

## GENERATION OF REACTIVE OXYGEN SPECIES IN RAT EPIDIDYMAL SPERMATOZOA AFTER CYCLOPHOSPHAMIDE TREATMENT

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### ABSTRACT

**Objective:** The aim of this study was to examine the effect of an anticancer drug cyclophosphamide (CPA) on generation of reactive oxygen species (ROS) in epididymal spermatozoa of Wistar rat, *Rattus norvegicus*.

**Methods:** For this purpose, the rats were injected intraperitoneally with different doses of CPA (5 mg, 15 mg, and 20 mg/Kg BW for 2 weeks).

**Results:** Treatment resulted into significantly increased level of ROS in CPA-treated groups when compared to the vehicle-treated control group.

**Conclusion:** The results revealed that CPA has deleterious effect on the sperm morphology and physiology, which is dose and duration dependent and at certain doses cause the production of a number of reactive molecules and free radicals derived from molecular oxygen consequently resulting into adverse effect on the sperm function and hence on reproduction.

**Key words:** Reactive oxygen species, Free radical, Cyclophosphamide, Spermatozoa.

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### INTRODUCTION

Reactive oxygen species (ROS) is a phrase used to describe a number of reactive molecules and free radicals derived from molecular oxygen or highly reactive oxidizing agents belonging to the class of free radicals [1-3]. Epididymal sperm maturation is considered as an essential process. Several modifications occur during transformation of immature testicular spermatozoa to mature epididymal gametes capable of fertilization. One of the modifications is changes in their capacity for ROS generation and tyrosine phosphorylation that are thought to be related to the ability of the spermatozoa to engage in the process of sperm capacitation [4-6]. ROS plays a significant role in sperm physiology. Production of ROS by spermatozoa has been associated with the loss of sperm motility, sperm hyperactivation, decreased capacity for sperm-oocyte fusion, and loss of fertility.

ROS such as the superoxide anion, hydrogen peroxide, and nitric oxide at low-level facilitate hyperactivation, capacitation, acrosome reaction, motility, fertilization, and oocyte adhesion of spermatozoa [7], but higher ROS damages a variety of biomolecules such as lipids, amino acids, carbohydrates, proteins, DNA, and adversely affect the sperm function [8,9].

Anticancer drug cyclophosphamide (CPA) belonging to the class of oxazaphosphorines is a bioactivated metabolite and alkylating agent that show cytostatic effects by forming covalent DNA adducts. Treatment with cytotoxic chemotherapy is associated with significant reproductive damage, and alkylating agents are the most common agent implicated in the development of infertility [10,11].

To prove the development of infertility due to anticancer drug CPA, the present study was conducted by generation of ROS in rat epididymal spermatozoa.

### METHODS

#### Chemicals

The anticancer drug CPA (Endoxan-N, CAS no. 50-18-0), with the chemical formula  $C_7H_{15}Cl_2N_2O_2P$  and molecular weight, 261.086 g/mol,

manufactured by Cadila Healthcare Limited, Goa. All other reagents were obtained from Sigma Chemical Company.

#### Experimental animals

Wistar albino rats (*Rattus norvegicus*) with average body weight of 250–300 g obtained from the Department of Biochemistry, R. T. M. Nagpur University, Nagpur, were used for the study. Animals were housed in polypropylene box type cages, bedded with rice husk under controlled environmental condition and humidity controlled with free access to food and water. All experimental procedures were carried out under strict compliance with the Institutional Animal Ethical Committee according to the guidelines of the Committee for the Purpose of Control and Supervision of Experimental Animals.

#### Treatments

Animals were allowed to acclimatize for a period of week before being treated. They were selected randomly and divided into two groups with six animals in each group, vehicle-treated control group and experimental group. The experimental group animals were administered with 5 mg, 15 mg, and 20 mg/Kg BW/day of CPA for 2 weeks intraperitoneally and the vehicle-treated group with equal volume of saline.

#### Spermatozoa sample preparation for ROS

The animals were sacrificed 24 h after the last day of each experiment. The cauda epididymis was removed, cleared of fat and connective tissue, rinsed in Dulbecco's phosphate-buffered saline (PBS) solution, and cut into pieces. The initial characteristics such as sperm count and motility were recorded. Each piece of tissue was transferred to small Petri dishes, containing 3ml of Dulbecco's PBS solution prewarmed to 37°C. The epididymis was incubated for 10 min to allow diffusion of the spermatozoa. The remaining tissues were then removed, and the cells were counted and adjusted to  $10 \times 10^6$  spermatozoa/ml.

#### Estimation of ROS by chemiluminescence assay

ROS levels of rat spermatozoa were measured using luminol-peroxidase-dependent chemiluminescence assay [12]. In this method, Luminol

(5-amino-2,3-dihydro-1,4-phthalazinedione) is used as a probe to assess ROS generation by spermatozoa. Chemiluminescence occurs when excited electrons return to ground state. With this protocol, sample was centrifuged at 300 rpm for 5 min, and after washing the pellet with PBS it was resuspended in the same washing media at a concentration of  $10 \times 10^6$  sperm per ml. 4  $\mu$ L of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) prepared as 5 mm stock in dimethyl sulfoxide, was added to the mixture and served as a probe. The luminol-dependent chemiluminescence was enhanced by the addition of horseradish peroxidase. Chemiluminescence is measured in the integration mode using a luminometer (Luminoskan Ascent - Thermo Scientific) at 37°C for 2 min after the luminol was added. ROS production was expressed as counted photons per minute (cpm)/ $10 \times 10^6$  spermatozoa.

### Statistical analysis

The data were analyzed statistically following the method adopted by Delgaard [13]. Standard deviation and probability test, i.e., t-test were employed to know the levels of significance. A statistical significance level of  $p < 0.05$  was accepted.

### RESULTS

Measurement of ROS generation in epididymal spermatozoa was undertaken using chemiluminescence. The spontaneous ROS generation exhibited by CPA-treated groups (5 mg, 15 mg, and 20 mg/ Kg BW for 2 weeks) was significantly greater than the level shown by the vehicle-treated control group (Fig. 1).

### DISCUSSION

ROS measurement appears to be a helpful tool in the initial evaluation and follow-up of infertile male patients because high oxidative stress seems to be strongly correlated with low fertility [14]. Adequate amount of ROS is required to acquire fertilizing capacities in male, but higher ROS level damages a variety of biomolecules such as lipids, amino acids, carbohydrates, proteins, and DNA and causes apoptosis in sperm, also adversely affect the sperm function leading to infertility in men [15,16].

The present study demonstrated the adverse effect of CPA on testicular gametogenic and androgenic activities. A significant difference in the level of ROS between the vehicle-treated control and rats treated with CPA was also described by Venkatesh *et al.* [17]. In addition, ROS may also affect the sperm axoneme by affecting microtubules assembly, inhibits mitochondrial function and affects the synthesis of DNA, RNA, and proteins [18], decreased axonemal protein phosphorylation, sperm immobilization (as in the present study dose 20 mg/Kg BW CPA), by the inhibition of glucose-6-phosphate dehydrogenase [19]. Moreover, ROS has also been implicated to cause mutation or polymorphism in both nuclear and mitochondrial DNA of spermatozoa [14]. Our results are also in consonance with the previous workers on CPA-induced testicular toxicity [20-24].

The administration of CPA may have resulted into electron leakage from defective sperm mitochondria at complexes I and II, and hence, could

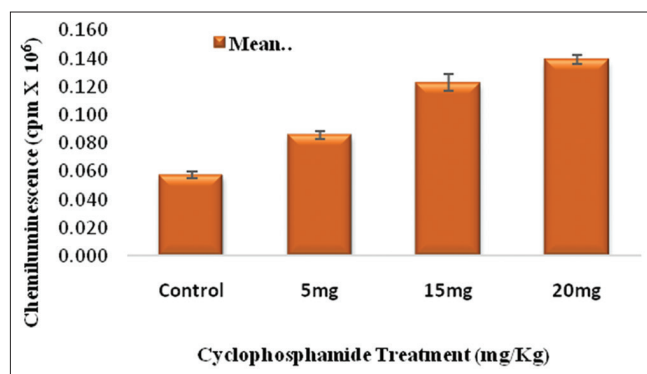


Fig. 1: Spontaneous generation of chemiluminescence by rat spermatozoa following cyclophosphamide treatment

be another potential source of oxygen radicals as described by Vernet *et al.* Baker and Aitken [12,25]. Our results are also in consonance with Anderson *et al.* [26] after the administration of estrogenic compounds in human spermatozoa, nickel-induced oxidative stress in testis of mice [27] after the effect of nitric oxide on human spermatozoa activity [28], or during infertility [17,29] or infiltration of leukocytes [30,31], during infections in the male genital tract, or in a condition of varicocele [32], or in patients with spinal cord injury [33] or chronic prostatitis [34], urogenital inflammation [35], or by short-term exposure to organic hydroperoxides *in vivo* in rat model since elevated levels of ROS can induce oxidative damage to DNA which is of potential risk to reproduction [36], ascorbic deficiency, and smoking may result into male reproductive dysfunctions [37] or tertiary butyl hydroperoxide [38], cryptorchidism [39], hypogonadism [40] or genetic nuclear, and mitochondrial [41].

### CONCLUSION

Our results demonstrate that the luminol-dependent chemiluminescence assay for ROS measurement is both accurate and reliable, and easy and rapid. Moreover, the obtained results also emphasizes that CPA has deleterious effect on the sperm morphology as observed in the sperm analysis [42] and physiology, which is dose and duration dependent and at certain doses cause the production of a number of reactive molecules and free radicals derived from molecular oxygen consequently resulting into adverse effect on the sperm function and hence on reproduction.

### AUTHOR'S CONTRIBUTIONS

Both the authors contributed to the study design, manuscript preparation, and critical revision of the manuscript and finalization of the manuscript. ZK responsible for laboratory experiments, data collection, and analysis. Both the authors agree with the content of the manuscript.

### CONFLICT OF INTEREST

There is no conflict of interests.

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