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S Pavithra
Ph.D., Scholar, Department of Veterinary Pharmacology and Toxicology, Indian Veterinary Research Institute, Bareilly, Uttar Pradesh, India

SP Preetha
Assistant Professor, Department of Veterinary Pharmacology and Toxicology, Madras Veterinary College, Tanuvas, Chennai, Tamil Nadu, India

M Thangapandiyan
Assistant Professor, Department of Veterinary Pathology, Madras Veterinary College, Tanuvas, Chennai, Tamil Nadu, India

A Karthikeyan
Ph.D., Scholar, Department of Veterinary Public Health and Epidemiology, Indian Veterinary Research Institute, Bareilly, Uttar Pradesh, India

Corresponding Author:
S Pavithra
Ph.D., Scholar, Department of Veterinary Pharmacology and Toxicology, Indian Veterinary Research Institute, Bareilly, Uttar Pradesh, India

Role of fractionated pectin powder capped zinc oxide nanoparticle in 5-fluorouracil induced testicular oxidative stress in rats

S Pavithra, SP Preetha, M Thangapandiyan and A Karthikeyan

Abstract
5-Fluorouracil (5-FU) is a widely used anticancer drug known to induce testicular toxicity. Oxidative stress plays a crucial role in causing infertility due to decreased sperm motility, viability, and sperm abnormalities. The present study was designed to evaluate the antioxidant properties of the Fractionated Pectin Powder (FPP) capped nano Zinc oxide (nZnO) in 5- Fluorouracil induced testicular toxicity in rats. Experimental rats were divided into four groups (n = 10) which received normal saline (Group I), 5-FU @ 20gm/kg b.wt (Group II), FPP capped nZnO @ 40mg/kg b.wt (Group III), 5-FU @20gm/kg b.wt and FPP capped nZnO @ 40mg/kg b.wt (Group IV). All treatments were given orally up to 14 days. On the 15th day of sacrifice tissues of testis and epididymis were collected and subjected to antioxidant assays. In Group II both testis and epididymis showed a significant (P< 0.01) decrease in the activities of antioxidants (SOD, CAT, GSH, and GPX) with an increase in lipid peroxidation (LPO). On treatment with FPP capped nZnO (Group IV) showed a significant (P< 0.01) increase in the activities of CAT, GSH, and GPX and a decrease in the activity of LPO. Treatment with FPP capped nZnO considerably restored the collapsed antioxidant status. This study highlights the protective role of FPP-capped nZnO against 5-FU induced oxidative stress to the testicular and epididymal cells.

Keywords: 5-Flourouracil, Oxidative stress, FPP capped nZnO, Antioxidant

Introduction
The anticancer drug 5-Fluorouracil (5-FU) an antimetabolite, widely used for the treatment of cancers is approved by FDA for the treatment of colorectal cancer and various gastrointestinal tumors [1-3]. 5-FU is known to induce testicular toxicity like germinal epithelial sloughing, cell killing, multinucleated cell formation, seminiferous tubular atrophy, spermatogonial damage, and arrest of spermatid development [4-8]. Reactive Oxygen Species (ROS) at controlled concentrations can act to mediate the process of capacitation, hyper activation, and acrosome reaction of the mammalian sperms crucial to the acquisition of fertilizing ability [9]. A reduction in the antioxidant status is associated with idiopathic infertility, while a shift of pro-oxidants can induce oxidative stress on the spermatozoa [10]. Chemotherapeutic agents induce a state of oxidative stress in the testis and are capable of compromising male infertility [11].
Pectins are complex polysaccharides of the plant cell wall and are generally recognized as safe as a direct human food ingredient [12]. Owing to the low biodegradability of plant pectin in the body fractionated pectin powder (FPP) was developed. Pectins are reported to have a protective effect against experimentally induced testicular toxicity [13-14]. Nanoparticles of ZnO had significantly improved the antioxidant status, sperm count, and testosterone levels in chemotherapy-induced testicular toxicity [15]. Hence, the present study was designed to explore the role of FPP capped nZnO against 5-FU induced testicular toxicity in rat models.

Materials and Methods
(a) Experimental protocol
Sale Wistar rats (6-7 weeks old), were maintained under standard laboratory conditions with an *ad libitum* supply of feed and purified water during the study period. Rats were acclimatized for one week and then randomized based on the body weight and distributed to different groups such that the mean body weight variation does not exceed 20%.

Groups	Treatments	No. of Rats
I	Control	10
II	5-Fluorouracil (5-FU) contro l@ 20mg/kg B.wt (p.o for 14 days)	10
III	Modified Pectin (FPP capped nZnO) control @ 40mg/kg b.wt (p.o for 14 days)	10
IV	5-FU @ 20mg/kg b.wt (p.o for 14 days) + FPP capped nZnO@ 40mg/kg b.wt (p.o for 14 days)	10

All treatments were given orally up to 14 days. After sacrificing the animals on the 15th day, sacrifice tissues of testis and epididymis were collected and washed in ice-cold normal saline. Then, it was blotted dry and stored at -20°C for further analysis. Testis was crushed in a tissue homogenizer (Heidolf, Germany) and 10% w/v testis homogenate was prepared with PBS (pH 7.4). This was used for the estimation of lipid peroxidation (LPO) and reduced glutathione (GSH). The remaining homogenate was centrifuged at 15,000 g for 1hr at 4°C. The supernatant thus obtained was used for the estimation of total protein (TP), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx).

(b) Estimation of total protein

The protein content of the tissue homogenate was measured by the method of Lowry *et al.* [16] based on the biuret reaction of protein with copper ion in an alkali medium. Briefly, 0.5 ml of the tissue homogenate was mixed with 0.5 ml of 10% Trichloroacetic Acid (TCA) and centrifuged for 10 min. The precipitate obtained was dissolved in 1.0 ml of 0.1 NaOH. From this, an aliquot was taken for protein estimation. An aliquot of 0.1 ml was mixed with 5 ml of alkaline copper reagent and allowed to stand at room temperature for 10 min. 0.5 ml of Folin's phenol reagent was added and the blue colour developed was read after 20 min at 640 nm.

(c) Estimation of Superoxide Dismutase (SOD)

The Superoxide Dismutase (SOD) activity was determined by the method described by Marklund and Marklund [17] based on the ability of SOD to inhibit the auto-oxidation of pyrogallol at pH 8.2. Briefly, the supernatant (0.5ml) obtained from the tissue homogenate was mixed with 0.25 ml of ethanol and 0.15 ml of chloroform. This mixture was centrifuged at 13,000 g for 15 min at 4°C to get supernatant which was used for the estimation of SOD activity. The above supernatant (0.5 ml) was mixed with 2 ml of 0.1 mm Tris-HCl buffer (pH 8.2), 1.5 ml of distilled water, and 0.5 ml of 2 mm pyrogallol in 0.05 M Tris-HCl buffer (pH 7.4). The assay mixture for the enzyme contained 2 ml of the buffer, 0.5 ml of pyrogallol, aliquots of the enzyme preparation, and water to give a final volume of 4 ml. The rate of inhibition of pyrogallol auto-oxidation after the addition of the enzyme was noted. One unit activity corresponds to the amount of enzyme required to give 50% inhibition of pyrogallol auto-oxidation. The enzyme activity was expressed as units/min/mg protein.

(d) Estimation of Catalase (CAT)

Catalase (CAT) activity was estimated by the method of Caliborne [18] based on the principle of decrease in absorbance at 240 nm due to decomposition of hydrogen peroxide (H_2O_2). Briefly, 0.2 ml of the supernatant obtained from the tissue homogenate was mixed with 1ml of 30 mM H_2O_2. The decrease in the absorbance was measured at 240 nm for 3 min. Enzyme activity was expressed as µM of H_2O_2 decomposed per min per mg of protein.

(e) Estimation of Glutathione Peroxidase (GPx)

The Glutathione Peroxidase (GPx) was determined by the method of Rotruck *et al*. [19] based on the reaction of GPx with H_2O_2 and GSH giving rise to oxidoreductase which forms a colour complex with 5, 5'dithio-bis 2-nitrobenzoate (DTNB). Its absorbance was measured at 412 nm. The reaction mixture consisting of 0.4 ml of 0.4 M phosphate buffer (pH 7.0), 0.1 ml of 10 mm sodium azide, 0.2 ml of 4 mM GSH, 0.1 ml of 2.5 mm H_2O_2, and 0.1 ml of tissue homogenate, was incubated at 37°C for 3 min and the reaction was terminated by the addition of 10% trichloroacetic acid (TCA). The mixture was centrifuged and the supernatant was used to determine the remaining GSH content. To 1 ml of the supernatant, 3 ml of 0.3 M disodium hydrogen phosphate solution, and 1 ml of 0.04% DTNB in 1% sodium citrate. The colour developed was read at 412 nm. Serial dilutions of GSH standards were prepared and treated in the same manner. The enzyme activity was expressed as µg of GSH consumed/min/mg protein.

(f) Estimation of Glutathione (GSH)

Total Reduced Glutathione **(**GSH) content in the liver tissue was estimated by the method of Moron *et al.* [20] based on the reaction with 5, 5' dithio-bis 2-nitrobenzoate (DTNB or Ellman's reagent) to give a yellow colour compound whose absorbance was measured at 412 nm. Briefly, to 0.5 ml of tissue homogenate, an equal volume of 5% TCA was added and proteins were precipitated. The precipitate was removed by centrifugation and to the aliquot of supernatant, 2.0 ml of 0.6 mM DTNB in 0.2 M sodium phosphate buffer (pH 8.0) and 0.8 ml of 0.2 M sodium phosphate buffer (pH 8.0) were added to make a final volume of 3 ml. The absorbance was measured at 412 nm and the values were expressed as mg GSH/g tissue.

(g) Estimation of Lipid peroxidation (LPO)

Estimation of Lipid peroxidation (LPO**)** in the tissue homogenate was carried out by the method of Yagi *et al.* [21] using thiobarbituric acid (TBA). Malondialdehyde (MDA), formed as the end product of the peroxidation of lipids, served as an index of the intensity of oxidative stress. MDA reacts with TBA to give the brilliant pink colour product. Briefly, 0.5 ml of homogenate was mixed with 1 ml of 20% TCA to which 2 ml of 46 mm TBA was added and heated for 30 min in a boiling water bath. Then, the reaction mixture was cooled and centrifuged at 1157g for 10 min. The absorbance of supernatant having brilliant pink colour was measured at 532 nm and the values were expressed as µm of MDA/g tissue.

Results

Activities of the antioxidants in the testicular tissues of experimental animals are presented in Table 1. Statistically significant ($P< 0.01$) decrease in the activities of antioxidants (SOD, CAT, GSH, and GPX) with the increase in lipid peroxidation (LPO) were observed in the 5-FU group as against the control group. On treatment with FPP capped nZnO in the 5-FU group, there was a significant ($P< 0.01$) increase in the activities of CAT, GSH, and GPX. LPO was significantly ($P< 0.01$) reduced.

The antioxidant status in the epididymal tissues of

experimental animals is presented in Table 2. A significant (P< 0.01) decrease in the activities of antioxidants (SOD, CAT, GSH, and GPX) and increased LPO were observed in the 5-FU group as against the control. In 5-FU+ FPP capped nZnO group, activities of CAT, GPX (P< 0.01), SOD, and GSH increased, while LPO (P< 0.01) was significantly reduced.

Table 1: Effect of drugs on antioxidant enzymes and lipid peroxidation of testis

Groups	SOD (U/mg protein)	CAT** (U/mg protein)	GPX** (µM of GSH utilized/min/mg protein)	GSH** (mg of reduced GSH/g of tissue)	LPO** (µM of MDA/g tissue)
Control	128.82^{b}±1.7	23.17^{c}±0.59	11.25^{c} ±0.34	0.41^{a}± 0.02	5.45^{a}±0.35
5-FU control	123.83^{a}±2.3	10.08^{a}±0.67	7.05^{a}±0.27	0.31^{a}±0.04	15.06^{c}±1.36
FPP capped nZnO control	138.72^{a}±12.03	23.00^{c}±0.38	10.45^{b}±0.24	0.33^{a}± 0.07	5.88^{a}±0.52
5-FU + FPP capped nZnO	139.50^{ab}±8.65	16.92^{b}±1.73	9.95^{b}±0.16	0.67^{b} ±0.88	10.36 ^{b}± 0.88

Table 2: Effect of drugs on antioxidant enzymes and lipid peroxidation of epididymis

Groups	SOD (U/mg protein)	CAT** (U/mg protein)	GPX** (µM of GSH utilized/min/mg protein)	GSH (mg of reduced GSH/g of tissue)	LPO** (µM of MDA/g tissue)
Control	548.84^{b}±19.48	8.08 ^{b}±1.0	12.22^{b} ±0.62	0.24^{a}±0.53	4.62^{a}±0.39
5-FU control	448.82^{a}±15.62	3.27^{a}±0.44	7.43^{a}±0.30	0.10^{b}±1.4	36.59^{b}±3.72
FPP capped nZnO control	512.82^{ab}±40.30	11.22^{b}± 2.35	11.30^{b}±0.37	0.20^{a}±0.03	9.46^{a}±0.93
5-FU + FPP capped nZnO	518.39^{ab} ±0.23	9.14 ^{b}± 0.17	11.06^{b}±0.59	0.26^{a} ±0.03	7.20 ^{a}± 1.18

Discussion

Reactive oxygen species (ROS) and oxidative stress play a crucial role in causing infertility due to decreased sperm motility, viability, and sperm abnormalities [22]. The collapse of the endogenous antioxidant machinery reflects the inability of testicular mitochondria, microsomes, and cytosol to eliminate the H_2O_2 generated in response to oxidative stress in the testis. Increased lipid peroxides may lead to disruption of functional intergrity of the cell organelles. Reduced GSH may be responsible for the apoptosis of the germ cells [23-24]. The plasma membranes of the spermatozoa contain excess polyunsaturated fatty acids (PUFA) and their cytoplasm contains low concentrations of scavenging enzymes, making them highly susceptible to oxidative stress induced damage [23-24]. Oxidative stress also damages the integrity of DNA in the sperm nucleus, accelerating the process of germ cell apoptosis leading to decline in sperm count [23-24]. It is also capable of drastically altering the steroidogenic capacity of Leydig cells associated with male infertility and apparently deteriorates the semen quality [25].

5-FU administration significantly decreased the activities of endogenous antioxidants with concurrent increase in lipid peroxides in the testis and epididymis. Especially, in the epididymis 10 fold increase in the LPO was noticed. This might be due to highly specific lipid composition of epididymis, which renders these cells more susceptible to ROS attack. Sikka [23] also reported enhanced LPO in the epididymis due to its specific lipid composition. Treatment with FPP capped nZnO considerably restored the collapsed antioxidant status and decreased the lipid peroxide in accordance with the earlier reports [13-15, 26-28].

Conclusion

The present study demonstrated that treatment with FPP capped nZnO significantly increased levels of endogenous antioxidants (SOD, CAT, GPx, and GSH) and decreased the lipid peroxides in the 5-FU administered group. It is obvious that FPP capped nZnO could be used as holistic approach for treating testicular toxicity. Further rigorous studies are needed to elucidate the concrete mechanisms of FPP capped nZnO in underlying pathway.

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