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Cyclophosphamide Induced Testicular Toxicity Mechanisms and Ameliorative Agents

Matam SriDeepthi[1], Kondampati Kasturi Devi[2*], Matukuamli Usha rani[2], Boinepally Ramya[2], and Beesam Srinu[2]

College of Veterinary Science, Warangal, PVNR Telangana Veterinary University, Rajendranagar, Hyderabad

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Abstract

Cyclophosphamide is an alkylating agent that is widely known for its anticancer and immunosuppressive properties used in various cancerous and autoimmune diseases. Acrolein and phosphoramide mustard, are its toxic metabolites, majorly involved in toxic effects such as induction of oxidative stress that can induce oxidative damage in tissues. The free radicles thus produced are implicated in gonad toxicity, hepatic and kidney disorders, hemorrhagic cystitis*etc*. Clinical efficacy of cyclophosphamide is restricted because of its toxicity. The reproductive system due to presence of rapidly dividing cells is highly vulnerable to cyclophosphamide toxicity. Especially male subjects treated with cyclophosphamide demonstrated severe alterations in testis and epididymis as well as disturbed gonadotropin and reproductive hormone levels. Several studies suggested the usage of medicinal plants with antioxidant properties along with cyclophosphamide could protect against its organ toxicity. Supplementation of the dietary antioxidant's during chemotherapy can reduce the drug induced toxic side effects and can further improvise the response

[*]*Corresponding author* : Email : kasturi120@gmail.com
[1]College of Veterinary Science, Rajendranagar
[2]College of Veterinary Science, Warangal
PVNR Telangana Veterinary University, Rajendranagar, Hyderabad

to therapy.

Key words: Cyclophosphamide, Testicular toxicity, Oxidative stress, Antioxidants

Cyclophosphamide is a most widely used nitrogen mustard in veterinary medicine as an antineoplastic drug and as a part of immunosuppressive therapy. It is a drug mostly used in the treatment of canine and feline lymphomas and in immune mediated disorders like organ transplant rejection, chronic autoimmune diseases etc., (Zanchi, 2015). It is anoxazaphorphorine derivative, which provides alkyl groups to covalently bind with DNA strands and cause disruption of cell growth, cell mitosis and differentiation activities. Cyclophosphamide is an inactive prodrug, requires metabolic activation through hepatic microsomal cytochrome P450 mixed function oxidase to form active metabolites. It is firstly transformed to 4-hydroxycyclophosphamide inequilibrium with aldophosphamide, the later is carried into target tissues where it gets spontaneously converted into phosphoramide mustard and acrolein. Phosphoramide mustard is effective and is responsible for therapeutic activity as alkylating agent whereas, acrolein contributes for side effects as it is hypothesized to generate oxidative free radicals like hydroxyl, peroxides, and superoxide radicals.

The male reproductive system consisting of rapidly proliferating cells is highly susceptible to the toxic effects of cyclophosphamide (Aguilar *et al*2001). Chronic administration of cyclophosphamide in male rats demonstrated various toxic effects that include oligospermia and azoospermia (Trasler *et al* 1987), reduced gonadotropin secretion and blood testosterone levels (Hoorweg *et al* 1992), variations in biochemical parameters, histology of testis and epididymis (Trasler *et al* 1988), all these leads to infertility.

The precise mechanism of cyclophosphamide induced testicular toxicity is not clear, but many studies shown that it can disrupt redox balance of tissues, biochemical and physiological alterations are due to oxidative stress (Das *et al* 2002). Toxic metabolite acrolein induces oxygen radical formation, increases lipid peroxidation at cell membrane as the region is rich in poly unsaturated fatty acids and contain low amount of antioxidant system (Kehrer and Biswal 2000). Such alterations in testis, induced by cyclophosphamide can lead to male infertility. Keeping in view, that male subject contributes for a half of genes for the developing progeny of next generation, he should be thoroughly considered for the male mediated developmental toxicity in off spring (Anderson 2005). Infertility associated with chemotherapeutic drug indication has a great impact on social life; hence prevention of germ cell toxicity has drawn much attention (Connolly *et al* 1992)

Many strategies have been considered to restore the fertility after cyclophosphamide induced testicular toxicity. Meistrich and Shetty (2008) considered suppression of gonadotropins and testosterone during cytotoxic therapy, but results of the study are uncertain. Numerous studies documented that synthetic and naturally occurring antioxidants can effectively ameliorate the cyclophosphamide induced gonadal toxic effects.

Natural plant derived products which are abundantly available in medicinal plants/ herbs provide crucial molecules for health benefits. Many of the researchers indicated that natural compounds with antioxidant properties may have the ability to ameliorate the testicular toxic effects induced by cyclophosphamide. In the recent years, bioactivities of phytoconstituents have drawn much attention, especially their anti and/or prooxidant and anti-inflammatory activity (Rice-Evans 2001).

In the current review, various mechanisms through which cyclophosphamide causes germ cell damage and possible interventions to restore the fertility in male subjects are discussed.

Cyclophosphamide induced testicular toxicity:

Effect on testis morphology:

Many studies indicated that cyclophosphamide treated subjects show significant decrease in testis weight (Elongovan *et al* 2006; Potnuri*et al* 2018; Iqubal*et al* 2020) indicating reduction in spermatogenic cell count and reproductive capabilities.The decrease in the activity might be due to reduced gonadotropin and testosterone

hormone release which effects spermatogenesis (Tawab *et al* 2014).

Effect of cyclophosphamide on biochemical parameters and hematology:

A significant decrease in the total white blood cells, platelets, red blood cells and increase in serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), serum glutamateoxaloacetic transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), acid phosphatase (ACP)urea and creatinine are reported (El-Naggar *et al* 2015).

Effect of cyclophosphamide on antioxidant activity:

Male reproductive organ testis is highly sensitive to oxidative stress due to presence of potential reactive oxygen species generating systems such as xanthine oxidase, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, mitochondrial electron transport chain and mitochondria membrane with high levels of poly unsaturated fatty acids. To overcome this, testis is naturally built up with an array of antioxidant systems comprising of enzymatic and non-enzymatic constituents. Upon the induction of oxidative stress in testis, as a part of defense strategy activation of enzymatic components arise from the NFκB mediated synthesis of mRNA for releasing enzymes super oxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione-S-transferase (GST) (Kaur *et al* 2006). Reactive oxygen species (ROS) is generated by an enzymes such as xanthine oxidase or NADPH oxidase, or as a by-product of cellular metabolism. Most common free radical formed during metabolism is superoxide anion (O_2^-). It is further converted into hydrogen peroxide (H_2O_2) by the enzyme SOD, to prevent the former from involving in the formation of highly destructive agent (OH^-) hydroxyl radical. The H_2O_2 generated has to eliminated by GPx and catalase enzymes, of which former is more predominating in the testes (Zini and Schlegel, 1996). On the other hand GST reductase catalyzes the conjugation of GSH disulfide back to GSH, to facilitate excretion from the cell. This reaction is essential for removal of toxic products formed during lipid peroxidation.

Cyclophosphamide enables the cell more susceptible by reducing the levels of antioxidant enzymes such as SOD, CAT, and GSH content along with glucose-6-phosphate dehydrogenase (G6PD), GSH reductase (Tawab *et al* 2014), vitamin E and C (Selvakumar *et al* 2005). Lipid peroxidation in the cell is measured by elevation of malondialdehyde (MDA) which is the break down product of poly unsaturated fatty acids oxidation (Türk *et al* 2010).

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that has a crucial role in the gene expression of proteins which are involved in cell adaptation and defense against oxidative stress (Dinkova-Kostova and Abramov 2015). Nrf2 regulates redox balance in the cell by controlling mitochondrial ROS production, sustaining the levels of reduced GSH, inducing expression of antioxidant proteins like thioredoxin and thioredoxin reductase and cytoprotective proteins like heme-oxygenase-1 and NAD(P)H dehydrogenase. Disruption in the function of Nrf2 makes the cell more susceptible to oxidative stress due to reduced formation of antioxidants, ant inflammatory and detoxifying proteins (Ma2013; Dinkova-Kostova and Abramov, 2015).

Metabolism of cyclophosphamide is majorly carried by cytochrome p450 isoenzymes CYP3A4 and CYP3A5, which are abundantly expressed in testis Sertoli cells (Westlind *et al* 2001). Cyclophosphamide upon enzymatic activation produces two active metabolites, of which phosphoramide responsible for the therapeutic effects, whereas acrolein is associated with oxidative and nitrosative stress. The later metabolite promotesoxidative damage, lipid peroxidation in the cell by depressing activity of GSH (James *et al* 1993). Acrolein promotes the induction of enzyme nitric oxide synthase (iNOS) results in subsequentsynthesis of nitric oxide and peroxynitrite along with decrease in activity of GPx (Ucar *et al* 2007). Occurrence of increased levels of acrolein in Sertoli cells significantly decreases activity of the antioxidant enzymes such as SOD, CAT and GSH-Px.

Protective agents against cyclophosphamide induced testicular damage

Literature from various studies suggest that compounds of natural or synthetic origin with antioxidant activity exhibited ameliorative or protective effect against cyclophosphamide induced gonadal toxicity.

α-lipoic acid

α-lipoic acid is a disulfide compound, synthesized in mitochondria. It serves as powerful antioxidant cofactor and has ability to scavenge the free radicals like singlet oxygen, hydrogen peroxide and can also chelate ferrous ions which take part in formation of hydroxyl radicals. Lipoic acid can amend the thiol deficiency via promoting the uptake of thiol containing amino acid, cysteine into the cells and GSH synthesis (Han *et al* 1997). Chronic administration of lipoic acid for a period of 10 weeks promotes mitochondrial lipid peroxidation and H_2O_2 formation in testis of rats. The prooxidant-antioxidant ratio is altered more towards prooxidants side in cyclophosphamide administered conditions. But upon exogenous pretreatment with lipoic acid significantly altered the antioxidant status and lipid peroxidation surge.

American ginseng

Ginseng plants both American and Asian ginseng, belongs to the family Araliaceae. Ginseng plants are known for many therapeutic uses such as tonic, stimulant, and aphrodisiac properties. Most of the pharmacological activities are attributed for the ginsengroots, rich in ginsenoside. Hosseini *et al* (2018) confirmed the cytoprotective role of *American ginseng* in cyclophosphamide induced toxicity by improving the testosterone levels, decreased intracellular production of hydrogen peroxide, malondialdehyde and DNA damage in testis. Ginseng also reversed the damage induced by cyclophosphamide in seminiferous tubules of male rats.

Rutin

Rutin, also called as rutoside is a flavonol found abundantly in tea leaves, apple and buck wheat. It is chemically a glycoside, consisting of quercitin and disaccharide rutinose. Antioxidant activity of rutin is demonstrated in several studies (Nakamura *et al* 2000). Protective effect of rutin in cyclophosphamide induced reproductive toxicity was studied by (Abarikwu *et al* 2012). Rutin significantly improved sperm count, sperm motility and reduced abnormal sperm count induced by cyclophosphamide. Further in testis and epididymis, rutin enhanced the activity of antioxidant systems such as super oxide dismutase, catalase, glutathione (GSH), glutathione peroxidase (GSH-Px). In testis, rutin increased the levels of testicular steroidogenic enzymes (3ß-hydroxysteroid dehydrogenase and 17ß-HSD) and maintained the activity of spermatogenesis marker enzymes indicating the restoration of spermatogenesis under cyclophosphamide induced damage.

Piper longum

Piper longum, commonly referred as "pippali" is an indigenous medicinal plant contains an alkaloid referred as piperine and piperlongumine. The plant has proven its efficacy as antitumor, protection against radiation during chemotherapy and immunomodulatory (Sunilla and Kuttan 2004; Sunilla and Kuttan 2005), antioxidant (Wakade *et al* 2008), bioenhancer (Harwansh *et al* 2014). Piperine and piperlongumine, phytochemicals can intercalate into DNA grooves to produce genome stability (Zahin *et al* 2018). Genoprotective effect of *Piper longum* against cyclophosphamide induced geno toxicity was studied by Yadav *et al* 2023. Their results demonstrated that co-treatment with *Piper longum* extract has maintained genome stability with significant reduction in cellular markers such as micronucleus formation, chromosomal aberrations along with protection from oxidative stress DNA damage and significant decrease in the levels of DNA double strand break marker – γH2AX i.e., phosphorylated version of histone HSAX.

Nerolidol

Nerolidol, is a sesquiterpene alcohol with numerous pharmacological activities such as antioxidant, antimicrobial and anti-inflammatory properties (Chan *et al* 2016). Iqbal *et al* 2020 explored the potential of nerolidol in cyclophosphamide induced gonado toxicity. They reported that nerolidol administered at a dose

of 400 mg/kg body weight restored the levels of catalase, thiobarbituric acid reactive substances (TBARS), nitric oxide levels, malondialdehyde and reduced the levels of inflammatory marker TNF-α. In addition, nerolidol restored the weight of the gonads, sperm functional markers such as sperm count, motility and levels of testosterone.

Astaxanthin

Astaxanthin, is a carotenoid pigment found in plants and marine animals such as fish, shrimps etc., (lai *et al* 2004). It is more potent in scavenging the free radicals such as peroxyl and singlet oxygen than other carotenoids. In addition to antiperoxidative property, it has cancer preventive activity and protection against metastasis (Kurihara *et al* 2002; Hix *et al* 2005), immunomodulatory (Naito *et al* 2004), antihypertensive (Hussein *et al* 2006), amelioration of metabolic syndrome by regulating insulin resistance (Hussein *et al* 2007). Tripathi and Jena (2008) investigated the protective role of astaxanthin in cyclophosphamide induced germ cell toxicity in male mice. Their study revealed that astaxanthin supplemented at a dose of 25mg/kg orally improved the testis weight, sperm count, protected germ cell integrity, prevented DNA damage due to cyclophosphamide, toxic metabolite acrolein induced redox imbalance and lipid peroxidation. Carotenoids such as astaxanthin can induce the activation of cytoprotective genes such as nrf-2 ARE pathway which are involved in increased expression of phase -II detoxification enzymes (Fahey *et al* 2005).

Crocin

Crocin, chemically is a digentiobiosyl ester of crocetin is a water soluble carotenoid found in dried stigmas of saffron (*Crocus sativus*) and in fruits of gardenia (*Gardenia jasminoides*). Previous studies on crocin indicate that it has antioxidant (Sapanidou *et al* 2015), antiproliferative effects (Aung *et al* 2007), antidepressant (Vahdati Hassani *et al* 2014), aphrodisiac activity (Hosseinzadeh *et al* 2008). The protective effects of crocin against cyclophosphamide induced testicular toxicity were investigated by Potnuri *et al* 2018. Their results indicated that crocin exhibited dose dependent gonad protective effect (10 mg/kg and 20 mg/kg per day for eight weeks) by preserving glutathione redox system, sperm quality, hormonal axis of steroidogenesis and spermatogenesis pathway. Further crocin reduced the testicular apoptosis by improving testosterone levels and prevented caspase 3 activation.

Conclusion

Despite being used as cancer chemo preventive and immunosuppressant cyclophosphamide induces free radical imbalance. Oxidative stress generated due production of reactive oxygen species in the germ cells can cause damage to cell membrane and functional integrity. Strategies aiming to restore the balance in the testicular environment can prevent the cyclophosphamide induced gonad toxicity.

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