https://doi.org/10.33451/florafauna.v31i2pp283-290
FLORA AND FAUNA ISSN 2456 - 9364 (Online)
2025 Vol. 31 No.2 PP 283-290 ISSN 0971 - 6920 (Print)

Exposure to cyclophosphamide induces male infertility in rats: A duration dependent investigation

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Received **:** 05.09.2025; ***Accepted*** : 28.10.2025

How to cite : Mukherjee A, Nirala SK, Bhadauria M.Exposure to cyclophosphamide induces male infertility in rats: A duration dependent investigation. *Flora and Fauna* 2025. 31(2) : 283-290

ABSTRACT

Though cyclophosphamide is used for treating a variety of cancers, a need is felt to identify its toxic effects on vital organs. In this investigation, detrimental effects of cyclophosphamide at varying duration of exposure were examined. A dose of 200 mg/kg of cyclophosphamide was administered intraperitonially at 12-, 24- and 48-hours duration in *Wistar* male rats. Total weight, testes weight, serum testosterone, epididymal sperm count, sperm motility and non-motility, and sperm morphology were observed to determine detrimental effect of cyclophosphamide. One-way ANOVA followed by Tukey's *post hoc* analysis was used to assess statistical significance at 5% level. Exposure to cyclophosphamide induces male infertility in duration dependent manner by significantly decreasing serum testosterone, epididymal sperm count and number of motile sperms, increasing non-motile sperms and inducing morphological abnormalities in sperm.

Figures : 04 References : 22 Tables : 03
KEY WORDS : Cancer, Cyclophosphamide, Exposure, Infertility, Wistar Rats

Introduction

Cancer is a vast category of diseases defined by uncontrolled cell proliferation and metastasis. It is one of the second leading causes of death and accounted for around 14. 57% of all deaths worldwide in 2021[22]. The treatment options generally include surgery, radiotherapy, chemotherapy, targeted biological therapies, *etc.*[15]. Chemotherapy has immense importance among treatment options against cancer.

Cyclophosphamide (CP), used as an antineoplastic agent to treat many malignant and non-malignant forms of cancer, is an alkylating agent with the ability to disrupts mitotic cycles[17]. Cyclophosphamide is used as an antineoplastic agent to treat malignant and non-malignant cancer due to its alkylating property to disrupt mitotic cycles[17]. It also causes serious damages to non-cancerous cells[6]. Acrolein, one of the by-products of cyclophosphamide, has been reported for its detrimental effects on metabolic responses and ability to produce oxidative stress in tissues[4,11,14]. Apart from extensive use of cyclophosphamide against a variety of cancer forms, a need is felt to identify its toxic effects on vital organs so that a suitable strategy may be adopted to treat cancer cases with minimal side effects. Thus, an attempt has been made to investigate deleterious effects of cyclophosphamide on reproductive organs at different durations of its exposure in rat model.

Materials and Methods

Chemicals:

Cyclophosphamide (Lot no. OMUBE) was purchased from Tokyo Chemical Industry Company Ltd. Japan and diagnostic kit to determine testosterone from Epitope Diagnostics Inc. (U.S.). Preparation, dose and route of exposure to cyclophosphamide were based on

TABLE-1 : Summary of experimental procedure

Groups	Treatments	Cyclophosphamide			
		Day 1 7 a.m.	Day 2 7 a.m.	Day 2 7 p.m.	
Group I (Control)	Vehicle	X	X	X	Euthanised Day 3 (7 a.m.)
Group II (12 hours)	CP (200 mg/kg)	X	X	✓	
Group III (24 hours)	CP (200 mg/kg)	X	✓	X	
Group IV (48 hours)	CP (200 mg/kg)	✓	X	X	

Abbreviation: CP = Cyclophosphamide

published literature [19].

Animals and ethical permissions

Wistar albino male rats (12 weeks old, b. wt. 150 ± 10g) were maintained under standard husbandry conditions with pelleted feed and water *ad libitum*, 25 ± 2° C temperature, 60–70% relative humidity, 12 hours light-dark cycle at all times. Experimental design was formed following guidelines set by CPCSEA and prior approval (341/IAEC/Pharmacy/2022) was obtained from Institutional Animal Ethics Committee (994/GO/Re/S/06/CPCSEA).

Experimental design

Twenty-four rats were randomly assigned into four groups with six animals in every group and treated as following and as summarised in Table-1.

Group I: Control, administered saline (*i.p.*)

Group II: 12 Hours duration, administered cyclophosphamide (200 mg/kg, *i.p.*), on day 2 at 7.00 pm.

Group III: 24 Hours duration, administered cyclophosphamide (200 mg/kg, *i.p.*), on day 2 at 7.00 am.

TABLE-2: Gravimetric analysis and testosterone level

Parameters	Control	12 Hrs	24 Hrs	48 Hrs	ANOVA F Value
Animal weight (g)	150 ± 8.25	153 ± 7.92	155 ± 8.14	145 ± 7.37	0.361
Testes weight (g)	1.01 ± 0.06	1.04 ± 0.07	1.08 ± 0.06	0.992 ± 0.06	0.487
Relative organ weight (g)	0.676 ± 0.08	0.680 ± 0.05	0.694 ± 0.06	0.685 ± 0.07	0.016
Testosterone (ng/ml)	48.1 ± 2.43	38.3 ± 1.95$^{\alpha}$	29.1 ± 1.50$^{\alpha\beta}$	22.3 ± 1.17$^{\alpha\beta\gamma}$	45.4$^{\$}$

Data are expressed as mean ± S.E. (n = 6). $^{\$}$Represents ANOVA significant at 5%. $^{\alpha}$Control vs 12 Hrs, 24 Hrs and 48 Hrs; $^{\beta}$12 Hrs vs 24 Hrs and 48 Hrs; $^{\gamma}$24 Hrs vs 48 Hrs for Tukey's HSD *post hoc* analysis at p d" 0.05.

TABLE-3: Toxic effects of cyclophosphamide on sperm count and motility

Parameters	Control	12 Hrs	24 Hrs	48 Hrs	ANOVA F Value
Count (10^6/ml)	4.45 ± 0.25	2.75 ± 0.15$^{\alpha}$	2.65 ± 0.15$^{\alpha}$	2.37 ± 0.13$^{\alpha}$	34.9$^{\$}$
Motility (%)	90.5 ± 5.00	65.5 ± 3.62$^{\alpha}$	63.5 ± 3.51$^{\alpha}$	61.0 ± 3.37$^{\alpha}$	14.6$^{\$}$
Non-motility (%)	9.50 ± 0.53	34.5 ± 1.91$^{\alpha}$	36.5 ± 2.02$^{\alpha}$	39.0 ± 2.16$^{\alpha}$	71.4$^{\$}$

Data are expressed as mean ± S.E. (n = 6). $^{\$}$Represents ANOVA significant at 5%. $^{\alpha}$Control vs 12 Hrs, 24 Hrs and 48 Hrs; $^{\beta}$12 Hrs vs 24 Hrs and 48 Hrs; $^{\gamma}$24 Hrs vs 48 Hrs for Tukey's HSD *post hoc* analysis at p d" 0.05.

Group IV: 48 Hours duration, administered cyclophosphamide (200 mg/kg, *i.p.*), on day 1 at 7.00 am.

The animals were euthanized on day 3 at 7.00 am after the completion of respective durations. Blood was drawn and testes along with epididymis was excised immediately and cleaned of adjoining fat for evaluation of organ weight and sperm related parameters.

Gravimetric analysis

Weight of testes from individual rats were noted to observe any change.

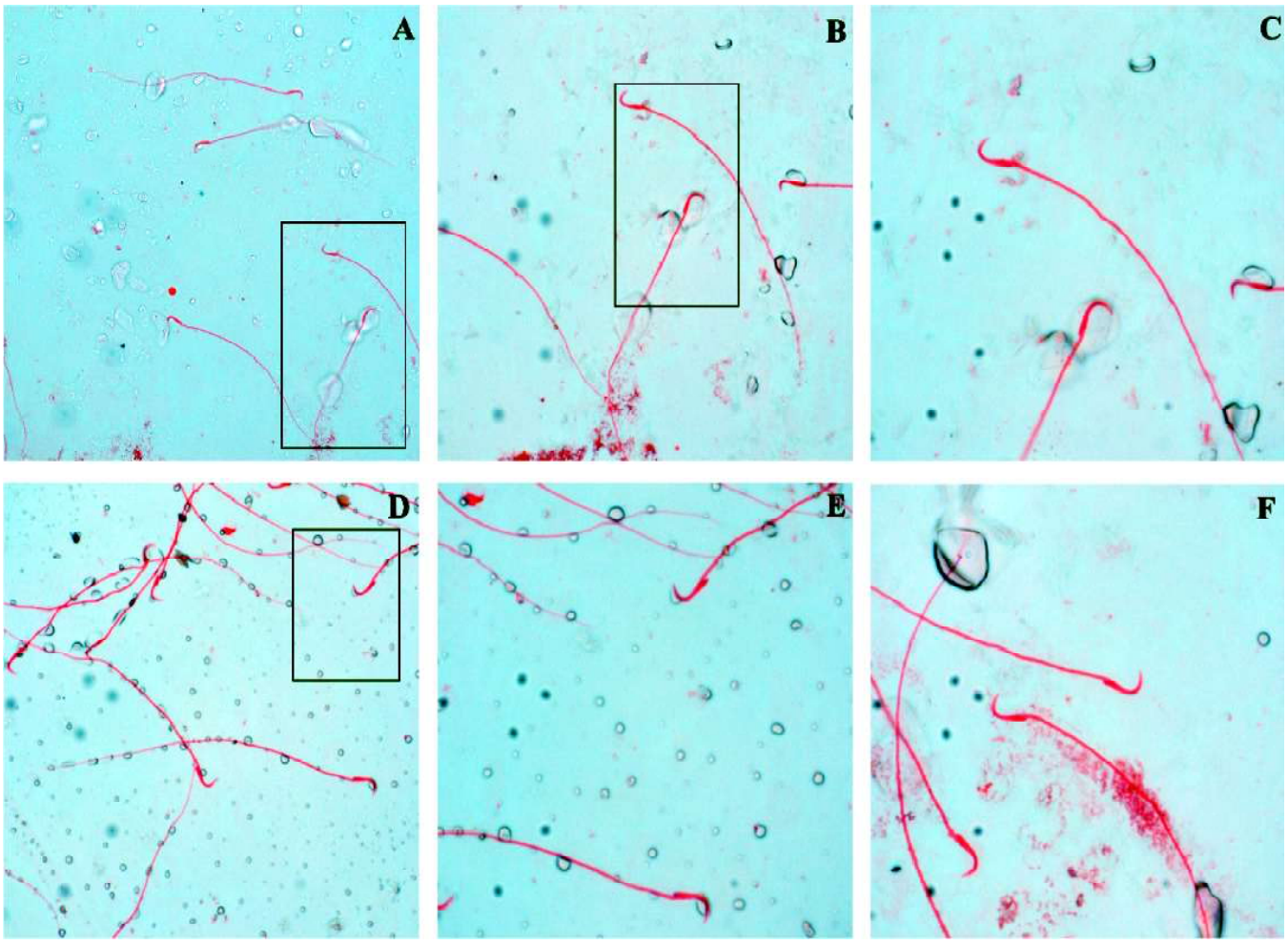

Fig. 1: Photomicrographs illustrating morphologically normal sperm
A at 600X; B & D at 945X; C, E & F at 1,500X

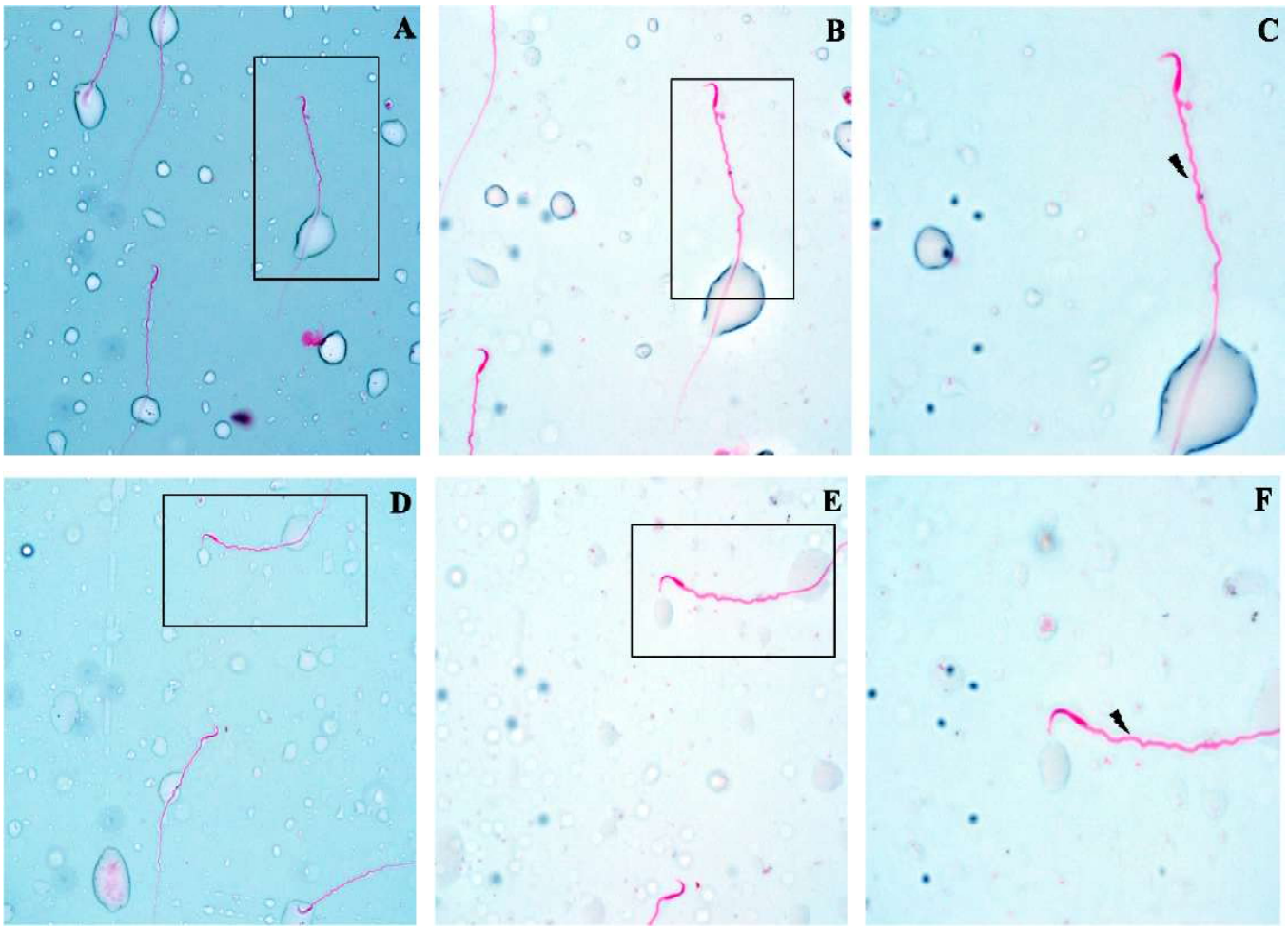

Fig. 2 : Photomicrographs illustrating morphological defects in sperm after 12 hours of cyclophosphamide exposure

A at 600X; B & D & E at 945X; C & F at 1,500X

Markings: Lightning bolt = wrinkled tail

Serum separation

Blood samples were collected in glass vials, kept for 30 minutes at room temperature to clot, and centrifuged at 500 Xg for 15 minutes for serum isolation. The serum was immediately used to assess the amount of testosterone present.

Determination of testosterone

Testosterone was determined by adding 50 µl of serum sample in testosterone-antigen magnetic particle solution and incubated for 20 minutes at 37° C. After incubation, 50 µl of acridinium ester labeled isomer was added and the mixture was again incubated for 10 minutes at 37° C. The reaction cuvette was washed three times with wash reagent and starter solution 1 and 2 were added. The reading was noted at 450 nm wavelength.

Sperm count

Caudal region of left epididymis was minced in a petri-plate containing 2 ml of saline (maintained at 37° C). This mixture was then filtered *via* a mesh large enough for passing of the sperms but no other cell debris. The filtrate was diluted 10 times with saline and loaded on a Neubauer haemocytometer for total sperm count. The four corner squares of 1 mm^3 (excluding the central square) was observed under light microscope for counting[12].

Spermatozoa per epididymis = $N \times 1.25 \times 10^5$

Sperm motility

For sperm motility and non-motility assessment, caudal part of the right epididymis was excised and macerated in test-tubes containing 2 ml saline (maintained at 37° C) for about 30-60 seconds to allow sperms to disperse from the tissue. Motility was observed immediately taking a smear under light microscope. The criterion for sample comparison was set at randomly chosen first 200 spermatozoids[9].

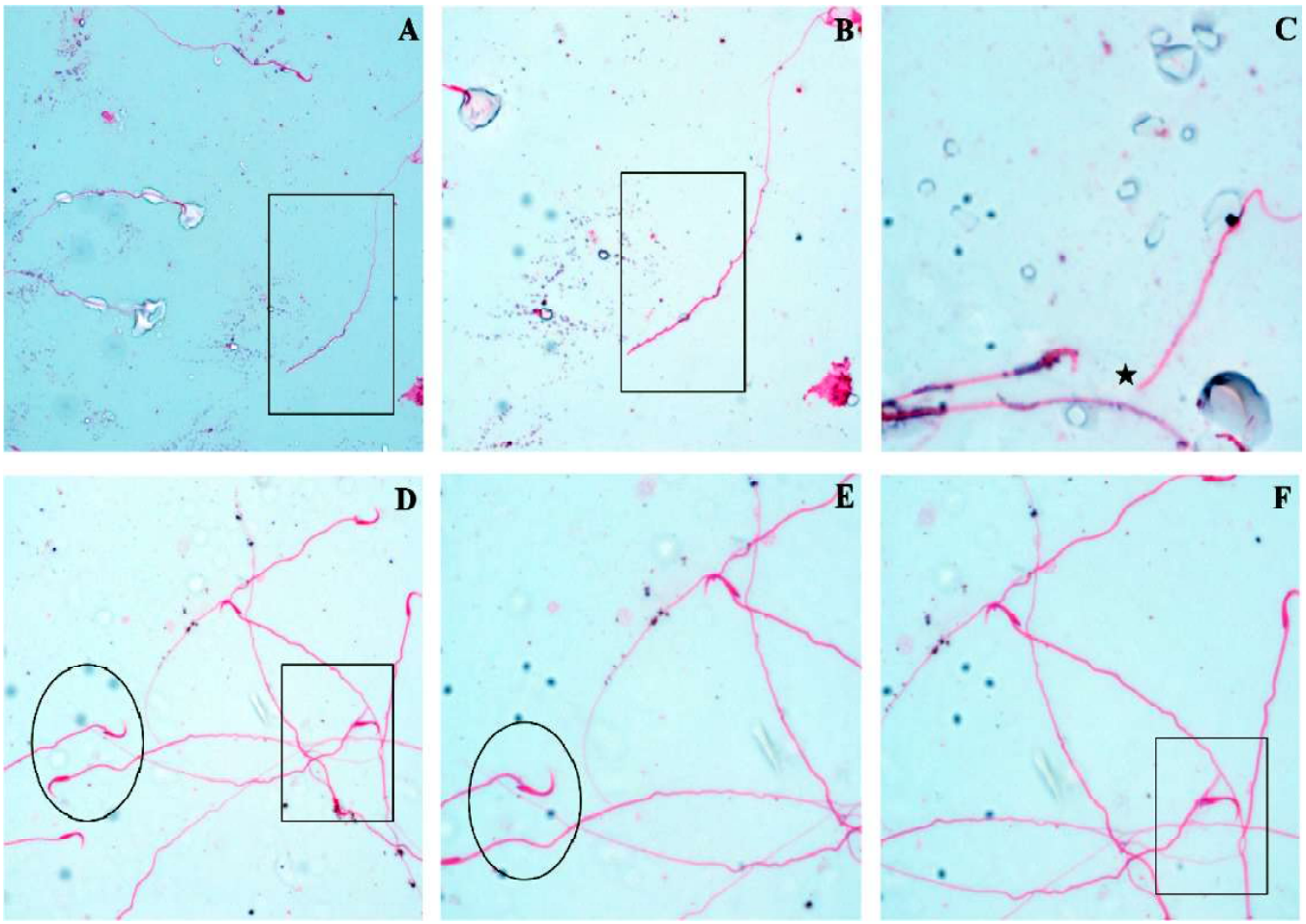

Fig. 3: Photomicrographs illustrating morphological defects in sperm after 24 hours of cyclophosphamide exposure

A & D at 600X; B & E at 945X; C, E & F at 1,500X

Markings: Star = Headless tail

Sperm morphology

For sperm morphology, about 10 µl suspension containing sperm (prepared for motility assessment) was taken in a microcentrifuge tube to which 20 µl of eosin Y (1%) solution was added and mixed thoroughly for 15 seconds. Slides containing thin smear of the mixture was prepared immediately and allowed to air dry, slides were mounted with mounting media and coverslips[2] and observed under light microscope.

Statistical analysis

Results are presented as mean ± standard error (S.E.) from six animals in every group. One-way analysis of variance (ANOVA) at 5% followed by Tukey's honestly significant difference (HSD) *post hoc* test at 5% level of significance was used to draw comparison between different groups[18].

Results and Discussion

Cyclophosphamide alone or in combination with other chemotherapeutics, is often used to treat various types of cancers, including breast cancer, leukaemia, lymphoma, *etc.* It interferes with DNA replication by cross-linking with DNA strands leading to apoptosis in rapidly dividing cells[3,5] and also causes significant side effects, including immunosuppression, myelosuppression and haemorrhagic cystitis[7]. Despite these risks, cyclophosphamide continues to play a pivotal role in cancer management protocols.

Metabolism of cyclophosphamide by cytochrome p450 isoenzymes CYP3A4 and CYP3A5[16], both are also expressed extensively in Sertoli cells[21] producing acrolein as one of the by-products. A highly reactive aldehyde, acrolein is well known for its ability to conjugate with cellular GSH and produce GS-propionaldehyde[13] ultimately causing depletion of GSH and leading to oxidative stress by increasing lipid peroxidation[1]. They are also known to covalently bind with DNA and protein and cause interstrand DNA and DNA-protein crosslinking[8].

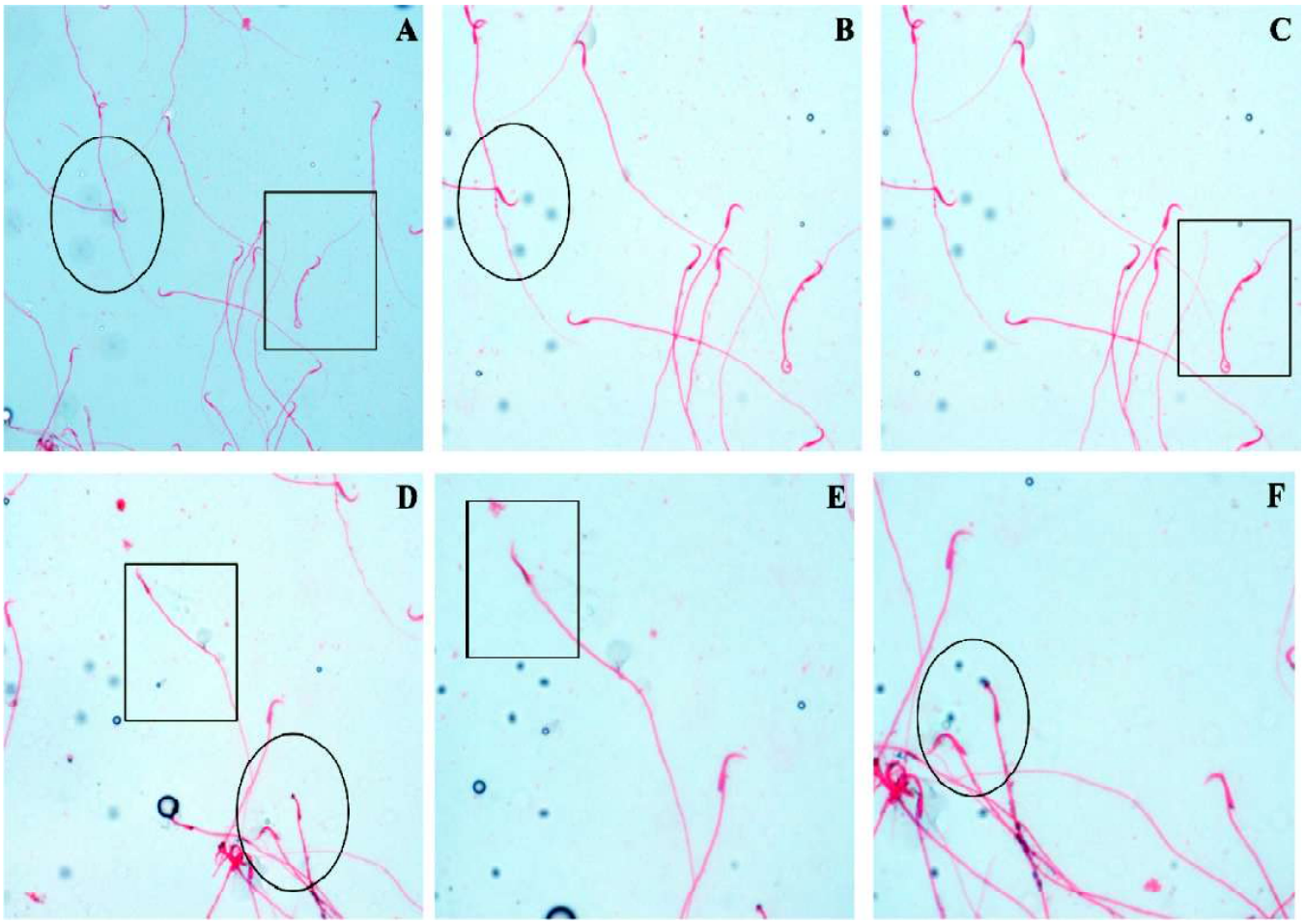

Fig. 4: Photomicrographs illustrating morphological defects in sperms after 48 hours of cyclophosphamide exposure

A at 600X; B, C & D at 945X; E & F at 1,500X

Table-2 shows that no significant changes occurred in body weight and wet weight of the testes after exposure to cyclophosphamide at different durations in comparison to control. Decrease in testosterone concentration was observed after exposure to cyclophosphamide at different durations, which corroborated previous findings of researchers[14]. This could be due to formation of acrolein, a metabolic by-product of cyclophosphamide, which inhibited synthesis of testosterone as reported by earlier researchers[20].

As shown in Table-3, exposure to cyclophosphamide at different durations significantly decreased total and motile number of sperms while significantly increased number of non-motile sperm as compared to control in duration dependent manner. This could happen as testicular tissues are highly vulnerable to free radicals produced by cyclophosphamide[6]. The polyunsaturated fatty acids (PUFAs) present in the mammalian sperm membrane are easy targets of the ROS to cause oxidative damages, which in turn increases membrane permeability and are reportedly associated to induce damage in sperm cells at various developmental stages and decrease in sperm count and motility[3,4,10]. Thus, longer the duration of exposure could decrease a greater number of motile sperm.

Morphological differences in different groups are illustrated (Figs. 1-4). Fig. 1(A-F) shows regular morphological features of the sperm of the control group, whereas groups exposed to cyclophosphamide for different durations show peculiar deformities in head and tail region both. The group impacted with exposure to cyclophosphamide for 12 hours (Fig. 2A-F) revealed shortening and heavy wrinkling of tails. The 24 hours duration group (Fig. 3A-F) retained wrinkling with bending at the neck region with headless tail sperm. The tails of the sperm exposed to cyclophosphamide for 48 hours (Fig. 4A-F) showed decrease in wrinkling while increase in length and thinning and dissolved tail ends and heads. These abnormalities in the sperm can be attributed to alkylating property of cyclophosphamide that caused DNA damage as stated by previous researchers[3,20] and indicated a shock to sperm by sudden exposure to

cyclophosphamide even at the least duration of exposure.

Conclusion

The results of this investigation conclude that exposure to cyclophosphamide produce toxicity on male reproductive organ. Decrease in number and motility of sperms, increase in morphological defects along with decrease in testosterone concentration may lead male infertility after treatment of cyclophosphamide. Thus, a suitable intervention is needed to avoid or minimize such side effects while treatment with cyclophosphamide.

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