



(RESEARCH ARTICLE)



## Hot-humid season restraint stress duration on sperm indices and oxidative biomarkers on rats

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### Abstract

Restraint stress is usually occasioned by excessive production of reactive oxygen species (ROS) thereby causing potential damage to the normal spermatozoa by inducing lipid peroxidation. This study investigated the possible time in which restraint stress induces maximum effects on sperm motility, concentration, and oxidative stress markers in male Wistar rats. Animals were randomly divided into three groups of three animals each (n=3). Group I- normal control (undisturbed), Group II- 3 h stress group, Group III- 6 h stress group. Restraint stress was induced by placing rats in specially constructed restraint meshes for both 3 and 6 hours (between 9.00-15.00 h) for 21 days. Testes homogenate were evaluated for Malondialdehyde (MDA), catalase (CAT), superoxide dismutase (SOD) and reduced glutathione (GSH). The 3 h stress group showed a significant decrease in sperm motility and concentration ( $5.4 \pm 0.24$ ) when compared to the normal control group ( $10.0 \pm 0.45$ ) and 6 h stress group ( $7.3 \pm 0.89$ ) ( $P < 0.05$ ). Furthermore, there was a significant increase in SOD and CAT activities in the normal control group ( $3.33 \pm 0.09$ ;  $60 \pm 1.20$ ) when compared to the 3 h and 6 h stress groups ( $2.13 \pm 0.09$ ;  $34 \pm 1.20$ ) and ( $2.37 \pm 0.09$ ;  $54 \pm 1.2$ ) respectively ( $P < 0.05$ ). Restraint stress for both 3 and 6 h induces oxidative stress which might lead to a decrease in sperm motility and concentration; however, 3 h of stress induces more oxidative damage among male Wistar rats.

**Keywords:** Biomarkers; Oxidative stress; Restraint stress; Sperm motility; Sperm concentration

### 1. Introduction

Stress threatens the normal body functions and may arise from environmental, physiological and psychological extraneous factors [1]. The psychological extraneous factor may be induced using restrainers which include cages or tubes, known as restraint or immobilization stress [2]. Restraint stress is a model of psychological stress associated with the induction of harm that affects internal organs [3]. The resultant effects it has on organ is been precipitated by the conditioned environment, considering the season variation that affects homeostasis one will consider seasons such as the hot-humid season (May-October), in Nigeria notably to have the highest recorded amount of average rainfall ( $155.9 \pm 26.2$  mm), high-temperature ( $30.2 \pm 0.6$  °C), high relative humidity ( $63.2 \pm 5.6$  %) and low evaporations rate ( $154.2 \pm 9.71$  mm) [4]. With these dual effect affecting several organs in the body, one is expected to ask the impact it may have on the continuous existence of man which the reproductive organ serves.

Over the years, the reproductive system in animals has been observed to be prone to the adverse effect of psychological stress, inducing harmful effects on both male and female reproductive organs, thereby threatening procreation [5][6].

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A study on female mice showed that restraint stress inhibits mouse implantation in a temporal window-dependent manner by impairing blastocyst activation, hatching, and uterine receptivity *via* down-regulating oestrogen and progesterone [6]. In males, restraint stress suppressed the male reproductive functions, inducing a rise in corticosterone, norepinephrine, and fall in serum testosterone concentration; thereby enhancing testicular germ cell apoptosis [7][8]. It has been reported to also induce a significant elevation in plasma adrenocorticotrophic hormone, prolactin, and progesterone; decrease follicle-stimulating hormone, luteinizing hormone and immunoreactive (IR-) inhibin; suppress sperm motility, increase the hypothalamus-pituitary-adrenal axis activity, and disturb hypothalamus-pituitary-gonadal axis activity [9]. One of the underpinned reactors in the aforementioned rampaging effect of stress is the free-radicals; these free radicals may have positive, negative or neutral effects. Reactive oxygen species (ROS) plays crucial roles in capacitation, acrosome reaction, and fertilization. However, during restraint stress, these reactive species are produced in excess leading to cellular damage by inducing lipid peroxidation and deoxyribonucleic acid (DNA) damage [10]. The present study is aimed at investigating the possible time in which restraint stress induces maximum effects on sperm motility, concentration, and oxidative stress markers in male Wistar rats.

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## 2. Material and methods

### 2.1. Animals

Twelve young adults male Wistar rats weighing between 140-150 g were utilized for the experiment [11] [12]. They were obtained from the animal house of the Department of Human Physiology, Faculty of Medicine, Ahmadu Bello University, Zaria, Nigeria, and were fed with rat chow and water *ad libitum*. All the procedures on the use of these animals were by Ahmadu Bello University Animal Ethical Committee (ABUAEC), with ethical clearance been issued.

### 2.2. Experimental groups

The rats were divided into three groups consisting of 3 animals each (n=3): The rats in Group I were kept undisturbed and served as the control group, those in Group II were exposed to stress (3 h stress), while those in Group III were exposed to stress (6 h stress) for 21 days.

### 2.3. Methodology

Rats in Group II were subjected to 3 h daily restraint stress and Group III for 6 h daily restraint stress both for 21 days. One rat per restraint wire mesh cage restrainer, of dimension 8 cm (length) x 4 cm (Breadth) x 4 cm (High) was used for the experiment, and a key lock and latch were used to secure the rat in restrainer from escaping. The study was carried out between 9.00-15.00 h with proper monitoring to avoid injury, as they were deprived of food and water, both during 3 h and 6 h restraint. At the end of 21 days, the animals were decapitated, and samples were collected to evaluate the effect of stress timing on sperm motility, concentration, and makers of oxidative stress of restraint male Wistar rats.

### 2.4. Sperm motility

The caudal epididymis was cut into small pieces and transferred into the Petri dishes containing 0.5 Tris buffer solution, and the sperm cells were allowed to swim out within five minutes, at 37 °C. An aliquot of this solution was observed under the light microscope using the magnification of 400 fold. The percentage of sperm cell motility was calculated using the number of live sperm cells over the total number of sperm cells, both motile and non-motile [13].

### 2.5. Sperm concentration

The epididymis was minced with anatomical scissors in 5 ml physiological saline, placed in a rocker for 10 mins and allowed to incubate at room temperature for 2 mins. After incubation, the supernatant fluid was diluted 1:100 with a solution containing 5g of sodium bicarbonate and 1mL formalin (35%). The total sperm number was determined by using improved Neuber's counting chamber (hemocytometer). Approximately, 10 ml of the diluted sperm suspension was transferred to each counting chamber of the hemocytometer and was allowed to stand for 5 min. This chamber was then placed under a binocular light microscope using an adjustment light source [14].

## 2.6. Assessment of oxidative stress bio-makers

### 2.6.1. Lipid peroxidation

The level of thiobarbituric-acid (TBA) reactive substance, malondialdehyde (MDA), as an index of lipid peroxidation was evaluated. Quantitative measurement of lipid peroxidation of MDA was determined using the NWLSS™ MDA assay kit (Northwest Life Sciences Specialties, Product NWK-MDA01, Vancouver WA, specificity: Malondialdehyde, Sensitivity: 0.08  $\mu$ M). The principle is based on the reaction of MDA with TBA; forming an MDA-TBA<sub>2</sub> adduct that absorbs strongly at 532 nm [15].

### 2.6.2. Superoxide dismutase (SOD) activity

The activity of superoxide dismutase (SOD) in the rat serum was determined using the NWLSS SOD assay kit (product NWK-SOD02, Specificity: Cu/Zn, Mn, and Fe Superoxide Dismutase, Sensitivity: 5 U/mL). The assay kit is based on the principle of superoxide inhibition of autoxidation of hematoxylin as described in this study [16].

### 2.6.3. Catalase activity

Catalase (CAT) activity was assessed using the NWLSS CAT activity assay kit (Product NWK-CAT01, Specificity: Catalase, Sensitivity: 6.0 Catalase/mL). Catalase enzyme activity was measured based on the principle of catalase consumption of the H<sub>2</sub>O<sub>2</sub> substrate at 240 nm

### 2.6.4. Glutathione peroxidase activity

Glutathione peroxidase (GPx) activity was assessed using NWLSS™ cGPx (GPx1) ELISA assay kit (Enzyme-Linked Immunosorbent Assay) (Product NWK-GPX02, specificity: Glutathione peroxidase, Sensitivity: 12.5 pg/ml). The NWLSS™ cGPx assay is based on ELISA, where sample GPx concentration is determined by comparing the 450 nm absorbance of sample wells to the absorbance of known standards [17].

## 2.7. Statistical analysis

The data obtained were expressed as mean  $\pm$  standard error of the mean (mean  $\pm$  SEM) and analyzed using analysis of variance (ANOVA) with Turkey's *posthoc* test. The statistical package used was Graphpad Prism version 5.0. (San Diego California, USA). Values of  $P < 0.05$  were considered significant.

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## 3. Results

### 3.1. Sperm motility

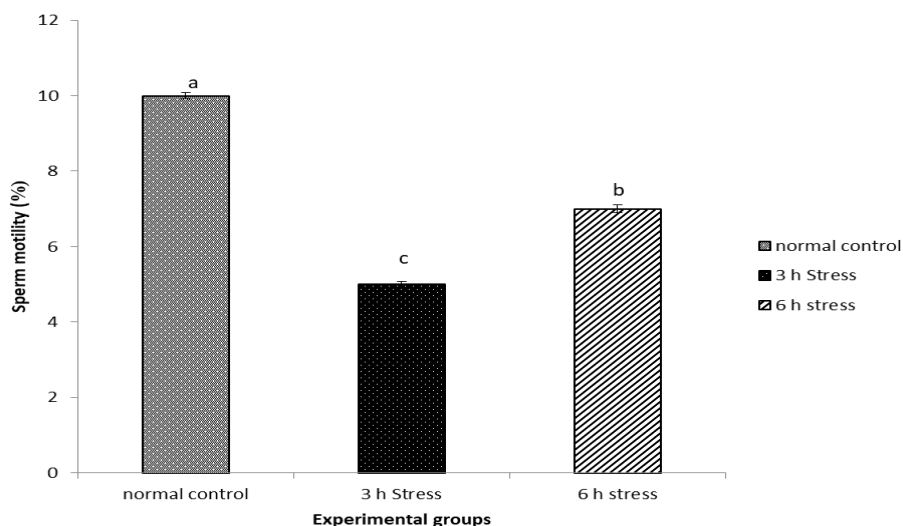
The results obtained showed a significant decrease in motile sperm of normal control ( $10.0 \pm 0.45$ ) when compared with both 3 h ( $5.4 \pm 0.24$ ) and 6 h ( $7.3 \pm 0.89$ ) stress groups ( $P < 0.05$ ), however, no significant difference was observed between 3 h and 6 h stress, as shown in Figure 1.

### 3.2. Sperm cell concentration

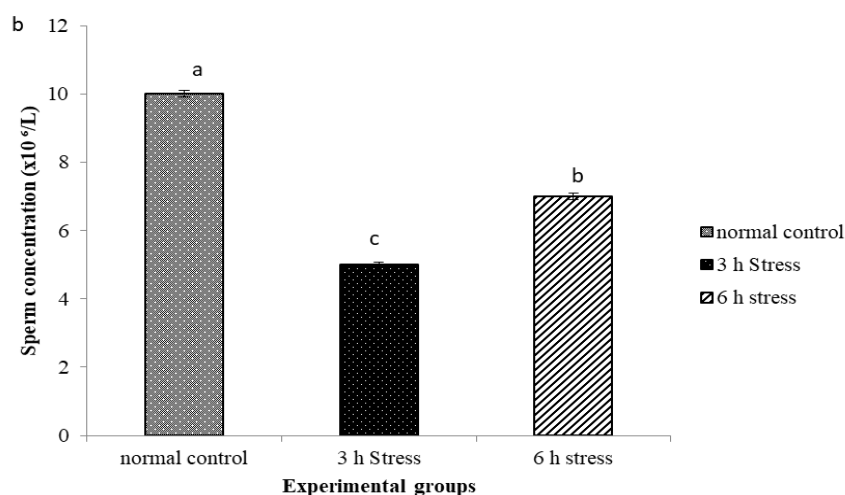
A significant decrease ( $P < 0.05$ ) was observed in sperm concentration in the 3 h stress group when compared with 6 h stress and normal control groups, as presented in Figure 2. Also, there was a significant increase in sperm concentration in the normal control group when compared to the 6 h stress group ( $P < 0.05$ ).

### 3.3. Effects of stress timing on oxidative stress biomarkers in restrained Wistar rats

A significant increase in the activities of SOD and CAT were observed in the normal group ( $3.33 \pm 0.09$ ;  $60 \pm 1.20$ ) and 6 h stress group ( $2.37 \pm 0.09$ ;  $54 \pm 1.2$ ) when compared to the 3 h stress group ( $2.13 \pm 0.09$ ;  $34 \pm 1.20$ ) ( $P < 0.05$ ) respectively, as presented in Table 1. Besides, there was a significant increase in the level of MDA ( $2.6 \pm 0.09$ ) in the 3 h stress group when compared to normal control ( $0.1 \pm 0.12$ ) ( $P < 0.05$ ).



**Figure 1** Effect of stress timing on sperm motility in Wistar rats subjected to restraint stress. Data are expressed, in mean ± SEM. a, b, c = means with different superscript letters are significantly different (P < 0.05).



**Figure 2** Effect of stress timing on sperm cell concentration in Wistar Rats subjected to restraint stress. Data are expressed, in mean ± SEM. a, b, c = means with different superscript letters are significantly different (P < 0.05).

**Table 1** Effects of stress timing on oxidative stress biomarkers in restrained Wistar rats

Protein (IU/L)	Group I	Group II	Group III
SOD	3.33±0.09a	2.13±0.09b	2.37±0.09a
CAT	60±1.20a	34±1.20b	54±1.2a
GSH	59±1.3a	38±1.2b	45±1.3
MDA	0.1±0.12b	2.6±0.09a	2.2±0.07

Data are expressed, in mean ± SEM. a, b = means with different superscript letters within rows are significantly different (P<0.05); Group I = control, Group II = 3 h stress group, Group III = 6 h stress group.

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#### 4. Discussion

Sperm motility, which is an indicator of the presence of active moving sperm in the ejaculate, is critical in ensuring the transportation of sperm cell through the vaginal to bring about fertilization; due the unstoppable exposure of stress which leads to exaggerated production of ROS; the motility of sperm is under constant threat [18]. Increase production of reactive species is being linked with exposure to psychological stress which is been mimicked in the laboratory by restraining animals [3] [19]. Findings from our study, has shown that there is a significant decrease in sperm motility in Wistar rats when exposed to restraint stress for 3h compared to the 6 h stress exposure (Figure 1). This finding correlates with a previous study [9], which demonstrated that acute restraint stress reduced sperm motility starting after 30 min. However, the decreased effect observed in the 6 h restraint group might be due to adaptation to stress stimulus. Besides, findings from our study (Figure 2) indicate that the sperm concentration in the 3 h stress group was significantly lower when compared to the normal control and 6 h stress groups [20].

Regarding the analysis of oxidative stress biomarkers (Table 1), the malondialdehyde level is a determinant of lipid peroxidation [21]. In this study, findings revealed that the malondialdehyde level was statistically higher in the 3 h stress group ( $2.6 \pm 0.09$ ) when compared to the normal control group ( $0.1 \pm 0.12$ ) ( $P < 0.05$ ). This result agrees with the findings reported from a previous study which showed that fewer time exposures of restraint stress for 30 days induces lipid peroxidation in albino rats [22]. In this present study, we observed that SOD activity was significantly lowered in the 3 h stress group when compared to normal control ( $P < 0.05$ ). This correlates with the result of an earlier study which showed that immobilization stress decreases the activity of SOD [23]. A significant decrease in catalase activity was observed in the 3 h stress group when compared to the normal control group ( $P < 0.05$ ). This finding is in contrast with the report from a previous study [24], which showed that 3 h of restraint stress increased catalase activity. Results obtained for reduced glutathione showed a lesser value in the 6 h stress group when compared with the 3 h stress group, and this agreed with the findings from an earlier study [25].

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#### 5. Conclusion

Restraint stress induces oxidative stress which may lead to decrease sperm motility and concentration. Findings obtained from this study showed that 3 hours of restraint stress alters sperm indices and biomarkers of oxidative stress when compared to 6 hours of restraint timing. We suggest that the alteration of some sperm indices and oxidative biomarkers in 6 h restraint timing may be due to the rats' adaptability to restraint stress when exposed for a longer period.

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#### Compliance with ethical standards

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##### *Disclosure of conflict of interest*

We declare that we have no conflict of interest.

##### *Statement of ethical approval*

All the procedures on the use of these animals were by Ahmadu Bello University Animal Ethical Committee (ABUAEC), with ethical clearance been issued.

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