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Research article

AMELIORATIVE EFFECT OF *PUNICA GRANATUM* ETHANOLIC EXTRACT IN CYCLOPHOSPHAMIDE INDUCED TESTICULAR TOXICITY IN MALE WISTAR RATS.

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ABSTRACT

Aim: To explore the potential role of *Punica granatum* ethanolic extract (PGEE) in Cyclophosphamide (CP) induced testicular toxicity.

Methods: Healthy male Wistar rats were allotted to 4 groups (N=6, each) Group I: Control, Group II: CP 15mg/kg twice a week, Group III: PGEE 100mg/kg, Group IV: CP and PGEE for 28 days. At the end of the treatment period, organ weight, body weight, epididymal sperm count, motility, morphology, SOD, catalase, GSH, ACP & testosterone level in the testis were evaluated.

Results: The CP treated rats showed toxicity evidenced by decreased organ and body weight, decreased sperm quality and testosterone level also increase in MDA and decrease in antioxidants SOD, GSH indicating oxidative stress. In contrast PGEE co-treatment with CP resulted in significant restoration of the above mentioned parameters.

Conclusion: These results indicate that PGEE attenuates CP induced testicular toxicity through its ROS scavenging activity.

Key words: Cyclophosphamide, testicular toxicity, oxidative stress, *Punica granatum*.

INTRODUCTION

Cyclophosphamide (CP) is an alkylating anticancer drug that is chemically related to nitrogen mustard(Charles M, et al, 1973).It is extensively used to treat a wide range of cancers, as an immunosuppressive agent following organ transplants and for treating various autoimmune disorders (Fleming RE, et al, 2001). Previous studies have reported cyclophosphamide exposure causes oligospermia and azoospermia in experimental animal models and in humans (Hales BE, et al, 1982).Cyclophosphamide has shown to produce severe testicular damage, which is characterized by spermatogenic damage, germ cell apoptosis, leydig cell dysfunction and testicular steroidogenic disorder. It has also been reported that cyclophosphamide administration induces oxidative stress and the generation of toxic reactive oxygen species (ROS) which may be responsible for its testicular toxicity. CP can cause a significant damage during a key point of sperm chromatin remodelling, thereby affecting the chromatin structure altering sperm nuclear components (Jianpingqiu, et al, 1995).

Considering the importance of this drug in the clinical practice, there arise a necessity of an agent, which can ameliorate the side effects of this alkylating agent, without compromising on its therapeutic benefits.

Fruits and dietary sources are a promising source of new therapeutic options. Pomegranate (*Punicagranatum*L.)is a nutrient dense, antioxidant rich fruit has been revered as a symbol of health and fertility. Pomegranate belonging to *Punicaceae* family is a large deciduous shrub or small tree in many tropical and subtropical countries (Salaheddin ME, et al, 1984). Pomegranate has been used in traditional medicine as remedy for a lot of symptoms like eye sore, scurvy, blood clotting, diarrhea (Jinnawat, et al, 2012).The recent interest for this fruit is not only because of the pleasant taste, but also due to various medicinal properties such as anti-atherogenic, antiparasitic, antimicrobial, antioxidant, anticarcinogenic, antiinflammatory , cardioprotective, anti-inflammatory effects etc (Debjit, et al, 2013). Additionally, many studies have reported that pomegranate and its derivatives have free radical scavenging and potent antioxidant activity (Rajan S, et al, 2011).

The current study was undertaken to characterize the effects of Cyclophosphamide on testes and to investigate whether pomegranate can ameliorate the damage induced by Cyclophosphamide on testes.

MATERIALS AND METHODS

Animals

All the animal experiment protocols were approved by the institutional animal ethical committee (IEAC) and the experiments on animals were performed in accordance with the CPCSEA guidelines. Wistar albino male rats were procured from the central animal house, K S Hegde Medical Academy. All animals were kept under controlled environmental conditions at room temperature (22+2^{o}C). Standard laboratory animal feed (purchased from commercial supplier) and water were provided *ad libitum*. Animals were acclimatized to the experimental conditions for a period of one week prior to the commencement of the experiment.

Preparation of *Punica granatum* ethanolic root extract (PGEE)

Punicagranatum fruits were purchased from the local market. It was authenticated by a botanist. It was peeled and shade dried. They were coarsely powdered. The powder was subjected to Soxhlet extraction with 95%ethyl alcohol for 72 hours at 70^{0}C. The resulting extract was concentrated and the solvent was evaporated using rotary evaporator and stored at 4^{o}C for future use. This was then dissolved in distilled water & administered orally to the rats (Mahgoub et al, 2009).

Study design

The animals were randomly segregated into the following 4 groups (n=6)
Group I: Distilled water (1 ml)
Group II: PGEE 100mg/kg
Group III: Cyclophosphamide 15mg/kg twice a week
Group IV: CP 15mg/kg twice a week and PGEE 100mg/kg
All the drugs were administered orally and the treatment period was 4 weeks which is the time taken to complete two spermatogenic cycle in rats. (Amar K Chandra, et al, 2011)
The selection of dose of CP was based on previous studies and PGEE was based on the pilot study.

Animal observation, body and organ weight

All the animals were regularly observed for signs of toxicity and survival rate was recorded for the entire experiment. The body weight of each animal was recorded before and after the study.
At the end of experimental period, the rats were anaesthetized with ketamine 150mg/kg body weight. The testes and epididymis were dissected out soon after sacrifice, cleared of fat and washed with saline, soaked on bloating paper and weights were recorded.

Sperm analysis

Determination of sperm characteristics

The epididymis was placed in a petri dish containing 1ml of pre-warmed phosphate buffer. The epididymis was cut into small portions to allow sperm to swim out and filtering through a mesh. One drop of sperm suspension was placed on a glass slide, covered with a cover slip. The motility of the epididymal sperm was evaluated microscopically within 2-3 minutes of their isolation from the epididymis and data were expressed as percentage of motile sperm of the total sperm counted.
Sperm count was conducted according the standard procedure with the aid of the Neubauer Haemocytometer under a microscope (Narayana K, et al, 2005). The spermatozoa count was obtained by counting the number of sperms in the four WBC chambers using haemocytometer.
The sperm suspension was mixed with one drop of 1% eosin Y and smears were prepared on clean glass slides, slides were viewed by bright-field microscope with magnification of 400x. Two hundred sperms was examined to determine the morphological abnormalities which were classified as amorphous, hook less, coiled, tail less and finally represented as percentage total abnormality (Narayana K et al, 2002).

Biochemical analysis

Preparation of tissue homogenate:

The testis and epididymis was washed with ice cold physiological saline and 0.5g of tissue was homogenized in 0.1M Tris-HCl buffer of pH 7.5 and centrifuged in a refrigerated centrifuge at 10,000g for 30min. The supernatant was used as enzyme source for various assays.

Total protein by Biuret method

Add 1.5 ml of Biuret reagent to 1ml of appropriately diluted tissue homogenate, mix and maintain at room temperature for 10min and measure the OD at 540nm with 5mg/mL BSA used as standard protein solution.

Superoxide dismutase [SOD] assay

The SOD was assayed by the method described by Beauchamp & Fridovoch, et al, 1971. The reaction mixture contained, 50mM phosphate buffer [pH 7.8], 10mM methionine, 56uM NBT and 2uM riboflavin, 0.1mM EDTA with suitably diluted tissue homogenate in a total volume of 1ml.

Illumination of the solution was done with a 15W fluorescent lamp. The absorbance was measured at 560 nm. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the rate of NBT reduction measured at 560nm.The values were expressed in Units/g tissue.

Acid phosphatase activity

The acid phospahatase was assayed according to method described by Zwiazek, J.J. The assay mixture containing 25 µl of enzyme extract in 2.0 ml of 0.6 mM p-nitrophenylphosphate (p-NPP) in 0.05 M acetate buffer, pH 5.0, was incubated at 37 °C for 10 min. Blank samples contained the enzyme extract and acetate buffer without p-NPP. After 10 min the reaction was stopped by adding 0.5 ml of 0.4N NaOH. Absorbance of the reaction mixture was measured spectrophotometrically at 410 nm (Maholtra S, et al, 1980).

TBARS determination:

MDA was assayed according to method described by H. Esterbauer .A portion of homogenate was mixed with 1 ml of 15% (w/v) trichloroacetic acid, 0.38% (w/v) thiobarbituric acid, 0.25 N HCl. The mixture was heated in a boiling waterbath for 15 min, and after centrifugation, the absorbance was measured at 535 nm (e=153 M-1 cm-1) (H. Esterbauer, et al, 1990).

Catalase assay:

The activity of catalase was determined according to modified method of (AebiH, 1983). To 2ml of the reaction mixture, 20 µl of appropriately diluted homogenate was added and absorbance was read at 240nm in Kinetic mode immediately at 0 min followed by intervals of 30 seconds for 2min (30±0.5°C). Change in absorbance was calculated as a function of time.

Reduced Glutathione (GSH) quantification

GSH was assayed according to method described by Ellman G, 1959. DTNB (5,5'-Dithiobis(2-nitrobenzoic acid), reacts with glutathione (GSH) to generate 2-nitro-5-thiobenzoic acid and glutathione disulfide (GSSG). Since 2-nitro-5-thiobenzoic acid is a yellow colored product, GSH concentration in a sample solution can be determined by the measurementat 412 nm.

Testosterone

Intratesticular testosterone was carried outusing LDN kit method out using ELISA method using LDN kit according to manufacturer's protocol.

Statistical analysis

Results are expressed as median ± interquartile range. The data were analyzed by means of Kreskal- Wallis test followed by intercomparisons by Mann-Whitney U test using the SPSS package for Windows 11. The p-value less than 0.05 were considered to be statistically significant.

RESULTS

Body and organ weight changes

The weights of testes and epididymis in rats after CP administration were found to be significantly lower ($p< 0.01$) than those of CP treated rats. There was no significant change in the weights of testes and epididymis in rats treated with PG alone. However co administration of PGEE and CP caused significant increase ($p<0.01$) in testes and epididymis weight in comparison with CP treated group. The decrease in testes size and weight reflects the testicular regeneration and toxicity of cyclophosphamide. (Table-1).

The CP treated group showed weakness, but there was no mortality. The weight of the CP treated group was significantly decreased after the experimental period unlike the control and PGEE treated group. The PGEE and CP co-treatment group rats were slightly heavier than the CP treated group. (Figure 1).

Table 1: Testicular and epididymal weight and sperm parameters after the treatment period.

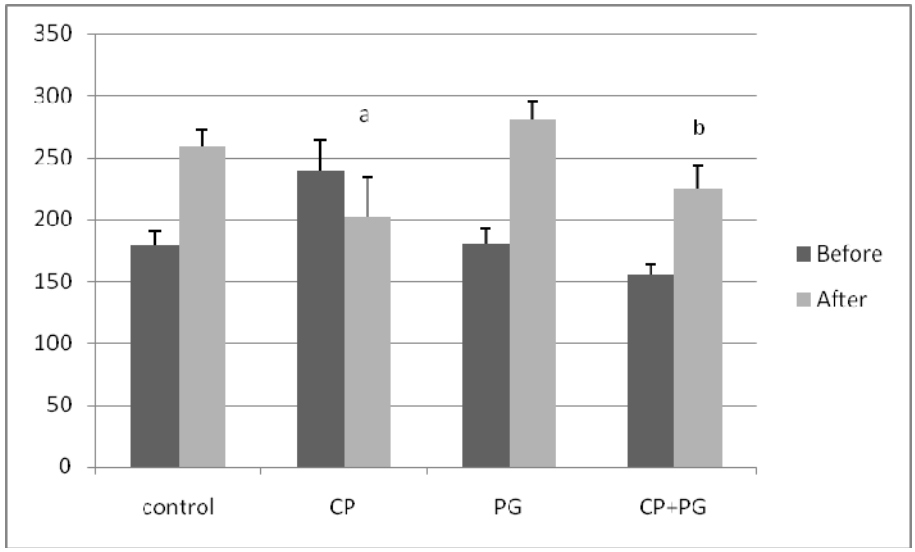
	Control	PGEE	CP	CP +PGEE
Testes weight (g)	3.18 ±0.87	3.2 ± 0.1	2.24 ±0.56[b]	2.6 ± 0.305[b]
Epididymis weight (g)	1.14 ± 0.207	1.195 ± 0.14	0.765 ±0.3[b]	0.78 ±0.29[b]
Sperm count ($x10^6$)	54±9	57.5±9.25[a]	20.67±10.7[b]	37.2±15.25[bc]
Sperm motility %	77±9.25	73.5±10	51±5.75[b]	62±7.25[bc]
Sperm abnormality %	20±2.5	18±3[a]	40.5±6.7[b]	31.5±7.25[c]

PGEE– Punica granatum ethanolic extract, CP- Cyclophosphamide

[a] $p<0.05$ as compared with control group

[b] $p<0.01$ as compared with control group

[c] $p<0.05$ as compared with CP group



P <0.05 as compared with (a) group I and (b) group II

Figure 1. Body weight of groups before and after treatment. Control indicates vehicle treated group. CP - Cyclophosphamide, CP+PGEE - Cyclophophamide+ Punica granatum.

Sperm characteristics

Treatment of male rats with CP caused a significant decrease ($p < 0.01$) in sperm count, motility while dead and abnormal sperms increased compared to those of control.

Co-administration of PGEE caused a significant increase in sperm count, sperm motility and improved morphology compared to control group (Table 1).

Biochemical analysis

Administration of CP alone significantly increased (p 0.01) level of MDA compared to control group indicating the oxidative stress caused by CP on testicular tissue. TheMDA level significantly decreased with co-treatment of PGEE and CP compared to control group. Also CP-treated animals showed a significant decrease in the level of antioxidants like reduced glutathione ($p<0.01$) and SOD ($p<0.05$) activities in the testes as compared to control group. Animals treated with Punica granatum extract also showed a significant increase in the activities of glutathione and SOD which is consistent with previous studies. However, co-treatment of PGEE with CP treated animals elicited a significant ($p<0.05$ to $p<0.01$) increase in the level of reduced glutathione and activity of SOD when data were compared with CP group animals, while level of testosterone was significantly decreased by CP treatment which was improved in co-treatment group. The biochemical changes observed in the testis of CP group include increase in acid phosphatase activities and decrease in proteins. (Table 2).

Table 2. Effect of *Punica granatum* ethanolic extract on biochemical parameters in cyclophosphamide induced toxicity

	Control	PGEE	CP	CP +PGEE
Protein mg/g tissue	96.93±13.6	100.6±9.17	54.28±6.57[b]	73.75±5.85[bc]
GSH µg/g tissue	194.74±80.22	214.42±56.85[a]	42±64.28b [b]	182.01±70.99[c]
SOD U/g tissue	151.46±55.13	156.30±31.91	228.31±87.24[a]	188.20±28.61[c]
CAT mmoles/ml	1.9277±0.167	1.915±1.57	1.635±0.55	2.06±1.12
MDA mmoles/g	134.09±48.64	141.39±20.58	336.42±36.09[b]	253.61±62.93[bc]
ACP U/g tissue	0.234±0.127	0.45±0.243	3.52±1.29[b]	2.44±0.64[b]
Testosterone ng/ml	15.76±4.2	16.5±6.4	7.86±1.4[b]	12±3.4[c]

[a] $p<0.05$ as compared with control group
[b] $p<0.01$ as compared with control group
[c] $p<0.05$ as compared with CP group

Co-administration of PGEE and CP decreased the enzyme activities to the control level indicating significant protection of germinal epithelium to CP toxicity

DISCUSSION

Testicular toxicity by chemotherapeutic agents is considered as inevitable side effect of cancer treatment in male patients. A strategy to diminish the side effects of anticancer drugs with preservation of chemotherapeutic efficacy is necessary. The present study reconfirmed the testicular toxicity of CP.

Cyclophosphamide has alkylating properties that result in nucleotide base mispairs and DNA/DNA or DNA/protein cross-linking that lead to major disruptions in nucleic acid function and the inhibition of DNA synthesis. (Crook T. R., 1986). Cyclophosphamide undergoes biotransformation by hepatic microsomal cytochrome P_{450} isoenzymes to aldophosphamide, which is spontaneously broken down to produce phosphoramide mustard and acrolein. Phosphoramide mustard is responsible for anti-cancer effects, while acrolein is associated with the toxic effects observed during CP therapy (Gerald A. LeBlanc, David J. Waxman, 1990).

As CP is an alkylating agent that causes more damage into rapidly dividing cells, the drug is expected to affect the seminiferous epithelium and reduce the number of spermatozoa produced. The number of spermatozoa in the cauda epididymis provides a good estimate of spermatozoa reserves.

Treatment with Cyclophosphamide in rats caused testicular toxicity resulting in obstruction of spermatogenesis, evidenced by reduction in testicular and body weight, sperm count, sperm motility, sperm morphology, testosterone level. So probably Cyclophosphamide causes testicular toxicity by destruction of germinal epithelium lining the seminiferous tubules and leydig cell dysfunction resulting in azoospermia. In this study, PGEE treatment protected against CP-induced adverse effects on sperm parameters.

Cyclophosphamide treatment has also been shown to cause oxidative stress (Myung Sook Oh, et al, 2007). Mammalian spermatozoa are rich in polyunsaturated fatty acids and thus very susceptible to ROS attack (R. John Aitken and Shaun D. Roman, 2008). Excessive ROS increases germ cell apoptosis and detrimental effect on spermatozoa resulting in decrease in sperm viability, sperm motility and increase in morphology defects with deleterious effects on sperm capacitation and acrosome defects. Lipid peroxidation (LPO) destroys the structure of lipid matrix in the membranes of spermatozoa, and it is associated with loss of motility and defects of membrane integrity(Dorota and Macie Kurpisz, 2004).Therefore stress could play an important role in the induction of sperm abnormalities, namely higher susceptibility of sperm DNA to denature and fragment. (M A Shalaby, et al, 2010). CP treated rats in our study had a significant increase in testes LPO and a significant drop in antioxidant levels suggesting the presence of free-radical toxic stress within the testes. Lipid peroxidation serves as an index of intensity of oxidative stress. The decrease in MDA level in PGEE and CP treated animals, compared with CP alone treated group demonstrates that PGEE treatment provided significant protection against lipid peroxidation induced by CP in the rat testes.

Also co-administration of PGEE with CP significantly restored GSH and antioxidant enzymes SOD in testes as compared with the CP group. Activities of antioxidative enzyme activity in tissues has always been used as a marker for tissue damage.(Miyun Tsai-Turton, et al, 2007) Hence increase in activity of MDA and decrease in SOD, catalase, GSH & ACP from this present study proved that CP caused oxidative & testicular injury.

The marked reduction in testicular weight by CP can be explained by decreased number of germ cells , atrophy of leydig cell and a significant lower rate of spermatogenesis. Reduction in weight of testes and accessory reproductive organs in CP treated rats reflects the reduced availability of androgens.

Testosterone is produced by leydig cell in the testes under the influence of LH. Intratesticular testosterone is an absolute prerequisite for normal spermatogenesis. The decrease in the mean testosterone levels of CP treated rat served as proof for the damage of testis. These results demonstrate that CP caused temporary interference of normal male reproductive system with treatment. Our findings also show significant decrease in protein levels, suggests that CP may interfere with protein synthesis.

The present study reported the testicular toxicity of CP as evidenced from decreased sperm quality, testicular weight, antioxidant levels and testosterone. Co administration of PGEE with CP reflects the effect of PG against CP induced oxidative stress in testes. Pomegranate juice is already reported to increase sperm quality, spermatogenic cell density, antioxidant activity and testosterone level in male rats (Gaffari Turk, et al, 2008). Studies have documented that pomegranate has protective effect on testes against carbon tetrachloride intoxication in rats. (Ampa Luangpirom, et al, 2013).

Various findings have supported a positive role of medicinal herbs to protect or prevent the side effect of many chemotherapeutic agents. Studies have already reported that antioxidant agents such as diallylsulphate (Sung-Hwan Kim, et al, 2013), melatonin (IlbeyYo, et al, 2009), alpha‑tocopherol‑succinate (Ghosh D, et al, 2002) have protective actions against CP-induced testicular toxicity.

Preliminary phytochemical investigation of PGEE has revealed the presence of glycosides, alkaloids, saponins, flavonoids, proteins and carbohydrates. From HPLC-LCMS analysis carried out on PGEE, it is postulated that the active components of PGEE are chlorogenic acid and rutin. Chorogenic acid is reported to have antioxidant, hepatoprotective, anti-inflammatory, antimicrobial properties.

It is already proved that chlorogenic acid is effective in preventing or treating the decline of male reproductive function caused by hormone-disrupting chemicals (Kweon Kim 2001). Another study reported pomegranate peel extract and rutin have abilities to preserve the activity of antioxidant enzymes and lysosomal membrane in Chlorpyrifos-ethyl-Induced oxidative stress in rats which may be referred to its role as antioxidants (Ebtesam M Al-Olayan, et al, 2014). So the attenuation of CP induced testicular toxicity can be due to its antioxidant property of its active components. Further studies are needed to decipher this activity and to confirm its protective role in other models of chemical induced testicular toxicity.

CONCLUSIONS

From the present study, it is suggested that cyclophosphamide treatment impairs male reproductive systems which is consistent with other studies. It appears, therefore, that CP treatment produces degenerative changes in the germ cells and inhibits androgen production and is partially protected by PGEE co- administration. It is suggested that *Punica granatum* or its active ingredients serves as a promising candidate for protection against CP induced testicular toxicity. .

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