, Vitamin E alleviates the effect of IGSN in Vit E+IGSN group by a significant increase of testosterone, FSH, LH level in comparison to IGSN group as shown in Figures 4-6 respectively. Moreover, Vit E group shows a significant increase of testosterone, LH level in comparison to the negative control group as shown in Figures 4-6 respectively, and significantly enhanced the

FSH level in comparison to negative control group.

DISCUSSION

The present study shows the toxic effect of IGSN (15 mg/kg) treatment represented by DNA damage (represented by Comet assay), histopathological and disturbance in different testicular hormones (testosterone, FSH and LH). Our results were in agreement with previous work, in which reported[21] that treatment of male rat with higher doses of IGSN (10 and 20 mg/ (kg day)) induced a significant decrease ($p<0.05$) in the weight of testis epididymis, ventral prostate, vas deferens and seminal vesicles and sex accessory tissues. Moreover, they assessed that in male rats treated with a dose of 20 mg/ (kg day) IGSN, there was a statistically significant decrease in the serum LH (38.5%), FSH (17%), cholesterol (35%), pregnenolone (31%) and testosterone (41%) levels ($p<0.05$) and a number of histopathological malformations which probably affected the production and maturation of the sperms as compared to control.

Recent research demonstrates that Irgasan has effects on the thyroid, estrogen, and testosterone hormones in laboratory animals, including mammals.[22-25] Moreover, IGSN treated goat epididymal sperms in vitro for 5 h results in decrease of sperm motility from 77 to 46% and sperm viability from 78% to 47% in a concentration-dependent manner.[26] In addition, IGSN treatment at different concentrations for different sampling time significantly decreased activities of testicular steroidogenic enzymes, 3β-HSD and 17β-HSD of goat sperms. The abnormalities observed in the testicular structures in the present study might be attributed either to the potential effect of IGSN on the pituitarygonadal pathway, or to the oxidative damaging effect of free radicals.

IGSN as an endocrine-disrupting chemical (EDC) in male rats has the potential to affect the pituitary-gonadal pathway at various levels because of its action at various steps of steroidogenesis: including reduction of LH and cholesterol production; depressed StAR protein expression and finally down-regulation of several key steroidogenic enzymes. That in turn impairs the androgen production and maintenance of sex accessory tissues.[21] An earlier report exists in the effect of polychlorinated biphenyls on testicular steroidogenesis through oxidative stress,[27] that might be a mechanism way for IGSN as an antiandrogenic chemical. The ROS production of IGSN was later reported in few recent researches, in which[28,29] revealed that 1 and 10 m MIGSN treatment on primary cultures of mouse neocortical neurons increased the production of ROS, that in turn this effect of IGSN was reversed by 10 mM of

the ROS scavenger, N-acetyl-L-cysteine. Moreover, it has been indirectly demonstrated that IGSN contaminants increase ROS production in aquatic organisms, such as mussels or Daphnia magna.[29-31] In addition, reported that 50 mM IGSN treatment directly increased the ROS formation and depleted the glutathione activity in rat neural stem cells.[32] The production of free radicals and oxidation of germ cells in the testis can reduce sperm concentration.[33,34]

Therefore, IGSN (15 mg/kg) treatment for 2 consecutive days for male mice increased oxidative stress production in testicular tissue. That was cleared in the presence of DNA damage by significant increase in tail length, %DNA in tail and tail moment in comparison with the negative control group. Moreover, IGSN treatment decreases different testicular hormones (testosterone, FSH and LH), that in turn affects testicular histological architecture. This hypothesis was confirmed by reversing IGSN effects by the oral pre-administration of the effective ROS scavenger, Vit E.

Our results were in agreement with previous studies, they have shown that antioxidants and vitamins C, E, and B can strengthen the bloodtestis barrier, protect and repair sperm DNA, and can be effective in treating male infertility by reducing the damage caused by free radicals.[33-35] In addition, vitamin E (100 mg/kg) treatment decreased the oxidative stress and the percentage of abnormal sperms in Busulfantreated mice and increased sperm concentration and the antioxidant activity.[36-38] Moreover, vitamin E, as an antioxidant, has the ability to reconstruct seminiferous tubules after damages caused by ozone gas and reduces the harmful effects of this gas on testicular tissue and strengthens the blood-testis barrier. This research showed vitamin E to be an effective antioxidant in dealing with external and toxic factors in testicular tissue.

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