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Reactive Oxygen Species (ROS) could be a causative factor for perfume-induced testicular toxicity in male rats

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Abstract

Fragrance materials have been implicated in male infertility. These ingredients are unsteady and tend to oxidize easily. The dearth of information regarding the mechanism of action in which it induces testicular toxicity prompted us to evaluate its possible mechanism pathway in animal model. Sixty adult male wistar rats were randomly divided into six groups of ten rats each. Group A and B rats (Controls) rats were exposed (6hrs day^{-1}) to 5 ml kg^{-1} body weight of normal saline for 56 days and 112 days via whole body inhalation respectively, Group C and D rats were exposed (6hrs day^{-1}) to 5 ml kg^{-1} body weight of one of the perfume designated as F^1 for a period of 56 days and 112 days via whole body inhalation respectively while Group E and Group F rats were exposed (6hrs day^{-1}) to 5 ml kg^{-1} body weight of one of the perfume designated as F^2 for a period of 56 days and 112 days via whole body inhalation respectively.

Result obtained showed a significant decrease in the activity level of SOD, CAT, GPx and GSH and a significant elevation in the level of MDA when compared to values from the control groups of rat. The decrease in level of these testicular enzymatic and testicular non-enzymatic antioxidants (SOD, CAT, GPx and GSH and MDA respectively) in group exposed for 112 days (D and F) were more significant ($P<0.005$) when compared to groups of rats exposed for 56 days (C and E). However, the GSH content in group E rats was not significantly ($P>0.05$) different from that of the control groups of rats.

From our study, we can only conclude that oxidative stress in testicular tissue might be responsible, at least in part, for perfume induced toxicity in animal model.

Keywords: Reactive Oxygen Species, Testicular toxicity, Male rats

1. Introduction

In 2001, Erin Weber a radio host lost her job for complaining she was allergic and sick from a co-worker's perfume [1].

Although most people complain about fragrance containing products, whether worn by someone else or displayed on shelves fragrance mainly affect the wearer [2]. Chances of developing allergic symptoms to fragrance ingredients later on is directly related to the level and frequency of exposure later in life [3].

About 100 fragrance ingredients have been reported to cause contact allergy [4].

In addition to reports associating these ingredients with various respiratory problems [5, 6, 7], contact allergy [8], fragrance components have been implicated in male infertility.

In our laboratory, we previously reported the pathologic effect of two Nigerian made perfume on the liver and testicular germ cells [10, 11]. Despite an avalanche of these reports, there has been a dearth of information on the possible mechanism of fragrance induced testicular toxicity. Because many fragrance chemicals are unsaturated volatile compounds (VOCs), they may react with indoor O_3 [ozone] to generate aldehydes, ketones and free radicals which causes oxidative stress [1].

Oxidative stress (OS) (which occur when the levels of free radical "engulf' the body's antioxidant defence system) is a state characterized by increase level of reactive oxygen species which results in cellular, tissue and organ degeneration [11, 12]. It must be reintegrated that although OS is fundamental in maintaining cellular homeostasis, intemperance OS results in cellular dysfunction.

OS has been one of the most investigated factors in medical science research and has virtually and numerously been implicated in various cases of male infertility [12].

It is not news that gametes are particularly vulnerable to oxidative insult. Plasma membrane of testicular cells contains lipids in the form of polyunsaturated fatty acids hence their vulnerability. It has been reported that in the presence of this particular lipid, ROS triggers a sequence of chemical reactions termed lipid peroxidation [13]. This is made worst as one of the main site of ROS production, the sperm plasma membrane lack cytoplasmic defences [14, 15].

The aldehyde 4-HNE, an alkylating agent and steady finish product of lipid peroxidation reacts with DNA and proteins thereby generating a variety of adducts capable of inducing specific cellular stress responses and apoptosis [16]. This is even pertinent as ROS has been shown to attack cells and tissues far from the site of free radical generation. The two main sites of ROS in semen are leukocytes and immature spermatozoa with the former normally present in the prostate and seminal vesicles and considered as the primary source of ROS [17-22].

To evaluate testicular oxidative stress, stable products like MDA, SOD, CAT, GSH, 4-HNE-modified proteins, 8-OHdG and nitrite/nitrate have been used as biomarkers.
In this study, we aimed to study the role of ROS in fragrance-induced testicular degeneration by evaluating testicular enzymatic and non-enzymatic antioxidants.

2. Materials and Methodology

In this study, two (2) commonly used perfumes in Nigeria designated as F1 and F2 were obtained from Bayous Cosmetics in Lagos on 23rd of April, 2012 and were kept under standard temperature. As described by Akunna *et al.*, [11], sixty adult male wistar rat (12-13 weeks old) weighing 190-220g were used for the study. The rats were randomly divided into six groups (A-F) of ten rats and the average weight difference between and within groups did not exceed ± 20% of the average weight of the sample population. Group A rats served as the first control (Control I) and were exposed (6hrs day^{-1}) to 5 ml kg^{-1} body weight of normal saline for 56 days. Group B rats served as the second control (Control II) group and were exposed (6hrs day^{-1}) to 5 ml kg^{-1} body weight of normal saline for a period of 112 days. Group C **(**Sub-chronic I**)** and Group D **(**Chronic I**)** rats were exposed (6hrs day^{-1}) to 5 ml kg^{-1} body weight of Fragrance I **(**F^1**)** for a period of 56 days and 112 days respectively. Group E (Sub-chronic II) and Group F **(**Chronic II**)** animals were exposed (6hrs day^{-1}) to 5 ml kg^{-1} body weight of Fragrance II (F^2) for a period of 56 days and 112 days respectively. The study is consistent with the standard of the use of laboratory animals. Small balls of cotton wool were soaked with the fragrance (Experimental Groups) and normal saline (Control Groups) respectively. The wools were then placed in a Petri dish inside the cages and covered with perforated plastic to prevent direct contact for an exposed duration of 6hrs day^{-1} throughout the period of study [11].

2.1 Animal sacrifice and sample collection

The rats were first weighed and then were sacrificed by cervical dislocation. The abdominal cavity was opened up through a midline abdominal incision to expose the reproductive organs. The testes were excised and trimmed of all fat. The testicular weights of each animal were evaluated with an electronic analytical and precision balance (BA 210S, d=0.0001- Sartoriusen GA, Goettingen, Germany). The testes volumes were measured by water displacement method. The two testes of each rat were measured and the average value obtained for each of the two parameters was regarded as one observation. One of the testes of each animal was fixed in 10% formol-saline for histological examination. Serum and the remaining testes of each animal were stored at – 25ºC for biochemical assays [11].

2.2 Assay of testicular enzymatic antioxidants

2.2.1Assay of catalase (CAT) activity

Catalse activity was measured according to the method of Aebi [23]. Testicular homogenate (0.1 ml) was pipetted into cuvette containing 1.9 ml of 50mM phosphate buffer, pH 7.0. Reaction was started by the addition of 1.0 ml of freshly prepared 30% (v/v) hydrogen peroxide (H_2O_2). The rate of decomposition of H_2O_2 was measured spectrophotometrically from changes in absorbance at 240 nm. Activity of enzyme was expressed as units /mg protein.

2.2.2 Assay of superoxide dismutase (SOD) activity

Superoxide dismutase activity was measured according to the method of Winterbourn *et al.* [24] as described by Rukmini *et al*. [25]. The principle of the assay was based on the ability of SOD to inhibit the reduction of nitro-blue tetrazolium (NBT). Briefly, the reaction mixture contained 2.7 ml of 0.067M phosphate buffer, pH 7.8, 0.05 ml of 0.12mM riboflavin, 0.1 ml of 1.5mM NBT, 0.05 ml of 0.01M methionine and 0.1 ml of enzyme samples. Uniform illumination of the tubes was ensured by placing it in air aluminum foil in a box with a 15W fluorescent lamp for 10 minutes. Control without the enzyme source was included. The absorbance was measured at 560nm. One unit of SOD was defined as the amount of enzyme required to inhibit the reduction of NBT by 50% under the specific conditions. Activity of enzyme was expressed as units /mg protein.

2.2.3 Assay of glutathione peroxidase (GPx) activity

Glutathione peroxidase activity was measured by the method described by Rotruck *et al*. [26]. The reaction mixture contained 2.0 ml of 0.4M Tris- HCl buffer, pH 7.0, 0.01 ml of 10mM sodium azide, 0.2 ml of enzyme, 0.2 ml of 10mM glutathione and 0.5 ml of 0.2mM. H_2O_2. The contents were incubated at 37^0C for 10 minutes followed by the termination of the reaction by the addition of 0.4 ml 10% (v/v) TCA, centrifuged at 5000 rpm for 5 minutes. The absorbance of the product was read at 430 nm. Activity of enzyme was expressed as units /mg protein.

2.3 Assay of testicular non-enzymatic antioxidants

2.3.1 Assay of testicular reduced glutathione (GSH) concentration

GSH was determined by the method of Ellman [27]. Testicular homogenate- supernatant (0.1 ml) was treated with 0.5 ml of Ellmans reagent (19.8 mg of 5, 5'-dithiobisnitro benzoic acid (DTNB) in 100 ml of 0.1% sodium nitrate) and 3.0 ml of phosphate buffer (0.2 M, pH 8.0). Distilled water (0.4 ml) was added. The mixture was thoroughly mixed and the absorbance was read at 412 nm. Reduced glutathione concentration was expressed as nmol/mg protein.

2.4 Estimation of lipid peroxidation (malondialdehyde)

Lipid peroxidation in the testicular tissue was estimated colorimetrically by thiobarbituric acid reactive substances (TBARS) method of Buege and Aust [28]. A principle component of TBARS being malondialdehyde (MDA), a product of lipid peroxidation. In brief, 0.1 ml of tissue homogenate in Tris-HCl buffer, pH 7.5 was treated with 2 ml of (1:1:1 ratio) TBA-TCA-HCl reagent (thiobarbituric acid 0.37%, 0.25 N HCl and 15% TCA) and placed in water bath for 15 min, cooled. The absorbance of clear supernatant was measured against reference blank at 535 nm. Concentration was calculated using the molar absorptivity of malondialdehyde which is 1.56 $x10^5$ M^{-1} cm^{-1} and expressed as nmol/mg protein.

3. Result and Discussion

In recent times experimental animal models have been employed in the study of oxidative stress and associated damage. However, to make clear the mechanisms pathway responsible for cellular degeneration and response, markers such as SOD, CAT, GSH, GPX and MDA are evaluated [29-31].

Unlike somatic cells, testicular cells are particularly susceptible to oxidative injury as a result of presence of high content of plasmalogens, sphingomyelins and polyunsaturated fatty acids (PUFA) with the latter being the main substrates for peroxidation [32].

Fragrances components have been shown to disrupt endocrine system, induce abnormal reproductive development in men and to cause decrease in testicular weights, seminiferous atrophy with vacuolization of sertoli cells and diminished sperm counts [11, 33-34].

We previously reported a decrease in sperm count, motility, normal sperm morphology and a significant increase in abnormal sperm morphology in rats exposed to fragrance [11].

SOD remains the first line of defence against ROS in the cell. In some species of mammals, the production of superoxide anion and SOD activity in spermatozoa remains similar [35]. However, there is excess production of superoxide anion and SOD in mice and humans. A reduction in the activity level of SOD indicates the occurrence of oxidative stress.

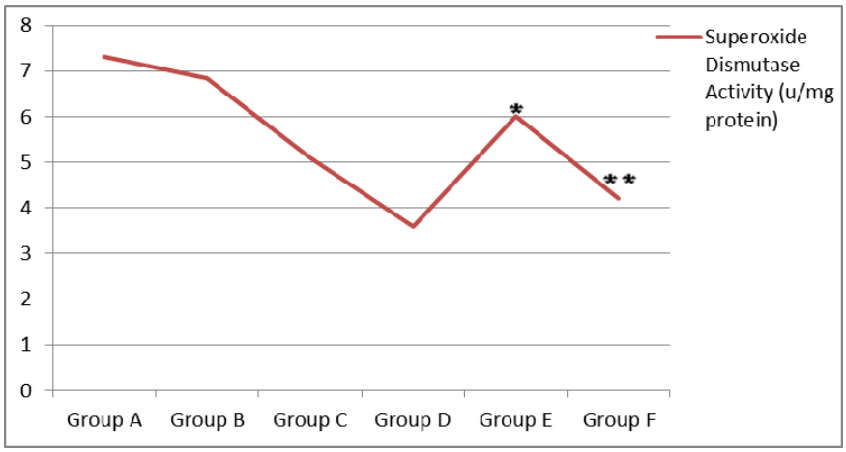

Fig 1: The effect of fragrance components (5 ml kg^{-1} b. wt for 56 days and 112 days) on testicular superoxide dismutase (SOD) activity. *P**<0.05, *P***<0.005 significantly different from control.

A: 5 ml kg^{-1} body weight normal saline (6hrs day^{-1}) for 56 days (Control I)
B: 5 ml kg^{-1} body weight of normal saline (6hrs day^{-1}) for 112 days (Control II)
C: 5 ml kg^{-1} body weight of F^{1} (6hrs day^{-1}) for 56 days (Sub-chronic I)
D: 5 ml kg^{-1} body weight of F^{1} (6hrs day^{-1}) for 112 days (chronic I)
E: 5 ml kg^{-1} body weight of F^{2} (6hrs day^{-1}) for 56 days (Sub-chronic II)
F: 5 ml kg^{-1} body weight of F^{2} (6hrs day^{-1}) for 112 days (Chronic II)

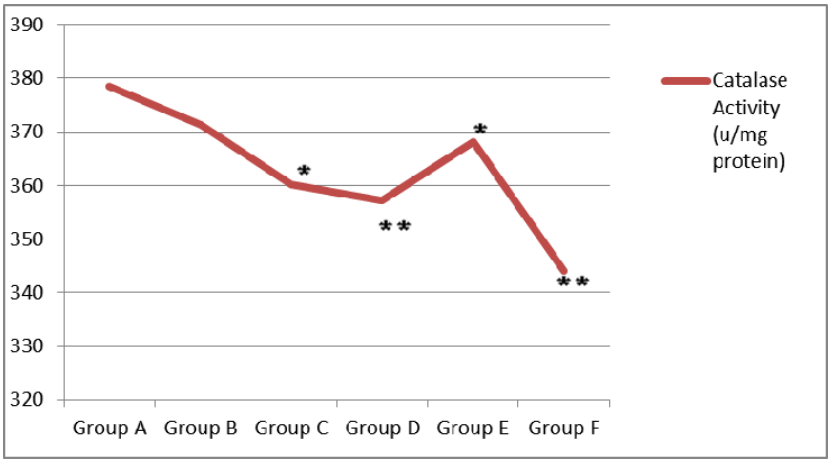

Fig 2: The effect of fragrance components (5 ml kg^{-1} b. wt for 56 days and 112 days) on testicular Catalase (CAT) activity. *P**<0.05, *P***<0.005 significantly different from control.

A: 5 ml kg^{-1} body weight normal saline (6hrs day^{-1}) for 56 days (Control I)
B: 5 ml kg^{-1} body weight of normal saline (6hrs day^{-1}) for 112 days (Control II)
C: 5 ml kg^{-1} body weight of F^{1} (6hrs day^{-1}) for 56 days (Sub-chronic I)
D: 5 ml kg^{-1} body weight of F^{1} (6hrs day^{-1}) for 112 days (chronic I)
E: 5 ml kg^{-1} body weight of F^{2} (6hrs day^{-1}) for 56 days (Sub-chronic II)
F: 5 ml kg^{-1} body weight of F^{2} (6hrs day^{-1}) for 112 days (Chronic II)

As shown in Figure 1 and 2, fragrance exposure to experimental rats resulted in a significant decrease in the activity level of SOD, CAT and GPx when compared to the control groups of rat. However, the decrease in level of these testicular enzymatic antioxidants in group D and F rats were more significant (*P<0.005*) when compared to that of rats in group C and E.

In the presence of external factors/toxins, extracellular superoxide anion can be converted to H_2O_2 and/or singlet oxygen which can diffuse across sperm membrane to induce lipid peroxidation hence reduction in sperm motility, concentration, and normal sperm morphology. Our findings are in parity with other reports [36].

Subsequently, the reduction in the activity level of CAT of rat exposed to fragrance might have allowed for additional conversion of peroxide to noxious hydroxyl radicals. This might have let to severe oxidative damage hence cellular apoptosis, viral proliferation and inflammatory reactions [37, 11].

GPx play an important role in protecting sperm membranes from the detrimental effects of lipid peroxidation [39-40].

Two different types of extracellular GPX isoenzymes within the epididymis have been reported: an epididymis-specific, selenium-independent GPX (GPX5) and plasma GPX (GPX3). These enzyme help by scavenging hydrogen peroxide from epididymal fluid [36].

GPx is secreted in the caput and cauda epididymides and constitutes 6 % of the secretory epididymal proteins [41-43].

In this study, fragrance exposure caused a significant decrease in the activity level of GPx when compared to the control groups of rat. However, the decrease in level of GPx in group D and F rats were more significant (*P<0.005*) when compared to that of rats in group C and E. Fragrance exposure could have induced testicular epididymal degeneration which could have resulted in the activity level of GPx seen in our study.

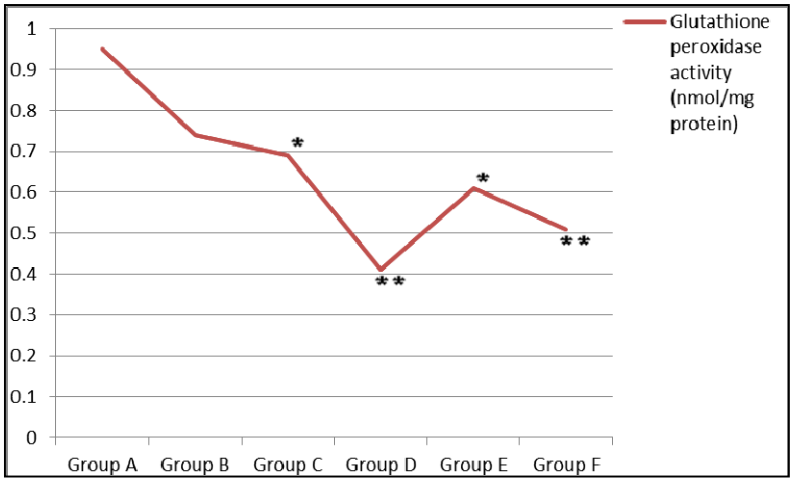

Fig 3: The effect of fragrance components (5 ml kg^{-1} b. wt for 56 days and 112 days) on testicular glutathione peroxidase (GPx) activity. *P**<0.05, *P***<0.005 significantly different from control.

A: 5 ml kg^{-1} body weight normal saline (6hrs day^{-1}) for 56 days (Control I)

B: 5 ml kg^{-1} body weight of normal saline (6hrs day^{-1}) for 112 days (Control II)

C: 5 ml kg^{-1} body weight of F^1 (6hrs day^{-1}) for 56 days (Sub-chronic I)

D: 5 ml kg^{-1} body weight of F^1 (6hrs day^{-1}) for 112 days (chronic I)

E: 5 ml kg^{-1} body weight of F^2 (6hrs day^{-1}) for 56 days (Sub-chronic II)

F: 5 ml kg^{-1} body weight of F^2 (6hrs day^{-1}) for 112 days (Chronic II)

Our report is consistent with findings indicating the sensitive nature of germinal epithelium to oxidative injury and cytotoxic agents [44-45].

We reported a significant (*P<0.005*) decrease in GSH content of rats in groups C, D and F when compared to that of the control groups of rat. However, the GSH content in group E rats was not significantly (*P*>0.05) different from that of the control groups of rats (Figure 4). GSH simultaneously catalyses the reduction of hydrogen peroxide to water and glutathione to oxidised glutathione [46]. Low activity of GSH evidenced in our study may be associated with fragrance exposure and an indication that fragrance induces toxicity via generation of free radicals [47].

In recent times, medical scientists have developed an interest in the pathological nature of oxidative stress and possible pathophysiology and cellular responses as a result of the fact that lipid peroxidation and oxidative damage to DNA and proteins occur at the cellular level [48].

Malondialdehyde, an endogenous genotoxic product of enzymatic and oxygen radical-induced lipid peroxidation remain one of the most frequently used biomarkers providing an indication of the overall lipid peroxidation level [49-51].

In this study, we estimated MDA by the thiobarbituric acid (TBA) test and results showed (Figure 5) that fragrance exposure to experimental rats resulted in a significant elevation in the level of MDA (a product of lipid peroxidation and an indication of oxidative stress) when compared to that of the control groups of rat. Nevertheless, the decrease observed in groups exposed for 112 days were more significant (*P<0.005*) when compared to groups exposed for 56 days (*P<0.05*) (C and E).

Our findings are in concordance with several other similar reports on lipid peroxidation and depletion of antioxidative defences of the body as a result of oxidative insult [52-54].

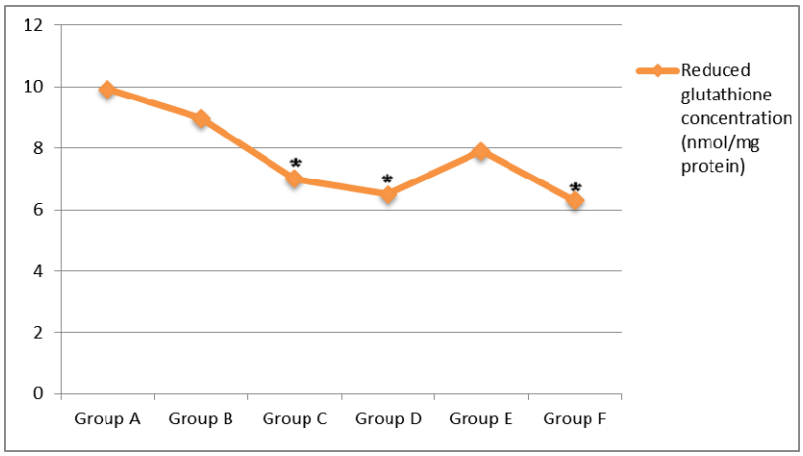

Fig 4: The effect of fragrance components (5 ml kg^{-1} b. wt for 56 days and 112 days) on testicular reduced glutathione peroxidase (GSH) activity. *P**<0.05, *P***<0.005 significantly different from control.

A: 5 ml kg^{-1} body weight normal saline (6hrs day^{-1}) for 56 days (Control I)
B: 5 ml kg^{-1} body weight of normal saline (6hrs day^{-1}) for 112 days (Control II)
C: 5 ml kg^{-1} body weight of F^{1} (6hrs day^{-1}) for 56 days (Sub-chronic I)
D: 5 ml kg^{-1} body weight of F^{1} (6hrs day^{-1}) for 112 days (chronic I)
E: 5 ml kg^{-1} body weight of F^{2} (6hrs day^{-1}) for 56 days (Sub-chronic II)
F: 5 ml kg^{-1} body weight of F^{2} (6hrs day^{-1}) for 112 days (Chronic II)

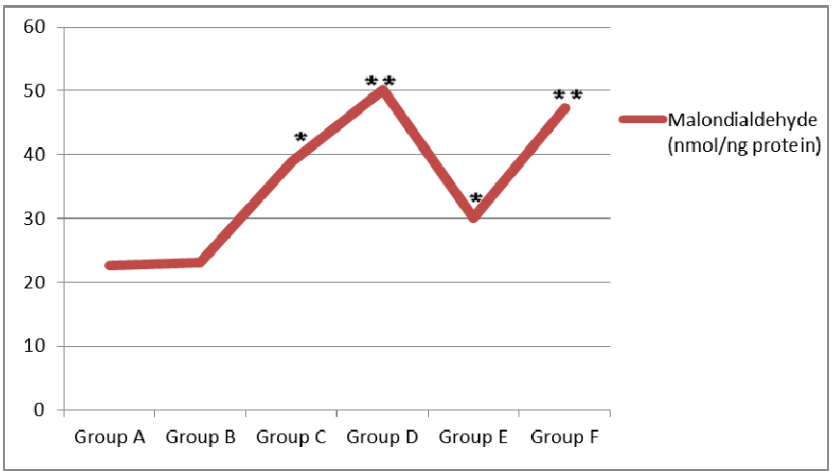

Fig 5: The effect of fragrance components (5 ml kg^{-1} b. wt for 56 days and 112 days) on testicular malondialdehyde (MDA) activity. *P**<0.05, *P***<0.005 significantly different from control.

A: 5 ml kg^{-1} body weight normal saline (6hrs day^{-1}) for 56 days (Control I)
B: 5 ml kg^{-1} body weight of normal saline (6hrs day^{-1}) for 112 days (Control II)
C: 5 ml kg^{-1} body weight of F^{1} (6hrs day^{-1}) for 56 days (Sub-chronic I)
D: 5 ml kg^{-1} body weight of F^{1} (6hrs day^{-1}) for 112 days (chronic I)
E: 5 ml kg^{-1} body weight of F^{2} (6hrs day^{-1}) for 56 days (Sub-chronic II)
F: 5 ml kg^{-1} body weight of F^{2} (6hrs day^{-1}) for 112 days (Chronic II)

For oxidative stress to occur, molecular oxygen is reduced to superoxide anion ($O_2^{\cdot-}$), which is converted to other forms of reactive oxygen species such as hydrogen peroxide (H_2O_2) and the more dangerous hydroxyl free radical (OH·) which then induce cellular membrane and macromolecular damage [55]. From our study, we can only conclude that oxidative stress in testicular tissue might be responsible, at least in part, for perfume induced toxicity in animal model.

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