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Protective effect of co-enzyme Q10 on testicular tissue and sperm parameters in adult male rats treated with Sunset Yellow FCF

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ABSTRACT

Objective: To determine the protective effect of co-enzyme Q10 (CoQ10) on testicular tissue and sperm parameters in male rats treated with Sunset Yellow FCF.**Methods:** Sixty male Sprague-Dawley rats were randomly divided into 6 groups of the control, CoQ10 (10 mg/kg/day), low dose of Sunset Yellow (2.5 mg/kg), high dose of Sunset Yellow (70 mg/kg), low dose of Sunset Yellow (2.5 mg/kg) plus CoQ10, and high dose of Sunset Yellow (70 mg/kg) plus CoQ10. The drugs were administered *via* daily oral gavages for 6 weeks. At the end of the experiment, sperm analysis, stereological and histological assessments of the testis were carried out.**Results:** The normal morphology (by 41.1%) and progressive spermatozoa (by 74.8%), testicle volume (by 33.4%), lumen volume (by 38.3%), interstitial tissue volume (by 44.7%), seminiferous tubule volume (by 40.7%), and number of spermatogonia (by 53.9%) and Leydig cells (by 70.7%) reduced in the rats that received high doses of Sunset Yellow in comparison to the control group. Nonetheless, all these alterations were recovered by CoQ10 treatment in the CoQ10 plus high dose of Sunset Yellow group. Furthermore, low doses of Sunset Yellow did not affect different parameters of the testis and sperm.**Conclusions:** CoQ10 could, to some extent, prevent structural changes of the testis induced by the high dose of Sunset Yellow.**KEYWORDS:** Sunset Yellow FCF; Co-enzyme Q10; Testicle; Sperm; Stereology

1. Introduction

Food additives are commonly used to improve the flavor, texture, taste, color, or appearance of foods[1]. Artificial food colors and

additives include different types of colors, pigments, or substances used to color beverages and foods. Excessive use of artificial food colors can develop kidney, urinary bladder, liver, and spleen malignancies both in humans and animals[2,3]. The excessive use of these food colors increases biochemical renal and hepatic oxidant markers. The other complications such as destructive effects on cellular immunity, genetic toxicity in the lymphocytes by a mutation in their nucleus, allergic reactions, and an increase in mobility,

Significance

Artificial food additives are commonly used to improve the flavor, texture, taste, color, or appearance of foods. Sunset Yellow FCF, an artificial food color, exerts severe side effects on the different organs. Although immunohistochemical and molecular assessments of the effects of Sunset Yellow FCF on the male reproductive system have been investigated in several studies, no efforts are to investigate the quantitative evaluations (including the volume of the testis components and the number of testicular cells) of the effects of Sunset Yellow FCF on testicular tissue. The study revealed that high dose of Sunset Yellow FCF caused structural changes in the testis and the use of CoQ10 decreased some undesirable effects of Sunset Yellow FCF.

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restlessness, and excitation in childhood are also seen with artificial food colors and additives excessive intake[4–6]. Sunset Yellow FCF is one of the most commonly used synthetic azo dyes, known by other names like C.I. 15985, Orange Yellow S, and FD&C Yellow 6 with a chemical formula of $C_{16}H_{10}N_2Na_2O_7S_2$ and molecular weight of 452.36 g/mol[7,8]. Synthetic Sunset Yellow, a water-soluble azo dye derived from crude oil products, is known as an additive food colorant used frequently in food industry because of its high stability, low production cost, and excellent color uniformity[2,9]. Sunset Yellow FCF is used in several food products, such as dairy products, snacks, grains, bakery ingredients, confectionery products, iced desserts, beverages, cosmetics products, and food supplements[3]. Several studies suggest that an animal's excessive exposure to the Sunset Yellow can develop histopathologic changes in the cerebral cortex, liver, and kidney[10,11]. In addition, a study reported an increase in the level of malondialdehyde (MDA) which reflects the fact that the level of anti-oxidants is declined. Other studies revealed the hepatic and renal parameters changes such as increased cholesterol, lipid, and urea[12].

Despite the toxic effects of Sunset Yellow on different organs such as the brain, liver, and kidney, the toxic effects of Sunset Yellow on the testicular tissue are still unclear. Therefore, research into drugs or substances to define the side effects of Sunset Yellow on the male reproductive system is necessary. Therefore, the main aim of the present study was to evaluate the effects of synthetic Sunset Yellow on the testicular structure. Furthermore, we aimed to explore a protective substance that could be easily consumed with the lowest side effects. Co-enzyme Q10 (CoQ10), also known as ubiquinone, is an active quinone redox compound with a hydrophobic tail and lipid solubility properties[13]. The CoQ10 can act as an intracellular antioxidant protecting the plasma membranes against peroxidation[14], and it regulates the cellular apoptosis and autophagy[15]. It has been reported that CoQ10 has anti-oxidative, anti-inflammatory, and anti-apoptotic effects in various models of testes toxicity[16–18]. Also, it has been observed that CoQ10 injection recovered the serum male hormones and treated testicular tissue toxicities after introducing aluminum chloride in animal models[19]. Therefore, the present study was designed to examine the protective effects of CoQ10 on the sperm quality, volume of the testicle, number of spermatogenic cells, number of Sertoli and Leydig cells, length and diameter of the tubule, and germinal epithelium height after the intake of Sunset Yellow. Stereological methods were applied to investigate structural changes in the testis. These methods provide quantitative and comparable data.

2. Materials and methods

2.1. Animals

In this study, 60 male Sprague-Dawley rats with a weight of 210–

270 g (10–12 weeks) were used. The rats had free access to food and water and were kept in a room with temperature (22 °C–24 °C) and a 12 hours light/12 hours dark cycle. The animals were purchased from the Experimental Animal Center of Shiraz University of Medical Sciences.

2.2. Study design

The animals were randomly divided into six experimental groups each containing 10 rats: the control (distilled water), CoQ10 (10 mg/kg/day)[20], low dose of Sunset Yellow (2.5 mg/kg/day = acceptable daily dose)[21], high dose of Sunset Yellow (70 mg/kg/day)[22], low dose of Sunset Yellow (2.5 mg/kg/day) plus CoQ10 (10 mg/kg/day), high dose of Sunset Yellow (70 mg/kg/day) plus CoQ10 (10 mg/kg/day).

CoQ10 [DarmanYab Darou (DYD), Tehran, Iran] and Sunset Yellow FCF (CAS Number 2783-94-0, Sigma-Aldrich, Germany) were used for experiments. Distilled water was the solvent for CoQ10 and Sunset Yellow FCF. Treatments were administered orally for six weeks.

2.3. Spermatozoa collection and count

Sperm samples were collected from the proximal part of the vas deferens and placed in a petri dish including 5 mL phosphate-buffered saline (PBS). The spermatozoa were allowed to diffuse into the solution and the suspension was shaken moderately at 37 °C for 5 min. The sperm samples were spread on a Neubauer hemocytometer; in each animal, about 200–300 spermatozoa heads were counted. The total spermatozoa count and motility were evaluated under a light microscope (Nikon E200, Nikon, Japan) using 100× magnification.

2.4. Spermatozoa motility

The spermatozoa suspension was placed on a slide warmed at 37 °C. The slides were examined in 10 randomly microscopic fields and 200–300 spermatozoa per rat were analyzed at a final magnification of ×1000. The motile spermatozoa were defined as the mean number of motile spermatozoa multiplied by 100 divided by the total number of spermatozoa. The motility of the spermatozoa was graded as: a) rapid progressive motility, b) slow progressive motility, c) non-progressive motility, and d) immotility. If more than 50% of the spermatozoa were graded as c or d, it was considered abnormal motility.

2.5. Spermatozoa morphology and viability

The spermatozoon suspension was placed on a slide and air-dried. The sample was stained with eosin-Y (assessment of sperm morphology and viability) for 5–10 min; then, the percentages of normal and abnormal spermatozoa were determined. The

abnormality was defined as those with an amorphous head, banana-head, pinhead or 2 heads, small head, 2 tails, or a broken or bent tail. The percentage of normal spermatozoa was evaluated as the mean number of normal spermatozoa $\times 100$ divided by the total number of normal and abnormal spermatozoa. On the other hand, the percentage of viable sperm has been assessed by eosin Y staining. Non-viable sperm stained red while live sperm stayed white.

2.6. Stereological study

At the end of the six weeks of treatment, the right testis was removed, cleaned entirely, and weighed. The primary volume “V” of the testis was estimated using the immersion method. Afterward, the testis tissue was immediately fixed in 4% formaldehyde in a buffered solution for 3 days. The orientated method was employed to obtain isotropic uniform random (IUR) sections. Briefly, the testis was placed on the ϕ -clock, a random number from 1 to 9 was chosen and the testis was split into two pieces in that direction. The cut surface of the first segment was placed on the 0-0 axis of the θ -clock and a random number was chosen; this segment was cut parallelly along the selected number. The second piece was located on the θ clock vertically, and cuts were made along the selected number. Eight to ten slabs were collected from each testis. To estimate the shrinkage, we punched a circular piece from a testis slab by a trocar with a 3 mm diameter. After tissue processing, the slabs and circular piece were embedded in paraffin blocks, sectioned, and stained with hematoxylin & eosin (H & E). The shrinkage (shr) volume (V) in the testis was estimated using the following formula:

$$V(\text{shr}) = 1 - (AA/AB)^{1.5}$$

Where AA and AB represent the circular zone of a slab after and before tissue processing and staining. After sectioning and staining, microscopic analyses were done by using a video microscopy system made up of a microscope (E-200, Nikon, Japan) that was linked to a video camera, a computer, and a monitor.

2.6.1. Estimation of the volume of the testis components

The “point-counting approach” was used to estimate the volume density “Vv (structure/testicle)” of the testis (including the germinal epithelium, interstitial tissue, and tubules). The following formula was used to determine the total volume of each structure:

$$V(\text{structure}) = Vv(\text{testicle/structure}) \times V(\text{testicle})$$

2.6.2. Estimation of the number of cells

To estimate the total number of Sertoli cells, Leydig cells, spermatogonia, spermatocytes, and round and long spermatids, we used sections with a thickness of 25 μm . An unbiased counting frame was superimposed on the images of testis sections on the monitor. The number of cells was evaluated using the “optical disector” method. Thus, the microscopic fields were scanned by moving the microscope stage at equal distances in the X and Y direction based

on systematic uniform random sampling. Movement in the Z direction was also performed using a microcator (MT12, Heidenhain, Germany) mounted on the microscope platform. The Z-axis distribution of cells sampled in different focal planes was plotted to determine the guard zones and disector height. The numerical density (Nv) was estimated using the following formula:

$$Nv(\text{cells/testis}) = \Sigma Q / (\Sigma A \times h) \times (t/BA)$$

Where ΣQ represents the number of the nuclei coming into focus in the disector height, ΣA is the total area of the unbiased counting frame in all fields, “h” indicates the height of the disector, “t” is the mean section thickness, and BA is the microtome setting.

The total number (N) of cells was evaluated using the following formula:

$$N(\text{cells}) = Nv(\text{cells/testis}) \times V(\text{epithelium}) \times [1 - d(\text{shr})]$$

2.6.3. Estimation of the tubules length

The length density (Lv) of the tubules was calculated as:

$$Lv = 2\Sigma Q / [\Sigma P \times (a/f)]$$

Where “ ΣQ ” was the total number of the tubule profiles counted per rat testis, (a/f) was the area of the counting frame, and “ ΣP ” was the total number of the frames counted in each animal. The total length of the tubule “L” was calculated using the following formula:

$$L(\text{tubules}) = LV(\text{tubules/testicle}) \times [1 - d(\text{shr})]^{2/3} \times V_{\text{testis}}$$

2.7. Statistical analysis

The data analysis was performed using PRISM.8 software version 8.4. (GraphPad Software Inc., San Diego, USA). Data were subjected to a normality test and conformed to a normal distribution; one-way analysis of variance (ANOVA), followed by Tukey’s *post hoc* test and Kruskal-Wallis tests were used for statistical comparisons. All values are expressed as the mean $\pm$ standard deviation (mean $\pm$ SD). The level of significance was set at $P < 0.05$.

2.8. Ethics statement

This study was performed in accordance with the animal care, and all experimental protocols were approved by the Institutional Ethics Committee (IEC) at Shiraz University of Medical Sciences (IR.SUMS.REC.1399.1094).

3. Results

3.1. Spermatozoa count and motility

The data showed that no change was observed in the parameters of spermatozoa in low-dose Sunset Yellow treated (2.5 mg/kg) animals in comparison to the control group. However, a significant decrease was observed (by 74.8%) in the percentage of progressive

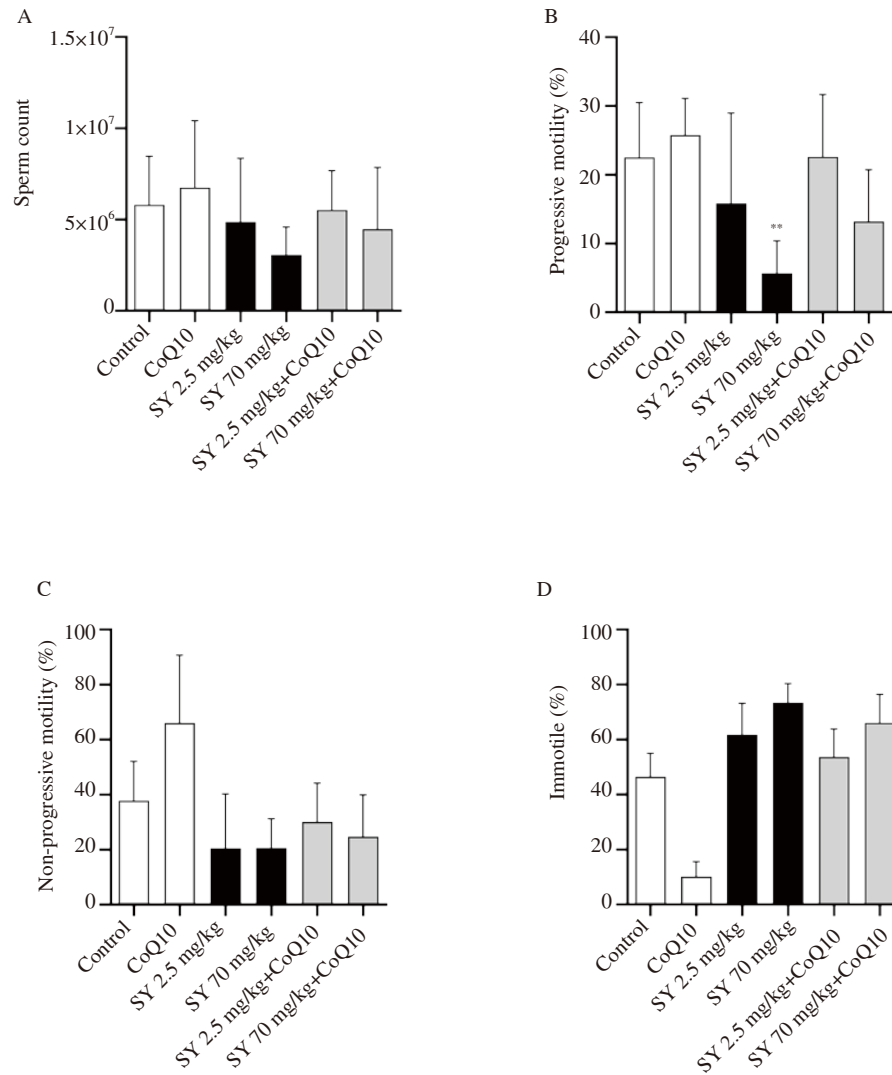


Figure 1. Effect of co-enzyme Q10 (CoQ10) on sperm parameters in adult male rats treated with Sunset Yellow (SY). ** $P<0.01$: Compared to the control group.

spermatozoa in the group that received the high dose of Sunset Yellow (70 mg/kg/day) compared to the control group ($P<0.01$). Nonetheless, these parameters returned to the control level in the rats that received the high dose of Sunset Yellow plus CoQ10. No significant differences were seen in the sperm count, percentage of non-progressive spermatozoa, and percentage of immotile spermatozoa in any of the groups compared to the control group (Figure 1).

3.2. Sperm morphology

The results showed a significant decrease (by 41.1%) in the percentage of normal morphology spermatozoa in the high dose of Sunset Yellow (by 70 mg/kg/day) compared to the control group ($P<0.01$). However, the percentage of normal morphology spermatozoa showed no significant differences between the high dose of Sunset Yellow plus CoQ10 group and the high dose of Sunset Yellow group. Also, the results revealed no statistically significant changes in the sperm morphology in other experimental

groups as compared to the control group (Figure 2A).

3.3. Sperm viability

There were no statistically significant changes in sperm viability between the various treatment groups as compared to the control group (Figure 2B).

3.4. The outcomes of stereological study

3.4.1. Volume of the testicle

The results indicated that the testicle volumes were reduced (by 33.4%) in the group that received high dose of Sunset Yellow (70 mg/kg) in comparison to the control group ($P<0.05$). The volume of the testicle showed no significant differences between the high dose of Sunset Yellow plus CoQ10 group and the high dose of Sunset Yellow group. Also, the results revealed no statistically significant changes in other experimental groups as compared to the control group (Figure 3A).

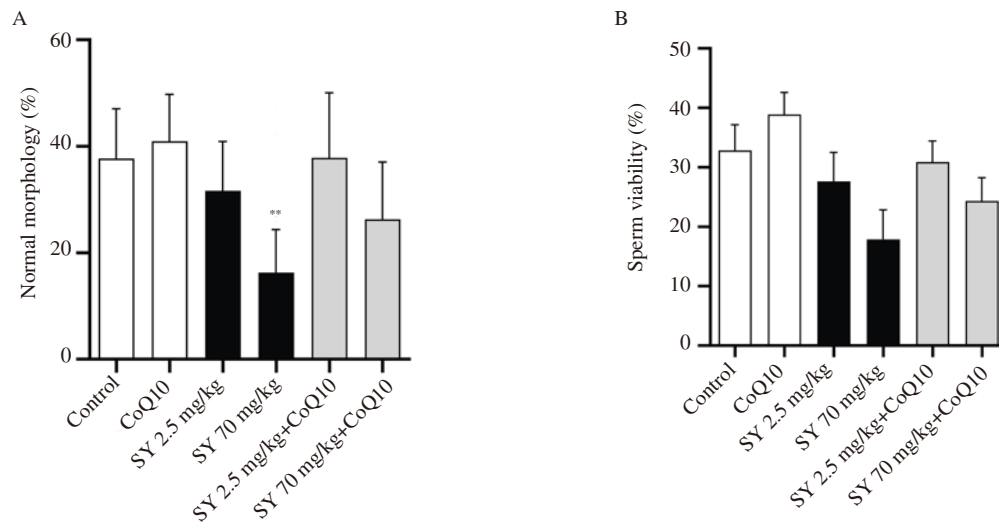


Figure 2. Effect of co-enzyme Q10 (CoQ10) on sperm normal morphology and viability in adult male rats treated with Sunset Yellow (SY). ** $P < 0.01$: Compared to the control group.

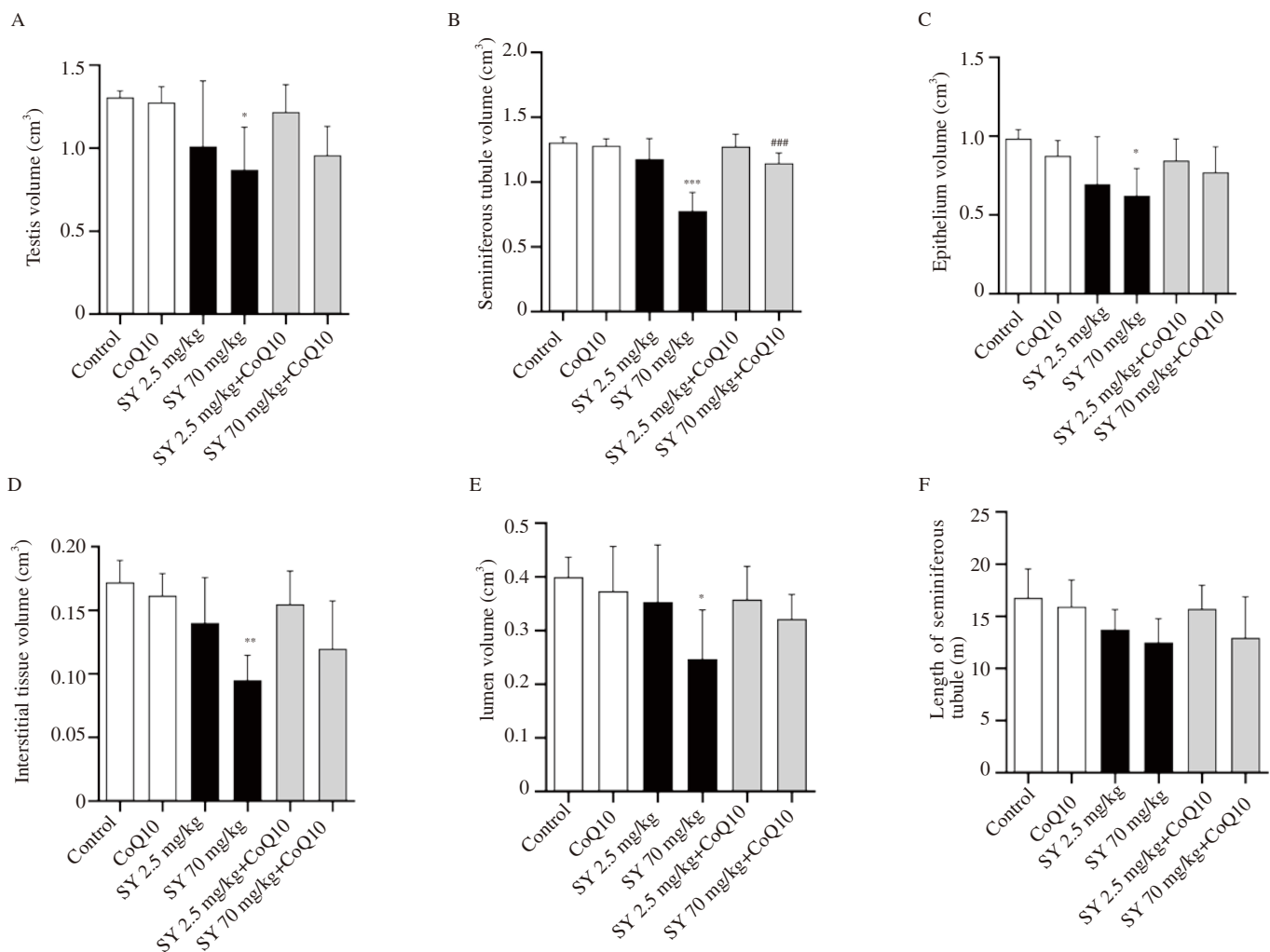


Figure 3. Stereological study results of co-enzyme Q10 (CoQ10) on adult male rats treated with Sunset Yellow (SY). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$: Compared to the control group; ### $P < 0.001$: Compared to the group of SY (70 mg/kg).

3.4.2. Volume of the seminiferous tubule

The tubule volumes in the group treated with high dose of Sunset Yellow (70 mg/kg) significantly reduced (by 40.7%) in comparison

with the control group ($P < 0.01$). However, the tubule volumes significantly recovered in the high dose of the Sunset Yellow plus CoQ10 group in comparison to the high dose of Sunset Yellow group

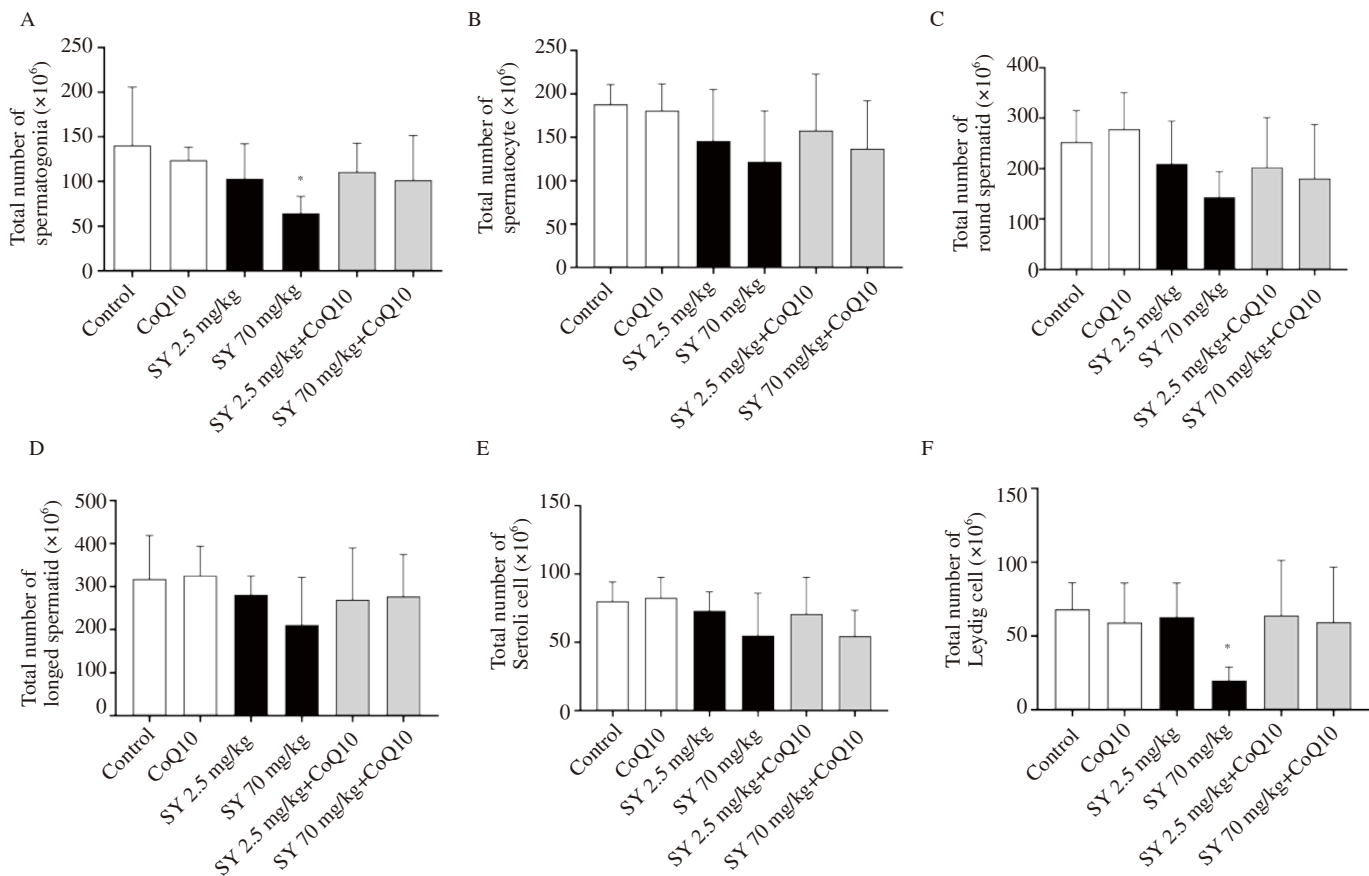


Figure 4. Effect of co-enzyme Q10 (CoQ10) on number of cells in the testis in adult male rats treated with Sunset Yellow (SY). * $P<0.05$: Compared to the control group.

($P<0.001$). There was no significant difference in this parameter between the other experimental groups as compared to the control group (Figure 3B).

3.4.3. Volume of the seminiferous tubule epithelium

According to the results, the volume of the seminiferous tubule epithelium in the animals exposed to high dose of Sunset Yellow (70 mg/kg) significantly decreased (by 36.7%) in comparison with the control group ($P<0.05$). However, this reduction was partially recovered in the high dose of Sunset Yellow plus CoQ10 animals, so that there was no significant difference in comparison to the control group (Figure 3C).

3.4.4. Volume of the interstitial tissue

The results also showed that the connective tissue volume greatly decreased (by 44.76%) in the group treated with high dose of Sunset Yellow (70 mg/kg) in comparison to the control groups ($P<0.001$). In addition, the connective tissue volume of the high dose of Sunset Yellow plus CoQ10 group was comparable with that of the control group but did not show significant differences in the high dose of Sunset Yellow group (Figure 3D).

3.4.5. Volume of the lumen

The results also indicated that the volume of the lumen decreased (by 38.32%) in the rats that received high dose of

Sunset Yellow (70 mg/kg), as compared to the control rats ($P<0.05$). However, the volume of the lumen returned to the control levels in the high dose of Sunset Yellow plus Q10 group in comparison to the high dose of Sunset Yellow group but did not show significant differences (Figure 3E).

3.4.6. Length of the seminiferous tubule

According to Figure 3F, no significant change was identified in the length of the seminiferous tubule in the low-dose and high-dose Sunset Yellow-treated rats compared to the control group.

3.4.7. Number of cells

The number of cells in the testis showed a significant loss in the spermatogonia (by 53.9%) (Figure 4A) and Leydig cells (by 70.7%) (Figure 4F) in the high dose of the Sunset Yellow group in comparison with the control group ($P<0.05$). According to our results, the number of spermatocytes, round spermatids, long spermatids, and Sertoli cells in the experiment groups did not reveal any significant difference comparing with the control group (Figure 4B-E).

3.5. Qualitative changes

A qualitative assessment of the testis has been showed in Figure 5. The testicular tissue sections in the high dose of Sunset Yellow group

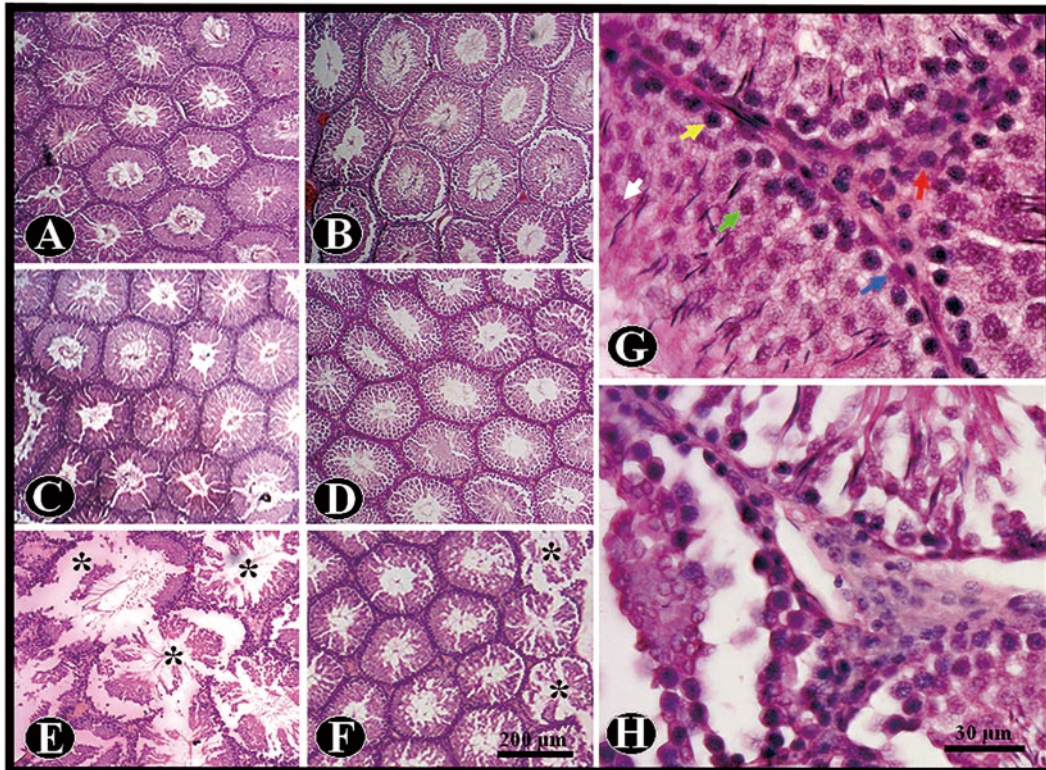


Figure 5. Photomicrograph of the testicular tissue stained with hematoxylin & eosin (H & E) in the control (A, G), CoQ10 (B), low dose of Sunset Yellow (SY) (C), low dose of SY+ CoQ10 (D), high dose of SY (E, H), and high dose of SY + CoQ10 (F) groups. The testicular cells, including spermatogonia (yellow arrow), spermatocytes (green arrow), spermatids (white arrow), Sertoli cells (blue arrow), and Leydig cells (red arrow), are represented in the control group (G). The images show the atrophied seminiferous tubules (E) (asterisk), epithelium height reduced, and many spermatogenic cells are lost in the group of high dose of SY (H).

revealed atrophy of seminiferous tubules, and reduction of number of testicular cell types than in the control group. Concomitant treatment of this group with CoQ10 improved these destructive effects. On the other hand, testicular cells and tubules maintained their normal form in the other groups.

4. Discussion

Our study revealed that the control and low dose of Sunset Yellow (2.5 mg/kg) groups always showed the same results, and low dose of Sunset Yellow had no significant effects on the testis and sperm different parameters. However, the high dose of Sunset Yellow induced structural changes in the rat testis and sperm parameters. Testicular damage induced by the high dose of Sunset Yellow treatment was characterized by the reduction of the volume of the testis, reduction of the spermatogonia and Leydig cell numbers, normal morphology, and progressive motility. Volume reduction of the seminiferous tubule in the present study was in agreement with the observations of Mathur *et al.* They reported that the diminished size of the degenerated tubule was the cause of the apparent increase in the size of the interstitium[10]. Our results also showed that the high dose of Sunset Yellow reduced the Leydig cell and spermatogonia

number. A decrease in the number of spermatogonia may be the result of a decrease in the number of Leydig because a decrease in the number of Leydig cells as testosterone-producing cells could affect the process of spermatogenesis as well as the proliferation and differentiation of the germ cells[23]. However, in contrast to our result, Mathur *et al* showed that Leydig cells presented a normal appearance[10]. Similar to our study, Bastaki *et al* indicated that Sunset Yellow did not affect the sperm count. Bastaki *et al* assessed Sunset Yellow effects on the sperm count, motility and viability in the rat in a 28-day toxicity study. The sperm quality evaluation presented no adverse effects on the sperm motility, epididymal sperm count, homogenization-resistant spermatid count, or sperm morphological development. Conversely, there was no difference in the sperm count after administration of Sunset Yellow[24]. Our study, consistent with Bastaki *et al*'s research, revealed that the daily intake of FDA-certified Sunset Yellow FCF did not affect the testicular parameters. Previous studies showed that azo dyes like Sunset Yellow significantly decreased the total antioxidant levels in azo dye-treated animals compared to the controls, so inducing oxidative stress might be a possible mechanism of toxicity by Sunset Yellow[11]. Moreover, oxidative stress also plays an influential role in the etiology of male infertility[25]. Overproduction of reactive oxygen species damages

the structure and quality of sperm cells and consequently causes infertility[26].

Another part of our study showed that the volume of the lumen, connective tissue, seminiferous tubule epithelium, seminiferous tubule, testicle, and the percentage of normal morphology spermatozoa, and progressive spermatozoa returned to the control levels by CoQ10 treatment in high dose of Sunset Yellow plus CoQ10 group. Furthermore, our study showed that CoQ10 had no adverse effect on the testis tissue and sperm parameters because the control and CoQ10 groups always showed similar results as the control group. Previous studies also reported the protective effects of CoQ10 on testicular histological changes. For instance, Delkhosh *et al* reported that CoQ10 had anti-oxidative, anti-inflammatory, and anti-apoptotic effects and could prevent testicular damage in the testicles of the mice exposed to scrotal hyperthermia[17]. In addition, according to Ramadan *et al*'s study, CoQ10 caused a significant recovery of the mice testes toxicity induced by the high magnetic field. In addition, they claimed that the effect of the magnetic field could be blocked by the free radicals scavenging activity of CoQ10[18]. Moloody *et al* assessed the effect of CoQ10 on the testicular tissue (sperm quality and histological changes) in rats treated with Busulfan. Their result showed that administration of CoQ10 in busulfan-treated animals improved the histological and sperm quality[27]. Karimi *et al* investigated the possible toxic effects of the Sunset Yellow and the protective effects of CoQ10 on testicular tight and gap junctions in rats by evaluating molecular, immunohistochemical, and histopathological changes. This study demonstrated the beneficial effects of CoQ10 on the blood-testis barrier cellular junctions and membrane proteins. In their study, they showed that the possible mechanism of the protective effects of CoQ10 on gap junctions and membrane proteins is through antioxidant defense[28]. These studies indicate that CoQ10 had the ability to prevent the testis toxicity through its antioxidative effects. Therefore, we hypothesized that supplementation with CoQ10 could reduce oxidative stress and related parameters such as the glutathione disulfide/glutathione ratio, MDA, or antioxidant activities induced by Sunset Yellow. Therefore, the protective effects of CoQ10 against the high dose of Sunset Yellow in this study could be explained by its antioxidant effects reported in previous studies. However, further studies are required to clarify the efficacy of different doses of CoQ10 on the male reproductive system. Therefore, it is recommended that the usage of repeated doses of CoQ10 and alteration of the dose of CoQ10 could be more useful.

There are some limitations. This study did not evaluate the amount of reproductive hormones and the level of antioxidant enzymes, so more detailed studies may be necessary to complete the present findings.

In conclusion, our results indicated that the high dose of Sunset Yellow produced structural changes in the rat testis, such as volume reduction of the testis, reduction of spermatogonia and Leydig cell

numbers, normal morphology, and progressive motility. However, the low dose of Sunset Yellow has almost similar effects as distilled water administrated to the control group. It seems that the use of CoQ10 decreases some undesirable effects of Sunset Yellow on the male reproductive system. However, further studies are required to clarify the efficacy of different doses of CoQ10 on the male reproductive system.

Conflict of interest statement

The authors declare no conflicts of interest.

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Authors' contributions

Rohollah Nazari carried out experimental study, data acquisition, data analysis, and statistical analysis; Somayeh Akbari performed statistical analysis and manuscript preparation; Maryam Naseh carried out intellectual content manuscript review and manuscript preparation; Shayan Yousufzai executed experimental study and data acquisition; Seyede Fatemeh Hosseini performed intellectual content manuscript review and literature review; Fatemeh Karimi performed design, literature review, manuscript editing, and review.

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