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KABUL VE ONAY SAYFASI

Muhamed Cemil BUŞİ'in Yüksek Lisans Tezi olarak hazırladığı "**Sıçanlarda siklofosfamid uygulamasına bağlı testis hasarının önlenmesinde ve/veya tedavi edilmesinde taurinin etkisi**" başlıklı bu çalışma Eskişehir Osmangazi Üniversitesi Lisansüstü Eğitim ve Öğretim Yönetmeliği'nin ilgili maddesi uyarınca değerlendirerek **"KABUL"** edilmiştir.

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Üye : **Prof. Dr. Fatma Sultan KILIÇ**

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Eskişehir Osmangazi Üniversitesi Sağlık Bilimleri Enstitüsü Yönetim Kurulu'nun …./…./ tarih ve ……/…….. sayılı kararı ile onaylanmıştır.

Prof Dr. Özkan ALATAŞ
Enstitü Müdürü

ÖZET

SIÇANLARDA SİKLOFOSFAMİD UYGULAMASINA BAĞLI TESTİS HASARININ ÖNLENMESİNDE VE/VEYA TEDAVİ EDİLMESİNDE TAURİN'İN ETKİSİ

Çalışmamızda sıçanlarda farklı dozlarda oral taurine uygulamasının intraperitoneal (i.p.) uygulanan siklofosfamide (CP) bağlı testis hasarı üzerine koruyucu ve/veya terapötik etkisi incelenmiştir. Bu amaçla toplam 40 adet Sprague-Dawley erkek sıçan her grupta 8'er hayvan olacak şekilde 5 gruba ayrılmıştır. Kontrol grubuna 14 gün boyunca SF oral olarak uygulanmıştır. Kontrol grubuna 8. gün i.p. SF uygulanmıştır. CP, tau75, tau150, tau300 gruplarına ise 8. günde 200 mg/kg tek doz i.p. CP enjeksiyonunun öncesinde 7 gün ve sonrasında 7 gün olmak üzere, CP grubunda SF ve tedavi gruplarında da 75 mg/kg, 150 mg/kg ve 300 mg/kg dozlarında taurine gavaj yoluyla uygulanmıştır. Son tedavi dozundan 24 saat sonra genel anestezi ve ötenazi sonrasında hayvanların testis dokuları alınmış ve birisi histolojik inceleme ve immunohistokimyasal apoptoz değerlendirmesi için diğeri de homojenat hazırlamak için kullanılmıştır. Morfometrik ölçümler için vücut ağırlıkları ve testis ağırlıkları değerlendirilmiştir. Testis homojenatında oksidatif stres ve sitokin düzeyleri değerlendirilmiştir. Çalışmamızda CP vücut ağırlığı önemli bir değişikliğe sebep olmuş ama testis ağırlığında önemli bir değişikliğe sebep olmamış, taurine tedavisi ise vücut ağırlığını anlamlı ölçüde azaltmıştır. Yine oksidatif stres belirteçleri gözlenmemiş ama sitokin düzeyleri açısından anlamlı bir değişiklik gözlenmiş olmakla birlikte histolojik olarak CP verilen grupta dejeneratif değişiklikler ve apoptotik bulgular olduğu ve bu bulguların taurine tedavisi ile artan dozlarla bağımlı birlikte iyileştiği saptanmıştır. Sonuç olarak, bu çalışmada taurine tedavisinin CP ile oluşturulan testis hasarı üzerine oksidatif stres ve inflamasyon aracılı olmaktan ziyade anti-dejeneratif ve anti-apoptotik etkiler gösterdiği saptanmıştır.

Anahtar kelimeler: Taurine, siklofosfamid, sıçan, testis hasarı

SUMMARY

THE EFFECT OF TAURINE ON THE PREVENTION AND / OR TREATMENT ON CYCLOPHOSPHAMIDE INDUCED TESTICULAR DAMAGE IN RATS

In our study, the protective and/or therapeutic effect of oral taurine treatment in different doses on cyclophosphamide-induced testicular injury was investigated in rats. For this purpose, a total of 40 male Sprague-Dawley rats were divided into 5 groups with 8 animals in each group. The control group was administered orally in SF for 14 days. Saline solution was applied to control group by ip on day 8. In CP, tau75, tau150 and tau300 groups, 200 mg / kg single dose of i.p CP was administered on Day 8 with administering SF, 75 mg / kg, 150 mg / kg and 300 mg / kg taurine by oral gavage for 7 days before and after the CP injection, respectively. After 24 hours of the last treatment dose, testicular tissues were removed in animals under Ketamine/Xylazine anesthesia and after euthanasia, and then one was used for histological examination and immunohistochemical apoptosis evaluation and the other was used to prepare testis homogenate. Body weights and testis weights were evaluated for morphometric measurements. Oxidative stress and cytokine levels were evaluated in testis homogenate. In our study, CP cause a significant change in body weight and not significant change in testis weight, whereas taurine treatment significantly decreased body weight. Although no significant change was observed in terms of oxidative stress markers but significant change in cytokine levels, degenerative changes and apoptotic findings were found in the CP group; which were improved significantly with doses of taurine treatment. In conclusion, in this study, it was found that treatment with taurine showed anti-degenerative and anti-apoptotic effects rather than affecting oxidative stress and inflammatory pathways in CP-induced testicular damage.

Key words: taurine, cyclophosphamide, rat, testicular injury

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SYMBOL AND ABBREVIATIONS INDEX

CP	Cyclophosphamide
SLE	Systemic Lupus Erythematosus
CL	Clearance
Vd	Volume of distribution
BBB	Blood-Brain Barrier
CYP	Cytochrome P450
CAA	Chloroacetaldehyde
ADH	Alcohol Dehydrogenase
AKR	Aldo-Keto Reductase
CEPM	Carboxy Ethyl Cyclophosphor amide
ALDH	Aldehyde Dehydrogenase
NAD	Nicotinamide Adenine Dinucleotide
GSH	Glutathione
GST	Glutathione S-Transferases
GSCY	Gluta thionyl cyclophosphamide
TEN	Toxic Epidermal Necrosis
POF	Premature Ovarian Failure
RA	Rheumatoid Arthritis
GPA	Granulomatosis with Polyangiitis
ROS	Reactive Oxygen Species
ATP	Adenosine-5-Triphosphate
AUC	Area Under Curve
Cmax	Maximum plasma concentration

T1/2	Plasma elimination half-life
Cl/F	Clearance/Bioavailability
NADH	Nicotinamide adenine dinucleotide
HOCl	Hypochlorous Acid
TauCl	Taurine Chloramine
CNS	Central Nervous System
TOS	Total Oxidant Status
OSI	Oxidative Stress Index
AU	Arbitrary Unit
IL	Interleukin
TAS	Total Antioxidant Status
BW	Body weight
TAU	Taurine
TNF-α	Tumor Necrosis Factor Alpha
IFNγ	Interferon gamma
İ.V.	Intravenous
µM	Micromolar
PJP	*Pneumocytis Jirovecci* Pneumonia
H_2O_2	Hydrogen peroxide
µg	Microgram
COX-2	cyclooxygenase-2

1.INTRODUCTION AND AIM

Cancer is not just a troublesome disease to treat but also all the drugs used to treat cancer have side effects which should be kept in mind. Among anticancer drugs, alkylating agents are so effective drugs for the treatment of cancer but with various side effects. One of the mostly used alkylating agent, cyclophosphamide (CP) is not only an important and helpful drug for treatment of cancer but also used for Hodgkin's disease because of its immunosuppressive properties. Unfortunately, besides the wide therapeutic uses of CP, it also causes several side effects in patients and experimental animals including hemorrhagic cystitis, cardiotoxicity and testicular toxicity.

Cyclophosphamide is a prodrug that is metabolized by the liver to both active and inactive compounds by the hepatic P450 system. CP is metabolized rapidly to aldophosphamide mustard 4-hydroxycyclophosphamide and acrolein that immediately interfere with cellular DNA synthesis result in cell death. The alkylation activity of CP to tumor cell DNA is responsible for its therapeutic activity. Nevertheless, acrolein trigger oxidative stress, which leads to DNA corruption in normal cells and induce above-mentioned toxicities in different organs (Kwolek-Mirek, Bednarska, Bartosz, Biliński. 2008).

One of its side effects that is so special and affecting the social life of the patient is the infertility in both males and females, but it affects men more than women. That's why we need to find some drugs to prevent and/or decrease this side effect, which will enhance the quality of life of the patient and prevent them from discontinuation of the drug because of this side effect.

This side effect of CP on fertility may be related to the production of reactive oxygen species such as superoxide anions and hydroxyl radicals, which induces an imbalance between oxidant and antioxidant system activities in many tissues, including the testes (Singh et al., 2015). The elevated oxidative stress leads to tissue damage and organ failure (Sarkar, Hazra, & Mandal, 2013).

There are some studies demonstrating that antioxidants can diminish CP-induced gonadotoxicity in male mice by diminishing the oxidative stress and enhance morphometric and morphological findings of gonadal injury. (Onaolapo1, Oladipo, Onaolapo, 2017)

On the other hand, antioxidant characteristics of taurine are confirmed by many previous studies, but taurine itself cannot remove the reactive oxygen species (ROS). Taurine shows an antioxidant effect by inhibiting ROS production due to increased activities of antioxidants (Sun Jang J, 2009). Several researches have shown that taurine protects cells versus oxidative stress (Schaffer, Azuma, Mozaffari, 2009; Erdamar, Turközkan, Ekremoğlu, Kurt, Yaman, 2007). Taurine is metabolized in vivo to taurine chloramine (TauCl) which inhibits the release of pro-inflammatory cytokines involving IL-6, IL-1ß, TNF-α and IL-8 (Kontny, et al., 2012; Marcinkiewicz, Kontny, 2014).

Cyclophosphamide has been suggested to induce DNA damage in germ cells and some of these cells are removed by apoptosis, which results in a decrease in the number of maturing spermatozoa (Trasler, Hales, Robaire, 1985; Hales Robaire, 1990; Hales, Crosman, Robaire, 1992; Trasler, Hales, Robaire, 1986). On the other hand, taurine has been reported to play a key role in cytoprotection and decrease the apoptosis (Das, Sil, 2012).

After looking for all these studies, the importance of our study is the potential to offer to the male patients the opportunity to use the cyclophosphamide without suffering from infertility. To the best of our knowledge, there was no previous study on the effect of taurine on testicular injury caused by CP in a rat model. Therefore, this study aimed to investigate the effects of taurine in the prevention and/or treatment of testicular injury due to CP administration in rats.

2. GENERAL INFORMATION

2.1. Cyclophosphamide

The oxazaphosphorine cyclophosphamide (2-[bis-(2-chloroethyl)amino]-tetrahydro-2H-1,3,2-oxazaphosphorine2-oxide, Cytoxan®, CP) is a widely used DNA-alkylating agent in cancer chemotherapy (Figure 2.1). CP was found to have antitumor activity in several animal models in the early 1940s (Gilman. 1963), In the 1950s, scientists at the Asta-Werkw Company in Germany synthesized an additional series of phosphorylated nitrogen mustards compound, Arnold, Bourseaux, and Brock reported that the compound with preclinical testing was CP (Arnold, Bourseaux, Brock, 1958).

Cyclophosphamide, a cyclic phosphamide ester, is an active alkylating agent that belongs to the group of nitrogen mustard. It introduces alkyl radicals into DNA strands of cells and prevent the growing of cancer cells. In the liver, it is metabolized by cytochrome P-450 enzymes to its active metabolite 4-hydroxycyclophosphamide, which forms aldophosphamide (Fenselau et al. 1977).

Cyclophosphamide is commonly used as an immunosuppressive medicine in combination with other immunosuppressant agents like prednisone, mycophenolic acid, or azathioprine to treat several autoimmune diseases such as systemic lupus erythematosus (SLE) (Barile-Fabris, et al.2005; Vasoo, Hughes. 2005) and rheumatoid arthritis (Leandro, Edwards, Cambridge. 2002; Edwards, Leandro, Cambridge, 2005).

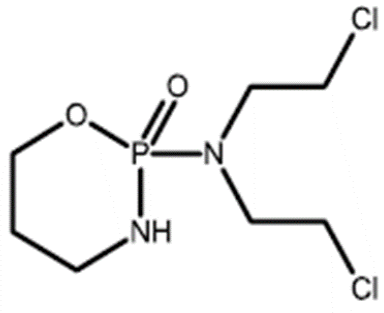


Fig 2.1. The chemical structure of cyclophosphamide

2.1.1. Indications

Cyclophosphamide is one of the mostly used alkylating agent. It has been effective in the treatment of both hematological and solid tumors. It is applied in combination with other antineoplastic drugs in the form of advanced neoplastic disease. CP shows potent immunosuppressive activity and is also used in the treatment of rheumatoid arthritis, Behcet's disease, nephrotic syndrome and some other autoimmune diseases (McEvoy, 2007).

2.1.2. Pharmacodynamic

The alkylating agents are among the oldest and most useful antineoplastic drugs; and the nitrogen mustards are the oldest alkylating agents in use in medicine.

Alkylating agents show their effects by different mechanisms that are inhibit cell reproduction by formation of irreversible covalent binding with the nucleic acids (DNA) and the specific type of chemical bonding involved is alkylation. After alkylation, DNA is unable to replicate and therefore unable to synthesize proteins and other essential cell metabolites. Also, they can cause interstrand and intrastrand cross-linking of DNA if two electrophilic groups are present (Fig. 2.2). Consequently, cell reproduction is inhibited, and the cell eventually dies from the inability to maintain its metabolic functions.

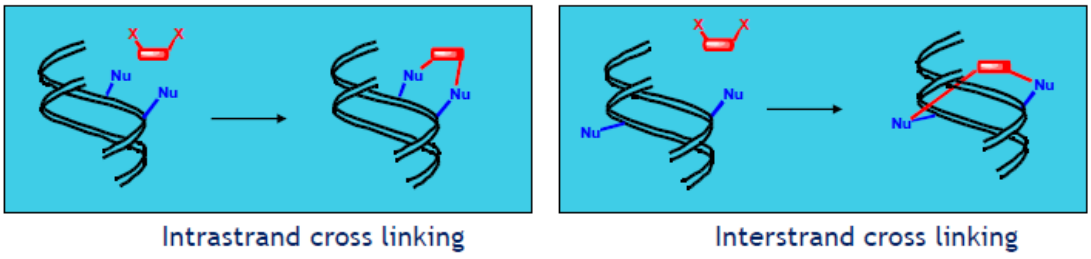


Fig 2.2. Anti-tumor agents interstrand and intrastrand cross-linking of DNA in the presence of two electrophilic groups.

The nitrogen mustards of CP are same other alkylating agents' mustards, work via the covalent bonding of highly reactive alkyl groups to nucleophilic groups of nucleic acids. Covalent binding to cellular proteins can also appear. After metabolic activation, bifunctional alkylating nitrogen mustards of CP are produced, which can react with the nitrogen-7 atom of purine bases in DNA, when they are flanked by adjacent guanines (Kohn, Hartley, Mattes. 1987; Lindley, et al.2002).

There is growing evidence that CP has important effects on the immune system. CP has been shown to modulate humoral and cell-mediated immunity (Colvin. 1999; Brodsky. 2002; Lindemann, et al.1989; Lacki, Mackiewicz, Leszczynski, Muller.1997; Lacki JK, Leszczynski P, Mackiewicz SH. 1996; Zhang, Georgiou, Mandel. 1993; Scambia, et al.1993) that leading to several beneficial effects which makes it to be used as an immunosuppressive drug. CP also found to diminish CD4+/CD25+ regulatory T cells and elevate T lymphocyte proliferation and T memory cells resulting in increased efficacy of antitumor immune responses in both animals and humans (Ikezawa, et al. 2005; Solomon, et al.2005; Lutsiak et al. 2005; Ghiringhelli, et al.2004; Lazzarino, et al.2005; Beyer, et al.2005).

2.1.3. Pharmacokinetics

Cyclophosphamide has two administration routes of orally and intravenous (i.v) and can be used alone or in combination with other medical drugs. Absorption, distribution, metabolism, and elimination may be affected by several factors including regimen and dosage, drug combination, route of administration and the patient's gender, age, renal and hepatic function as well as genetic factors. It is recommended to take the drug on an empty stomach, but in some cases, it may be taken with food to decrease the gastrointestinal irritation (Fraiser, Kanekal, and Kehrer. 1991). The time to reach the peak plasma concentration is about 1-2 h (Crom, et al. 1987).

The t½ of CP ranges from 3.2-7.6 hours with total body clearance (CL) values of ~2.5 to 4.0 L/h/m^2 (Boddy, Yule. 2000). CP metabolism is conceivably

more rapid in children that's may be because of increased CP activity (Yule. et al.1996).

Following intravenous administration, CP is distributed with a volume of distribution of 30-50 L, that is close to the total body water, suggesting that the distribution of CP occurs with minimal tissue binding.

2.1.3.1. Absorption

Cyclophosphamide is usually considered as water-soluble product and is well soluble with saline or alcohol as monohydrate and can be readily administered orally. CP is commonly given orally at doses (75−200 mg/day) when used for immunosuppression and as a component of the CMF (CP, methotrexate and fluorouracil) regimen which is used for breast cancer (Gheuens, Slee, De Bruijn. 1990; De Bruijn, et al.1988).

Cyclophosphamide is well absorbed in the stomach, CP reached after 1 hour the peak concentration following oral drug administration and 5 min-2 h after i.v administration. The oral bioavailability of CP is 85−100% (Gheuens, et al.1990; De Bruijn, et al.1988; Wagner, Fenneberg. 1984a; Stewart, Morgan, Verma, Maroun, Thibault. 1995; Wagner, Fenneberg.1984b), and a part of the drug metabolized in liver and gut due to the first-pass effect. At higher doses (0.7 g/m2), CP has an oral bioavailability about 87.7% (Matthias, Sohr, Preiss, Brockmann. 1984).

2.1.3.2. Distribution

Cyclophosphamide distributed immediately throughout the body after oral or intravenous administration, and show ~20% (0−30%) plasma protein binding without dose-dependent changes. Several metabolites of CP are protein bound more than 60%, like 4-hydroxy-CP (<67%) (Moore. 1991). CP is not structurally changed by blood plasma. The volume of distribution is closely to the total body water volume about (30 to 50 L), the volume of distribution (Vd) of CP is elevated in obese patients, which leads to a higher elimination half-life (t½ß) (Powis, Reece, Ahmann, Ingle. 1987). In some studies, it has been suggested that CP enters brain and into cerebrospinal fluid through

blood-brain barrier (BBB) with varying cerebrospinal fluid to plasma ratios ranging from 0.2 to 4 (Neuwelt, Barnett, Frenkel. 1984; Yule, Price, Pearson, Boddy. 1997; Hommes, Aerts, Bahr, Schulten. 1983). Some CP active metabolites found to have low penetration into brain which is may be because of their higher polarity and increased the plasma protein binding (Hommes, et al. 1983). That's may explain the lower neurotoxicity associated with the intravenous administration route of CP.

2.1.3.3. Metabolism

Cyclophosphamide metabolic pathways in humans are shown in Fig. (2.3). As a prodrug, CP is metabolized to active alkylating mustard by liver enzymes in the body.

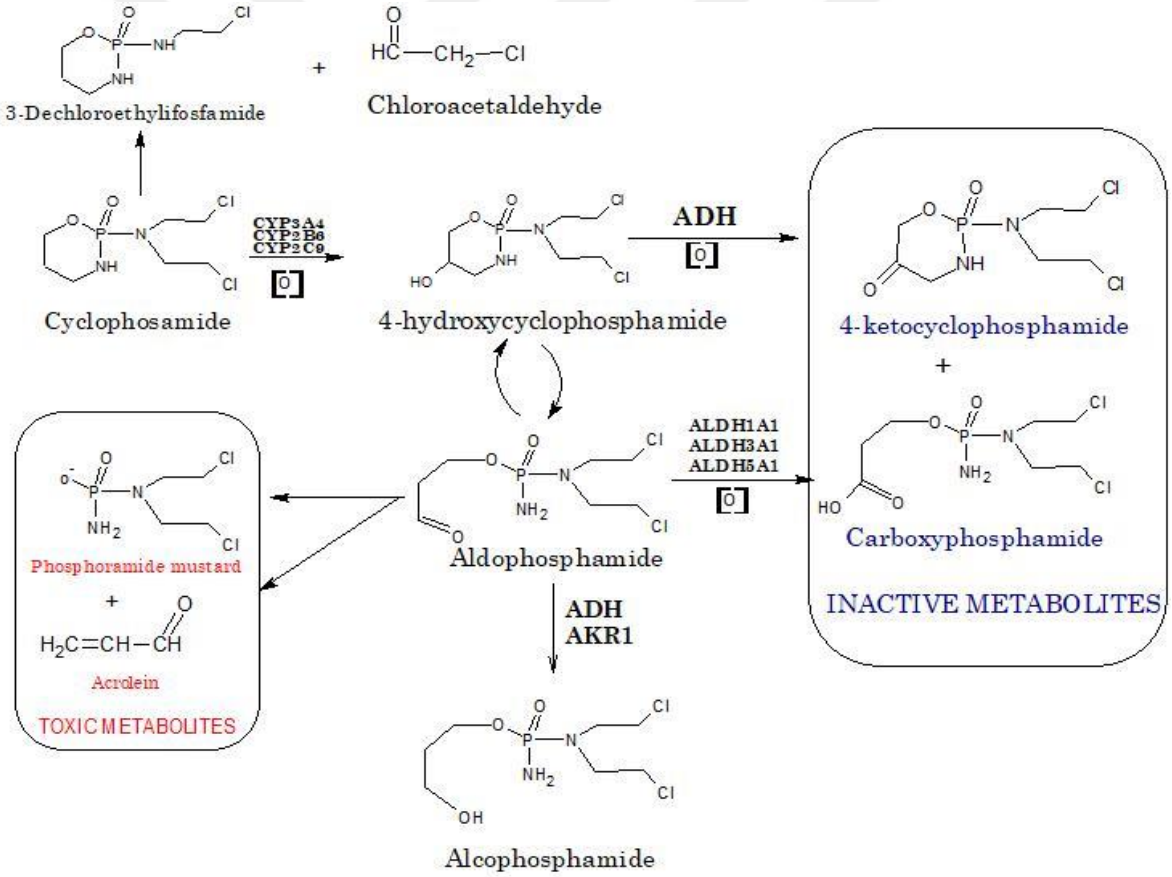


Fig 2.3. Metabolism of cyclophosphamide.

2.1.3.3.1. Activation

The liver is the main activation center for CP. After administration, approximately 75% of the CP is activated in liver by microsomal cytochrome P450s into the active metabolites of 4-hydroxycyclophosphamide, aldophosphamide, phosphoramide mustard, and acrolein, and the inactive metabolites which are 4-keto-cyclophosphamide, carboxyphosphamide and nornitrogen mustard. (Perry. 1992; Miller, Chandler, Ippoliti. 1994).

The initial activation of CP is 4-hydroxylation at C^4 of oxazaphosphorine ring to form 4-hydroycyclophosphamide (4-OH-CP) (Colvin, Brundrett, Kan, Jardine, Fenselau. 1976) Multiple cytochrome P450 (CYP) enzymes including CYP2B6, CYP2C9, and CYP3A4 in the liver are responsible for CP 4-hydroxylation (Chen, et al. 2004; Chang, Weber, Crespi, Waxman. 1993; Ren, Yang, Kalhorn, Slattery. 1997; Huang, Roy, Waxman. 2000). CYP2B6 is the major contributor (a mean of ~45% of total metabolism) for the activation of CP with the highest intrinsic clearance in vitro and in vivo, compared with 25% for CYP3A4 and 12% for CYP2C9 (Chen, et al. 2004; Huang, et al. 2000; Roy, Yu, Crespi, Waxman. 1999). Other cytochromes involving CYP2C8, CYP2A6, and CYP2C19 also make a lesser contribution to CP 4-hydroxylation (Chang, et al. 1993).

The metabolism of CP is primary happened in the liver through the drug is activated and eliminated, but metabolism may occur in other sites, involving the erythrocytes (Dockham, Sreerama, Sladek. 1997), kidneys (Aleksa, et al. 2005) and tumor itself (Schwartz, Chen, Waxman. 2003). Several CYPs including CYP1A1, 2A6, 2B6, 2C8/9, and 3A4 present in a variety of tumors like those originating from the central nervous system, lung, breast, ovaries, colon, kidney, and prostate, but their relative levels to the normal tissue are low(Murray, Melvin, Burke. 1995; Yu et al. 2001; Kivisto et al. 1995). Because CYP3A4 and CYP2B6 are the main enzymes for CP activation, their intratumoral level can be a useful predictive marker for the efficacy of CP treatment.

2.1.3.3.2. Oxidation (N-dechloroethylation)

In opposite, the inactivation pathway of CP include minor (~10%) side-chain oxidation (N-dechloroethylation) mainly by CYP3A4/3A5 and, to a lesser extent, by CYP2B6 to form 3-dechloroethylifosfamide and the byproduct chloroacetaldehyde (CAA) which is neurotoxic and nephrotoxic (Ren, Yang, Kalhorn, Slattery. 1997).

2.1.3.3.3. Detoxification

In the circulation and enters tumor cells 4-OH-CP is a major metabolite of CP separated through its tautomer aldophosphamide (an aldehyde intermediate) via spontaneous ß-elimination to generate final cytotoxic phosphoramide mustard (N,N-bis-2-(2-chloroethyl)phosphorodiamidic acid) and an equimolar amount of the byproduct acrolein (a highly electrophilic α,ß-unsaturated aldehyde)(Fenselau et al. 1977; Hohorst, Draeger, Peter, Voelcker. 1976; Colvin. 1999).

On the other hand, aldophosphamide by alcohol dehydrogenase (ADH) and aldo-keto reductase (AKR1) enzymes can also be oxidized to create the alcophosphamide alternatively, mainly by cytosolic aldehyde dehydrogenase (ALDH1A1), and to a lesser extent, by ALDH3A1 and ALDH5A1, 4-OH-CPA can be detoxified to O-carboxyethylcyclophosphoramide (CEPM) (Dockham, Sreerama, Sladek. 1997; von Eitzen, Meier-Tackmann, Agarwal, Goedde. 1994; Sladek, Dockham, Lee. 1991; Manthey, Sladek. 1989; Hipkens, Struck, Gurtoo. 1981; Domeyer, Sladek. 1980; Cox, Phillips, Thomas. 1976; Yule et al.1995; Yule, Price, McMahon, Pearson, Boddy. 2004; Moreb et al. 2005).

Aldehyde dehydrogenase enzyme catalyzes the conversion of a wide range of aldehydes to the corresponding acid through a nicotinamide adenine dinucleotide (NAD+)-dependent irreversible reaction. Additionally, 4-OH-CPA is oxidized by ADH to the non-toxic 4-keto-CP (Yule et al.1995; Yule et al. 2004; Lelieveld, van Putten. 1976; Boddy, Furtun, Sardas, Sardas, Idle. 1992; Jarman. 1973; Hohorst, Ziemann, Brock. 1971), but to a lesser extent compared with CEPM formation. Furthermore, 4-OH-CPA is exposed to reversible dehydration to generate iminocyclophosphamide that is additionally

conjugated with intracellular glutathione (GSH) via glutathione S-transferases GSTA1, A2, M1, and P1, giving rise to non-toxic 4-glutathionylcyclophosphamide (GSCY) (Dirven, van Ommen, van Bladeren. 1994; Dirven, Venekamp, van Ommen, van Bladeren. 1994).

The resulting phosphoramide mustard is known as a bifunctional alkylator of DNA and the final cytotoxic metabolite of CP (Struck, Kirk, Witt, Laster. 1975). The alkylation includes the formation of the intermediate phosphoramide aziridinium ion via an intramolecular nucleophilic attack (cyclization reaction) of the nitrogen on the ß-carbon of a chloroethyl chain (Ludeman. 1999). Cellular thiols (e.g., GSH) and other nucleophiles react quickly with phosphoramide aziridinium ions, which results in thioether products (Gamcsik, Dolan, Andersson, Murray. 1999). CEPM is one of the main stable metabolites of CP, which are easily measured in the plasma and urine (Joqueviel et al. 1998). On the other hand, acrolein is a greatly reactive aldehyde and covalently binds to several macromolecules in the cell and subsequently disturbs the organ functions and causes organ toxicity (Brock, Stekar, Pohl, Niemeyer, Scheffler.1979; Kehrer, Biswal. 2000). The detoxification is occurred by conjugation with GSH via GSTs present in hepatocytes (Gurtoo, Hipkens, Sharma. 1981) and this may result in depletion of intracellular GSH and injuries of the hepatocytes (DeLeve. 1996). The reaction between GSH and acrolein includes nucleophilic addition at the ß-carbon atom, which generates stable thioether compounds (Ramu, Fraiser, Mamiya, Ahmed, Kehrer. 1995; Ramu, Perry, Ahmed, Pakenham, Kehrer. 1996). (Figure 2.4).

2.1.3.4. Elimination

Cyclophosphamide is mainly (70%) eliminated in the urine in various metabolites forms and a lesser extent, in the feces (Boddy, Yule. 2003). However, following CP administration, in the urine only ~10-20% is excreted in the unchanged form (Fasola et al. 1991; Juma, Rogers, Trounce. 1979) and 4% is excreted in the bile (Dooley, James, Rogers, Stuart-Harris. 1982).

The main inactive metabolite of CP present in the urine is CEPM, while 4-keto-CP is just a minor metabolite in the urine sample of patients (<1%) (Hadidi, Idle. 1988). In cancer patients, the renal clearance of CP was reported as 15–44 ml/min (Chen et al 1995; Yule et al.1996; Busse et al. 1997). On the other hand, renal clearance of CP metabolites including dechloroethylifosfamide, 4-OH-CP, CEPM and keto-CP in cancer patients was reported to be 60.6 ± 9.0, 3.2 ± 1.0, 7.0 ± 4.5 and 1.3 ± 0.8 m1/min, respectively (Busse et al. 1997). therapy with in the course of high-dose of CP (100 mg/kg), although the amount of CP eliminated in urine was found to be correlated to the urine flow, this correlation was not present with conventional-dose therapy (500 mg/m2) (Busse et al. 1997). After all, in a study, no correlation was found between the renal excretion of CP metabolites and urine flow with either conventional or high dose therapy (Busse et al. 1997).

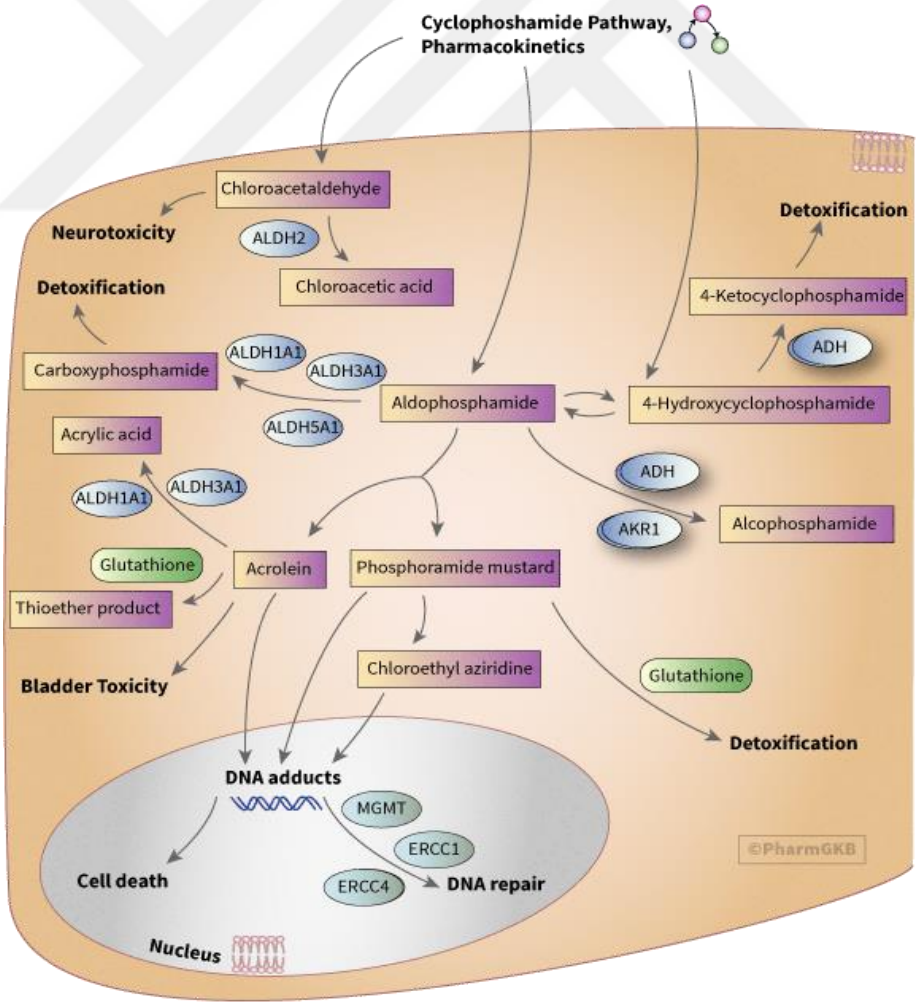


Fig 2.4. Pharmacokinetics of cyclophosphamide.

2.1.4. Pharmacogenetic

Glutathione S-transferases (GST) are the enzymes of Phase II and detoxifies several chemicals and drugs including chemotherapeutic agents and their metabolites. The GSTs involved in the detoxification of CP include GSTM1, GSTT1, GSTA1 and GSTP1 (Choi, Nowell, Blanco, Ambrosone. 2006). Decreased activity of these enzymes results in the decrease of detoxification of active CP and increase in duration of action of the drug. Theoretically, this should lead to increased drug side effects but, should also the enhanced survival.

On the other hand, aldehyde dehydrogenases, primarily ALDH1A1 and ALDH3A1 mediates the oxidation of two active and inactive (aldophosphamide and acrolein, respectively) metabolites of CP (Yang et al. 2005). Although these enzymes have been also reported to have genetic polymorphisms, the functional effects of these polymorphisms are not documented well observed (Spence et al. 2003; Vasiliou, Pappa. 2000).

2.1.5. Side Effects

Toxicity of CP depends on different factors like cumulative dose, which is a main causing factor for toxicity, and various genetic polymorphisms.

Dependent neutropenia is quite common appending to dose-related, and patients may also suffer from thrombocytopenia and anemia.

During CP treatment, immunosuppression and serious infections can occur and these infections may necessitate the reduction of dose and/or discontinuation of the drug. Common appeared side effect in Amenorrhea, gonadal suppression, azoospermia, oligospermia, oogenetic disorders and sterility are the major endocrine and metabolic side effects of CP treatment.

Most common gastrointestinal system side effects are non-specific including abdominal pain, anorexia, nausea/vomiting (dose-related), diarrhea, mucositis and stomatitis. On the other hand, a more specific side effect in genitourinary system is hemorrhagic cystitis.

2.1.5.1. Genotoxic damage

The genotoxic effects of CP on males depend on what is the level of germ cell development at the time of the drug exposure. There are some studies showing that postmeiotic germ cells are more susceptible developmental stage to the adverse effects of CP (Trasler, Hales, Robaire. 1986).

Alkylating agents have highly electrophilic groups and form covalent bonds to nucleophilic groups in DNA (e.g. 7-N of guanine), which prevents the replication of DNA and transcription. When CP binds covalently to DNA, DNA damage is induced in several forms including strand breaks, DNA-DNA cross-linking, both inter-strand and intra-strand, and DNA-protein cross-linking (Colvin .1999). On the other hand, the reaction of phosphoramide mustard with another guanine moiety in an opposite or same DNA strand may result in formation of crosslinks (Springer, Colvin, Colvin, Ludeman. 1998).

In previous studies with low-dose CP for 9 weeks in male rats, exposure of the spermatogonia to the drug in earlier stages resulted in a high incidence of malformations and growth retardation in fetuses (Trasler, Hales, Robaire .1987), as well as chromosomal aneuploidy (Barton, Wyrobek, Hill, Robaire, Hales . 2003). Moreover, increased preimplantation loss was observed following a 4–6-week CP treatment; this effect was found to be related to the effect of CP on germ cells first exposed as early spermatids and spermatocytes (Trasler et al .1986). During the development of mouse spermatocytes, a single injection of high-dose CP was found to result in heritable translocations (Sotomayor, Cumming .1975).

2.1.5.2. Effect on bone marrow

Myelosuppression resulting in leucopenia, neutropenia, thrombocytopenia, and anemia are considered as the most important hematological toxicity of the CP. Despite that, the leucopenia and granulocytopenia may render the patients susceptible to pathogenic bacterial or opportunistic infections. Infectious complications, including life-threatening septicemia are more commonly seen in patients treated with high-dose CP (Buckner et al. 1972; Horn, Phebus, Blatt. 1990). Although CP is generally

considered as being platelet-sparing, thrombocytopenia can also be a significant complication and can lead to an increased potential for spontaneous bleeding episodes.

2.1.5.3. Effects on skin and hair

The patients treated with CP can suffer from alopecia (more than 10%) and it is usually reversible after discontinuation of the drug and start to occur after taking the drug by 3-6 weeks (Qiu, Zhang, Zhao, Zhou. 2017).

For skin, it is so rare to have any disorder but rash, dermatitis, nail discoloration, skin discoloration can be seen with an incidence of 0.01% to 0.1%. Stevens-Johnson syndrome (SJS), toxic epidermal necrosis (TEN), pruritus and erythema, can also be seen rarely (less than 0.01%) (Heng, Allen.1991; Frangogiannis et al.1996; Trautmann, Klein, Kämpgen, Bröcker. 1998).

2.1.5.4. Effects on fertility

Cyclophosphamide is toxic for gonadal organs in both males and females and the incidence of gonadal dysfunction is dependent upon age, sex, and CP dose (Byrne et al. 1987)

In females, CP may lead to infertility, premature menopause and premature ovarian failure (POF). Treatment-induced damage includes the depletion of ovarian follicles and shrinkage and fibrosis of the ovaries. Some patients can have amenorrhea during CP treatment which may subsequently recover. On the opposite side, some females who have apparent preservation of ovarian function during and after treatment, premature ovarian insufficiency can develop years later. It has been suggested that women treated before the age of 25 are at lower risk of infertility than those treated after the age of 30 (Gourley et al.1996; Boumpas et al.1993; Watson, Taylor, Rance, Bain. 1986; Wang, Wang, Bosco. 1995; Huong et al.2002; Clowse et al.2011; Harward et al.2013). On the other hand, women treated successfully with CP can conceive and deliver healthy children (Wang et al. 1995; Huong et al.2002; Ramsey-Goldman, Mientus, Kutzer Mulvihill, Medsger .1993).

In male genitourinary system, CP reduce the sperm count and with high dose and treatment duration lead to irreversible azoospermia (Watson, Rance, Bain.1985; Guesry, Lenoir, Broyer. 1978). Researches in animals indicate that CP is excessively toxic to primordial and antral follicles (Plowchalk & Mattison 1991). All these effects are dependent on the dose and time of treatment. Libido and sexual capability are mainly not affected. Testicular injury is reported to appear in boys and men after 7 to 9 g of CP and to occur more often with comparable doses of IV versus oral CP (Jahnukainen, Ehmcke, Hou, Schlatt. 2011).

2.1.5.5. Malignant tumors

Cyclophosphamide was found to be mutagenic and clastogenic in several in vitro and in vivo genetic toxicology researches. Secondary malignancies urinary tract cancer, myelodysplasia, acute leukemias, lymphomas, thyroid cancer, and sarcomas have been showed in patients under a therapy containing CP. The bladder cancer can be decreased through avoiding of hemorrhagic cystitis. Patients who have rheumatoid arthritis (RA) and other systemic autoimmune diseases are at higher risk of developing lymphoma independent of treatment. However, treatment via CP improve the risk of leukemia, skin cancer, hematologic malignancies, and other malignancies (Lohrmann. 1984; Vasquez, Kavanaugh, Schneider, Wacholtz, Lipsky. 1992; Radis et al. 1995; Bernatsky, Clarke, Suissa. 2008). Mechanisms of alkylating agent-induced malignancy include direct chromosomal damage and decreased immune surveillance. The time of therapy is a significant risk factor, with the incidence being most in patients treated for more than to three years (Radis et al. 1995).

2.1.5.6. Infection

Treatment with CP leads to susceptibility to infections via inducing bone marrow depression, which lead to neutropenia or lymphopenia, Main bacterial or fungal infections can appear in the absence of neutropenia. This has been estimated to appear in 5 to 21 % of patients treated with CP for

granulomatosis with polyangiitis (GPA) or lupus nephritis. (Mok et al. 2006; Hoffman et al. 1992; Boumpas et al. 1992; Austin et al. 1986).

Although infection by *Pneumocystis jirovecii* is a serious complication of treatment with CP, the frequency is only <1 %. Especially when CP is used in combination with glucocorticoids (Vernovsky, Dellaripa. 2000), prophylaxis is recommended for almost all patients.

2.1.5.7. Hemorrhagic cystitis

Hemorrhagic cystitis in patients on CP treatment as an immunosuppressive agent for rheumatologic disease is determined by the route of administration. The incidence was found to be higher with use of continuous daily oral CP, because the treatment duration and total cumulative dose are higher in comparison with the intermittent intravenous exposure (Monach, Arnold, Merkel. 2010). In some cohort studies on patients with GPA, the incidence of hemorrhagic cystitis ranged from 12 to 41% (Reinhold-Keller et al. 2000; Talar-Williams et al. 1996; Stillwell TJ, Benson RC Jr, DeRemee RA, McDonald TJ, Weiland LH. 1988). The patients in all these studies were exposed to cumulative CP doses ranging from 50 to 100 g.

On the opposite side, newer CP regimens including intravenous CP administration have decreased the cumulative CP dose markedly, which lead to lower rates of hemorrhagic cystitis (Monach et al.2010). As an example, in a patient with lupus nephritis on six-monthly doses of IV CP at a dose of 1250 mg/month, total cumulative dose will be only 7.5 g. Nonetheless, patients requiring multiple doses of oral CP, may ultimately reach cumulative doses associated with bladder toxicity. Few cases of hemorrhagic cystitis also have been documented in clinical trials and case reports of patients treated by intermittent IV CP for rheumatic diseases (Monach et al. 2010).

2.1.5.8. Testicular damage

Testicular germ cell damage is a major adverse effect of CP and has been proved in previous experimental and clinical studies. Decreased testicular weight, transient oligospermia, and histological and biochemical changes have

been reported as the main adverse effects on the male reproductive system (Lu, Mei, Wang, Zheng, XueF, Xu DH. 2015; Elangovan N, Chiou TJ, Tzeng WF, Chu ST.2006).

Although the exact mechanism of reproductive toxicity induced by CP treatment is not well documented, previous studies suggested that CP can impair the redox balance in tissues resulting in physiological and biochemical changes caused by oxidative stress (Lu et al. 2015; Selvakumar, Prahalathan, Sudharsan, Varalakshmi. 2005a).

Previous studies have showed that CP changes the composition of sperm head basic proteins and sperm chromatin structure, as well as increases abnormal sperm rate, and result in biochemical and histological deteriorations in testis (Selvakumar, Prahalathan, Sudharsan, Varalakshmi. 2006; Codrington, Hales, Robaire. 2007; Ilbey et al. 2009). Oxidative stress-induced biochemical and physiological damage has been suggested to be responsible for CP toxicity in testis and spermatozoa (Selvakumar et al.2005b; Ahmadi, Hosseinimehr, Naghshvar, Hajir, Ghahremani. 2008). Because mitochondrial membrane of spermatozoa is rich in polyunsaturated fatty acids and poor in antioxidants, this compartment has been suggested to be more susceptible to lipid peroxidation (Aitken, McLaughlin. 2007; Agarwal, Makker, Sharma. 2008). On the other hand, in morphologically abnormal spermatozoa, mitochondria and plasma membranes produce reactive oxygen species (ROS) (Agarwal et al. 2009).

In the testis, because the cells within the seminiferous tubules of the germinal epithelium are those having highest mitotic and meiotic indices, they are most susceptible to the CP-induced toxic effects. Although during the first weeks of CP treatment, a decline can be seen in sperm count, it usually takes 2-3 months for azoospermia to occur. Antineoplastic agents including CP are most toxic to the rapidly proliferating cells such as type B spermatogoniz in testis; because they effect the sperm cells during the cell division. On the other hand, the severity and duration of testicular damage induced by CP treatment is mostly associated with the number of type A spermatogonia that are

affected. Spermatogenesis may recover nearly 12 weeks following the treatment in the case of stem cells remaining intact within the tubules. (Hayes, Bubley. 2015).

Spermatogenesis and steroidogenesis are main sources of ROS in controlled oxidative stress status. Testis have antioxidant system which protects the cell from oxidative damage in such this situation. Some exposures to environmental contaminants lead to an imbalance in pro-oxidant/ antioxidant levels and thereby stimulate the uncontrolled ROS productions. This can subsequently activate extrinsic (Fas and FasL) and intrinsic (mitochondrial) apoptotic pathways, thereby leading to apoptotic damage to the testis. (Mathur, D'Cruz. 2011). (Figure 2.5).

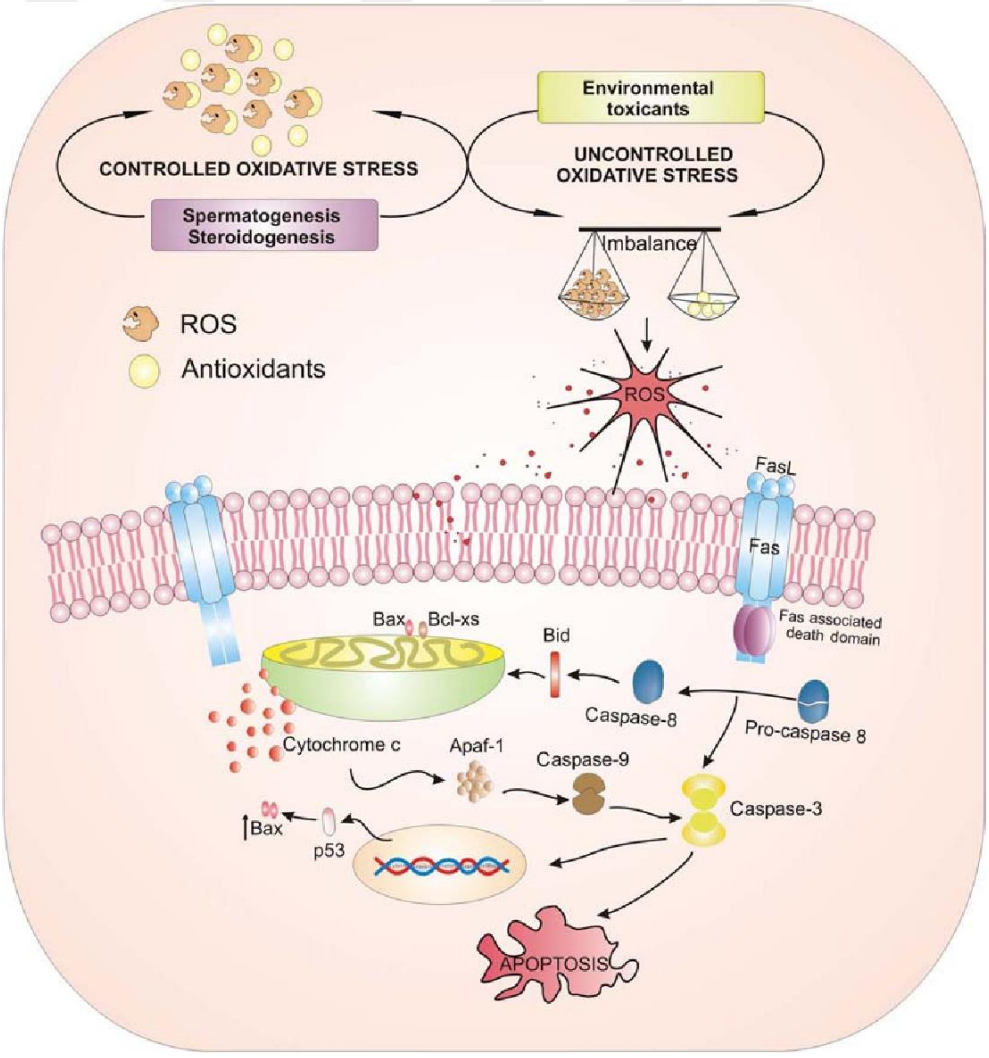


Fig 2.5. A model showing the effect of controlled and uncontrolled oxidative stress on male reproduction. (Mathur, D'Cruz. 2011).

2.1.5.9. Cardiotoxicity

Myopericarditis, myocarditis, pericardial effusion involving cardiac tamponade, and congestive heart failure which can be life-threatening in some cases are the main cardiac complications reported in patients on CP treatment. Supraventricular arrhythmias with atrial fibrillation/flutter as well as ventricular arrhythmias with severe QT prolongation were also found to be associated with CP use (Ejaz, Raza, Maroof, Haider. 2018)

Although cardiotoxicity has been suggested to be dependent mainly on CP dose, advanced age, previous radiation treatment to the cardiac region and concomitant treatment by other cardiotoxic agents are the other risk factors (Gottdiener, Appelbaum, Ferrans, Deisseroth, Ziegler. 1981).

2.2. Oxidative stress

2.2.1. Definition of Oxidative stress

Oxidative stress can be explained as an imbalance between the generation of free radicals and the capability of the body to neutralize their damaging effects through antioxidants.

Oxygen is frequently referred to have double faces, because of producing both beneficial effects and potentially damaging side-effects for biological systems. Oxygen take part in high-energy electron transfers because of it is reactivity, and accordingly participates by oxidative phosphorylation in the production of large quantities of adenosine-5-triphosphate (ATP). Although this is crucial for the evolvement of the multicellular organisms, it also attack to the several biological molecules including proteins, lipids or DNA. Although human body is constantly under the attack of ROS, an unique and complex antioxidant defense system mainly balances this attack. In the case of perturbation of this pro-oxidant–antioxidant balance in favor of the former, oxidative stress occurs and have potential damages to the cells. (Halliwell, Gutteridge. 1999).

2.2.2. The effect of cyclophosphamide on oxidative stress

The therapeutic activity of CP depends on the alkylating activity of drug on tumor cell DNA. On the other hand, acrolein, one of its metabolites, induces oxidative stress, leading to DNA damage in normal cells and various degrees of toxicity on target organs including bladder, heart and lungs. Immediately after production, acrolein enters the cell where it activates the intracellular ROS production and nitric oxide synthesis, resulting in peroxynitrite production which has damaging effects to the lipids, proteins, and DNA (Korkmaz, Topal, Oter. 2007). (Figure 2.6).

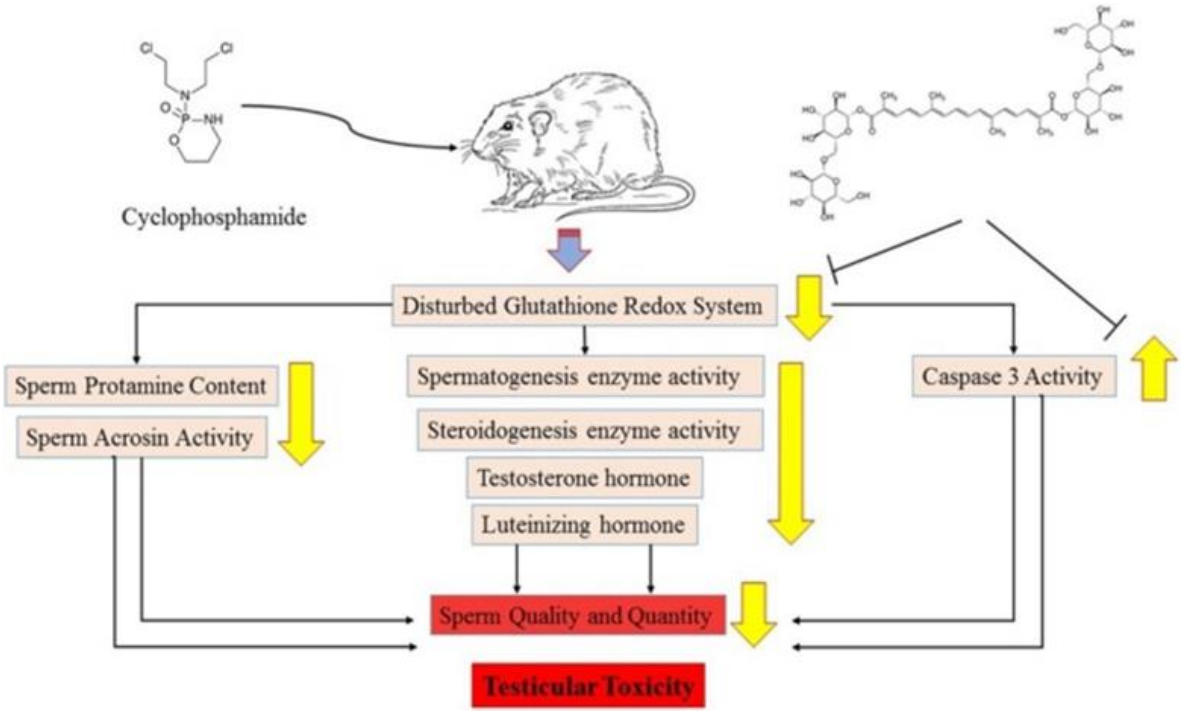


Fig 2.6. The sequence effect of cyclophosphamide on oxidative stress.

2.3. Apoptosis

Apoptosis called as programmed cell death and mainly have distinct morphological characteristics and biochemical processes that need energy. Apoptosis is a crucial component of various processes in human such as normal cell growth and development, functioning of the immune system, embryonic development and chemical-induced cell death. (Elmore. 2007).

Apoptosis is markedly seen during the developmental stage and is a balancing mechanism that maintains the cell population within the tissues. On the other hand, apoptosis also appears as a defense mechanism for example in immune reactions or when cells are damaged due to a disease or harmful agent (Norbury and Hickson, 2001). Although there are numerous physiological and pathological stimulus triggering the apoptosis, the same stimulus does not necessarily result in apoptosis in all cells. For example, radiation therapy or anticancer drugs result in apoptotic death in some cell via DNA damage. On the other hand, corticosteroids can result in apoptotic death in only limited cell types such as thymocytes with several other cells remaining unaffected or even stimulated. (Elmore, 2007).

In some situations, the choice of cell to die via apoptosis or necrosis is determined by the type and the degree of injurious stimuli. For example, although the low doses of such stimuli including hypoxia, heat, radiation, and cytotoxic drugs usually induce apoptosis, the higher doses of the same stimuli may result in necrosis. At last, apoptosis is a clearly energy-dependent process that include the activation of a group of cysteine proteases called "caspases" and a complex cascade of events that link the initiating stimuli to the final demise of the cell.

Apoptosis is induced through multiple pathways. As it explained in Figure 2.7, after activation of the initiator Casp-8 and -9, the caspase cascade leads to cell death by more activation of death effector molecules. (A) Receptor-mediated induction of apoptosis. (B) Stress, irradiation, and inflammation act on mitochondria through intermediary proapoptotic Bcl-2 family members like Bax, leading to blocking the antiapoptotic activity of Bcl-2. (C) Cytotoxic cells introduce granzyme molecules inside the target cell by multimerization of the perforin molecule. The dashed arrows in the figure 2.7 indicate secondary effects of activated caspases. PARP, poly (ADP-ribose) polymerase; MAPK, mitogen-activated protein kinase. (Gavrilescu and Denkers.2003). (Figure 2.7).

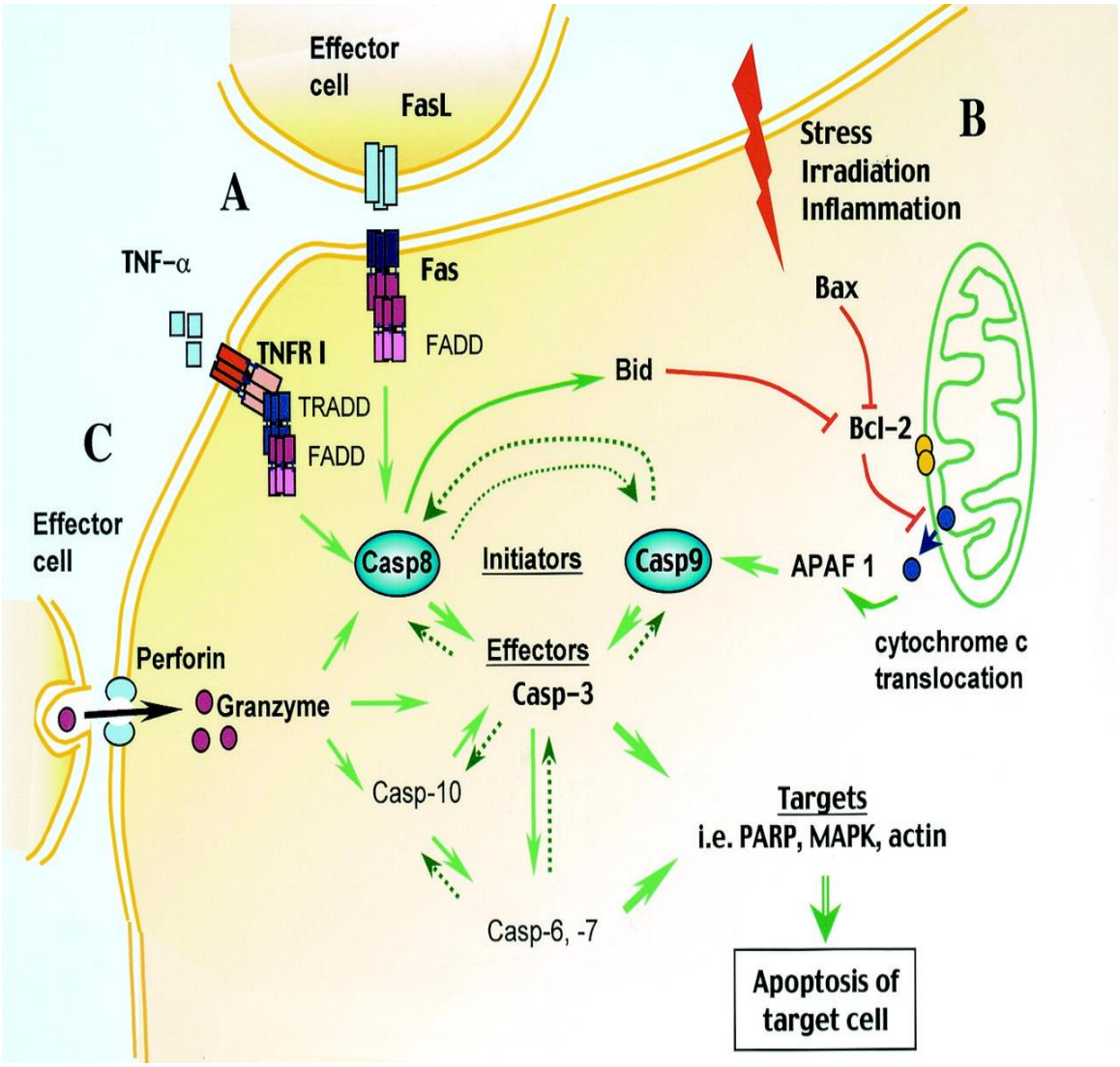


Fig 2.7. Apoptosis induced pathways (Gavrilescu and Denkers.2003)

2.3.1. The effect of cyclophosphamide on apoptosis

Cyclophosphamide has been suggested to induce DNA damage in germ cells and some of these cells are removed by apoptosis, which results in a decrease in the number of maturing spermatozoa (Trasler, Hales, Robaire. 1985; Hales, Robaire. 1990; Hales, Crosman, Robaire. 1992; Trasler et al. 1986). CP has been also found to induce apoptosis mainly in spermatogonia and spermatocytes. It should be noted that apoptosis may prevent the premeiotic germ cells from transmitting their DNA damage to future generations.

2.4. Taurine

Taurine is a thiol-containing amino acid and was isolated from bovine bile nearly 150 years ago. Taurine can be taken from the diet, mainly by eggs, meat, and seafood, with higher taurine concentrations in the heart and retina, and less in the brain, kidneys, intestine, and skeletal muscle. (Birdsall. 1998)

Taurine, 2-aminoethanesulfonic acid, is considered as an endogenous end-metabolite which distributed in several body tissues at high concentration. Also is an amino acid with sulfur group and is synthesized from cysteine. It is excreted unchanged from human body without any further metabolism. Many biological functions of taurine have been studied mainly on skeletal muscle, retina central nervous system and cardiovascular system. The cytoprotective action of taurine is the main therapeutic effect in humans including various mechanisms such as antioxidation, energy production, neuromodulation and many others. Therefore, taurine has beneficial effects in various diseases ranging from those of the central nervous system (CNS), cardiovascular system and skeletal muscle. (Schaffer and Kim. 2018). (Figure 2.8).

Because it is a naturally occurring substance, few adverse side effects have been reported in therapeutic and/or supplemental use of taurine. On the other hand, although clinical studies on taurine is very limited, it has already been approved by different studies for use in patients with congestive heart failure.

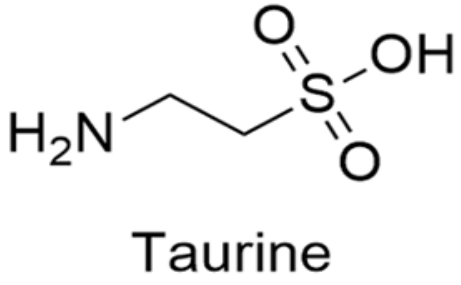


Fig 2.8. Taurine chemical structure

Taurine (2-aminoethane sulfonic acid) synthesized from cysteine in the organism (figure 2.7) and is a colorless, water-soluble, non-protein-containing amino acid with a molecular weight of 125 Daltons and is freely available. (Kendler. 1989; Jacobsen, Smith, 1968).

Due to the sulphonate group, taurine has a strong acidic property, has a pKa value of 1.5 and has a zwitterionic structure at physiological pH. (Huxtable, Sebring, 1986; Sturman, Hepner, Hofmann, Thomas, 1975).

2.4.1. Pharmacokinetics

A study about the pharmacokinetics of taurine in eight healthy male volunteers (median age 27.5 years, range 22–45) following oral administration of 4 g of taurine. Maximum plasma taurine concentration (Cmax) was measured at 1.5 ± 0.6 h after administration as 86.1 ± 19.0 mg/L (0.69 ± 0.15 mmol). Plasma elimination half-life ($T_{1/2}$) and the ratio of clearance/bioavailability (Cl/F) were 1.0 ± 0.3 hr and 21.1 ± 7.8 L/hr., respectively (Ghandforoush-Sattari, Mashayekhi, Krishna, Thompson, Routledge. 2010).

Taurine is highly bound to plasma proteins and retained in the plasma fraction (Tang et al. 2015).

2.4.1.1. Absorption

Taurine oral administration was studied for different dose-dependent values of area under curve (AUC), Cmax and Tmax and at a dose of 1-30 mg/kg ranged from 89-3452 mcg min/L, 2-15.7 mcg min/ml and 15 min respectively (Nielsen, Bjerg, Ulaganathan, Holm. 2017). More researches in healthy individuals gave an AUC, Cmax and Tmax in the range of 116-284.5 mg h/L, 59-112.6 mg/L and 1-2.5 h (Ghandforoush-Sattari et al. 2010).

2.4.1.2. Distribution

In mammals, taurine distributes nearly all-over the body, with tissue concentrations are being in the micromole per gram wet weight. Body fluids, such as plasma, cerebrospinal fluid, and extracellular fluid, contain much lower concentrations, usually in the range of l0-100 μM. The highest

concentrations are typically found mainly in the heart or brain, but taurine is present in the musculature massively

The distribution of taurine was studied with a two-compartment model and each one of the compartments gave a range for the volume of distribution (Vd) of 299-353 ml/kg in compartment 1 and 4608-8374 ml/kg in compartment 2 in mice (Nielsen et al. 2017). Further studies in healthy individuals gave a Vd that ranged from 19.8 to 40.7 L. (Ghandforoush-Sattari et al. 2010).

2.4.1.3. Metabolism

Mammals metabolize sulfur amino acids through cysteine to cysteine sulfinate to sulfate. Also metabolize cysteine sulfinate to taurine. However, the capacity to do this is highly variable by species. On the other hand, the mammals that cannot decarboxylate enough cysteine sulfinate should take the taurine from dietary sources. Taurine is excreted in different forms can eliminate unchanged or as taurocholate form or related to the bile salts. Because of the loss of the ability to conjugate taurine in some mammals they cannot form bile salts (Huxtable. 1992) (Figure 2.9).

In mammalian, taurine synthesis usually appears in the pancreas through the cysteine sulfonic acid pathway. In this pathway, via the enzyme cysteine dioxygenase the sulfhydryl group of cysteine is oxidized to cysteine sulfonic acid. Then, by a sulfinoalanine decarboxylase cysteine sulfonic acid is decarboxylated and hypotaurine is produced. Taurine is conjugated to generate the bile salts sodium taurochenodeoxycholate and sodium taurocholate. This metabolism occur in hepatocytes and is the way of bile acids to recovered from the intestine are converted to bile salts before being released again into the bile. The low pKa (1. 5) of taurine's sulfonic acid group approve that this moiety is negatively charged in the pH ranges normally found in the intestinal tract. (Lehninger. 2005; Salway. 2004; De la et al. 2010).

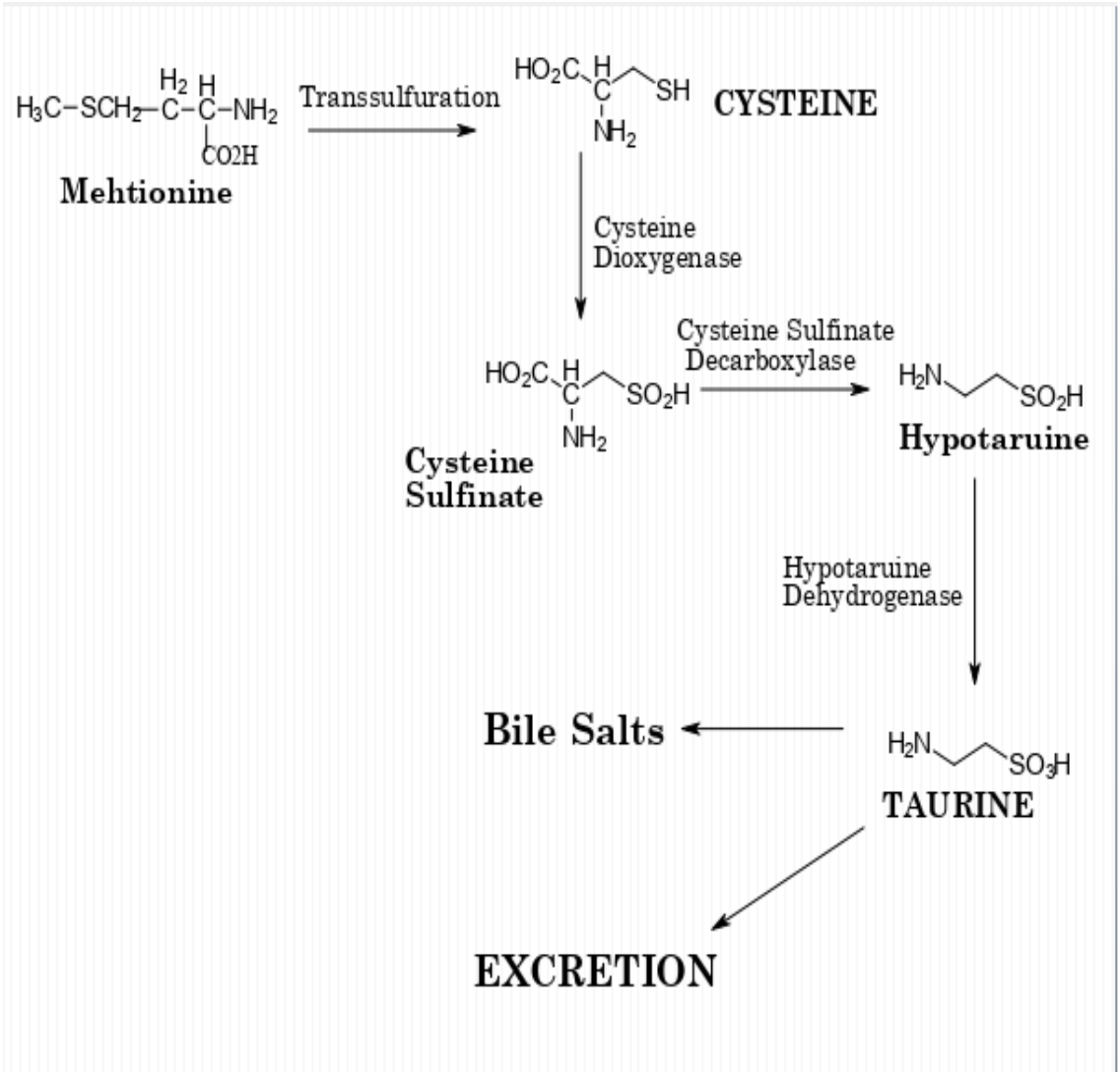


Fig 2.9. Hepatic metabolism of sulfur in mammals (Huxtable. 1992).

2.4.1.4. Elimination

Enterohepatic cycle of taurine can explain the elimination route via the gut and is the main elimination route of taurine is thought it. (van Stijn et al. 2012)

In healthy individuals using of taurine as oral administration route gave a plasma elimination half-life that ranged about 0.7-1.4 h. (Ghandforoush-Sattari et al. 2010) after the measurement of clearance rate was showed to be dose-dependent where in a dose of 1 mg/kg, it presents a clearance rate of 11.7 ml/min/kg, 10 mg/kg generates a clearance rate of 18.7 ml/min/kg and a dose of 30 mg/kg reports a clearance rate of 9.4 ml/min/kg. (Nielsen et al. 2017) More other studies about a clearance rate in healthy individuals produce that ranged about 14 to 34.4 L/h. (Ghandforoush-Sattari et al. 2010).

2.4.2. Pharmacodynamic

Taurine has been tested as potential pharmacological agents in many pathological cases also has lot of physiological functions, entering in cell volume regulation and inhibitory neuromodulation. These that's have increased the interest about how much taurine can be use as a therapeutic agent potentially.

In tissues, taurine has thought to work an antioxidant in cell protection and have found beneficial effects on cardiovascular system. These days, taurine has been approved in several countries like Japan for the treatment of congestive heart failure and shows promised future in the treatment of other diseases.

Furthermore, taurine is actually effective in the treatment of different mitochondrial disease, mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes, and shows an attractive future for the treatment of metabolic diseases, starting by diabetes, and inflammatory diseases like arthritis (Schaffer and Kim. 2018).

2.4.3. General effects of taurine

Because taurine usually an inert molecule with no reactive groups and is sulfur-containing amino acid, it is not including in the protein synthesis. Beside its well-known of his function to conjugation with bile acids, taurine has also several physiological functions divided into nonmetabolic effects including osmoregulation, calcium homeostasis and phospholipid-protein interactions, and metabolic effects including antioxidation, radioprotection, cysteine detoxification and xenobiotic conjugation (Bouckenooghe, Remacle, Reusens. 2006; El Mesallamy, El-Demerdash, Hammad, El Magdoub. 2010). Taurine has been suggested to have beneficial effects in several diseases including heart failure epilepsy and cystic fibrosis (Caine, Geracioti. 2016). There are also some studies reporting that taurine can biosynthesized also via male reproductive organs (Li, Ling, Fan, Zhang, Cui. 2006). (Figure 2.10).

2.4.3.1. The Cytoprotective Activity of Taurine

Taurine have many functions in mammals and attracted attention because of its cytoprotective actions (Schaffer, Shimada-Takaura, Jong, Ito, Takahashi. 2016). Because of these effects, taurine is now used in nutritional supplements, energy drinks and infant formula. It also drew attention about its potential therapeutic uses. After all, most of those researches on taurine-mediated reversal of several pathologies in animals result in many clinical studies suggesting a promising future for taurine therapy in many diseases (Ginguay, De Bandt, Cynober. 2016). There are also promising studies showing the nutritional value of taurine (McCarty, 2013).

On the other hand, taurine is classified to be conditionally essential nutrient or a functional nutrient in man (Gaull, 1986; Gaull, 1989; Bouckenooghe, Remacle, Reusens. 2006). At the same time, taurine supplementation is also linked to decreased body mass index (Yamori et al., 2010) and reduced levels of inflammatory markers in obese women (Rosa, Freitas, Deminice, Jordao, Marchini. 2014). Cytoprotective action of taurine may contribute to an improvement in the clinical and nutritional health of humans.

2.4.3.1.1. Antioxidant activity

Most of researches nowadays have uncovered novel mechanisms about taurine-mediated cytoprotection. One of the most important cytoprotective function of taurine is antioxidant activity, which is defined by various distinct events. Taurine is a well-known anti-inflammatory agent and neutralizes the hypochlorous acid which oxidizes the neutrophils. The reaction between taurine and hypochlorous acid produces the taurine chloramine that interferes with the inflammatory process (Kim and Cha, 2014; Marcinkiewicz and Kontny 2014). Besides, taurine directly decreases the superoxide synthesis via the mitochondria (Jong, Azuma, Schaffer. 2012; Schaffer, Jong, Ito, Azuma. 2014). However, the production of the taurine conjugate is decreased in some mitochondrial diseases, which in turn suppresses the expression of several mitochondria encoded proteins including NADH-ubiquinone oxidoreductase chain 6 (ND6) (Schaffer et al., 2014). ND6 is required for maximal complex I activity and a decreased ND6 biosynthesis diminishes the complex I activity and the utilization of NADH by the respiratory chain and thus the mitochondrial ATP synthesis but it increases the superoxide production by the same respiratory chain (Jong et al., 2012; Schaffer et al., 2016; Shetewy et al., 2016). It is well-known that mitochondrial oxidative stress damages several macromolecules within the mitochondria, but above all it can alter the mitochondrial permeability and trigger mitochondria-dependent apoptosis (Ricci et al., 2008, Shetewy et al., 2016).

Oxidative stress is a main response in cases of tissue damage such as aging, infection, acute and chronic inflammation and cancer. At the site of inflammation, activated leukocytes such as neutrophils, macrophages, and eosinophils produce ROS that mediates oxidative stress. In the case of infections, although ROS play a beneficial role in host defense, they also induce tissue injury (Weiss 1989; Smith 1994). There are several antioxidants involving in the prevention of ROS-induced cell damage and oxidative reduction of various molecules including high molecular compounds such as lipids, proteins and DNA. In summary, antioxidants act through one of the

mechanisms including decreased ROS formation, neutralization of ROS and/or inhibiting the action of ROS (Schaffer, Azuma, Mozaffari. 2009).

Because it is present at high concentrations particularly in tissues exposed to increased levels of oxidants, taurine has been suggested to play a key role in the defense against oxidative stress (Green, Fellman, Eicher, Pratt. 1991; Jeon et al. 2009; Oliveira et al. 2010). Although, there are many reports on the antioxidant capacity of taurine, the underlying mechanism remains unclear. The best-known antioxidant effect of taurine is neutralization of hypochlorous acid (HOCl), which is a highly toxic oxidant (Weiss, Klein, Slivka, Wei.1982). This effect of taurine also explains the anti-inflammatory activity of taurine, because the reaction of taurine with HOCl generates taurine chloramine (TauCl), which is a more stable (not membrane-permeable) and less toxic anti-inflammatory mediator (Weiss et al. 1982; Thomas 1979; Marcinkiewicz and Kontny 2012).

2.4.3.1.2. Regulation of gene expression

The genetic changes induced by taurine were previously investigated by Park et al. (Park et al. 2006). More recently, it has been found that taurine sensitive genes contribute to various cellular functions such as cell cycle progression, cell signaling, death and survival, amino acid metabolism, protein biosynthesis, protein folding and aging (Ito et al. 2014). Also, several changes in the content of transcription factors have been found (Schaffer et al., 2016).

2.4.3.1.3 Osmoregulation

Taurine is present in quietly high concentration within most cells. Intracellular taurine level has been suggested to increase with the increasing osmotic load and to decrease in response to hypo-osmotic stress. This is the main mechanism protecting the cell from excessive stretching caused by imbalances in osmotic pressure. Because taurine functions as an organic osmolyte, it also regulates the level of another osmolytes, the Na+. Unlike the taurine that is a neutral zwitterion, Na+ carries a charge and also play a role in many cellular functions including transport and membrane potential (Schaffer, Solodushko, Kakhniashvili. 2002).

Taurine was found in high concentration in mammalian sperm and seminal fluid, and it is suggested to have an osmoprotective function. In hamsters, fluids from reproductive tract were found to be hyperosmolar with values of up to 400 mosmol/kg (Johnson, Howards. 1977). On the other hand, in chimpanzee, hypoosmotic stress killed the sperms, which was prevented by the addition of 2 mM taurine (Ozasa, Gould.1982).

After all, it is necessary to show that taurine affects either water movement or ion fluxes sufficiently to restore osmotic equilibrium across the cell membrane to establish a clear osmoregulatory function in mammals.

2.4.3.1.4. Regulation of quality control processes

Taurine also regulates quality control processes, such as the ubiquitin-proteasome system and autophagy. These processes either renew damaged cells and subcellular organelles or eliminate them through degradation or cell death. In cells with taurine deficiency, a decreased the activity of the proteasome which will leads to an accumulation of ubiquitinated proteins, an effect abolished by the mitochondrial specific antioxidant (Jong, Ito, Schaffer. 2015).

Autophagy is a statement that allows damaged cells and organelles to accumulate it was found that is also associated with diminished taurine deficiency (Jong et al., 2015).

Inactivation of these quality control processes is highly damaging to cells and tissue. That said, excessive autophagy is also damaging because it can elevate cell death. Beside that not more much studies about examining the effect of taurine treatment on autophagy, the actions of taurine are compatible with its cytoprotective activity, as taurine attenuates toxin-mediated autophagy (Li et al., 2012; Bai et al., 2016).

2.4.3.2.Taurine Effect on Pathology and Disease

Since the first discovery in 1827, many functions of taurine have been studied particularly on skeletal muscle, and the central nervous and cardiovascular systems. The cytoprotective effects of taurine improves both the

clinical and nutritional health of humans via various mechanisms, including antioxidation, Ca2+ homeostasis, energy production, osmoregulation and neuromodulation. All these cytoprotective effects, alone or in combination, act to improve the pathology and/or symptoms of the various diseases of CNS, cardiovascular system and skeletal muscle. Many patients with CNS-related diseases, including stroke, neurodegenerative diseases and epilepsy benefit from taurine therapy. Taurine is also used for the certain inflammatory and cardiovascular diseases, such as diabetes mellitus, arthritis, hypertension, atherosclerosis, heart failure, ischemia-reperfusion injury and myocardial arrhythmias.

Because taurine is a naturally occurring substance, it has relatively few adverse effects and plays a key functional role in most mammalian cells. Thus, the potential of taurine as an effective therapeutic agent and a nutritional supplement is extremely attractive for many diseases. Although clinical evaluation of taurine has been limited to a few diseases, it has already been approved for use in congestive heart failure.

2.4.3.2.1. Role of taurine in inflammatory diseases

The characteristic features of the acute inflammatory response, which can usually continue several days, are vascular dilation, microvascular leakage and leukocyte recruitment. Adhesion factors facilitating the interaction between leukocytes and activated endothelium result in the recruitment of leucocytes. Damaged or diseased tissue, as well as various irritants and pathogen organisms trigger an acute inflammatory response, in which the damage is and the microorganisms and harmful agents are removed by the immune system. During acute inflammation, after adherence of the leukocytes to the endothelium, to transmigration occurs into the interstitium across the endothelium. Neutrophils are recruited to the site of inflammation via the chemotactic factors and cytokines. Activation of leukocytes, in particular neutrophils and mononuclear phagocytes, result in the secretion of various proinflammatory mediators such as including lytic enzymes, ROS and lysosomal granule constituents. The release of these proinflammatory

mediators into phagolysosomes result in the destruction of the pathogen organisms. However, when they are released into the extracellular space, tissue damage can occur. In rheumatoid arthritis, a disease characterized by synovial inflammation and proliferation, bone erosions and articular cartilage loss, activation of the inflammatory process is an important component of the autoimmune disorder contributing to joint deformation, erosion of bone and damage of the cartilage-bone interface.

The taurine content in the neutrophils is quite high and represents nearly 50% of the total free amino acid pool. In neutrophils, the two main functions of taurine are anti-inflammatory and antioxidant effects. As mentioned above, ROS are produced by the neutrophils to kill the pathogens and one of those ROS is hypochlorous acid (HOCl). Myeloperoxidase enzyme catalyzes the formation of taurine chloramine (TauCl) from taurine and HOCl. Because TauCl is a less potent oxidant than HOCl, the neutralization of HOCl via taurine is an important antioxidant mechanism. This myelperoxidase-catalyzed reaction is also responsible for the anti-inflammatory activity of taurine, as TauCl inhibits the production of several proinflammatory cytokines (Marcinkiewicz, Grabowska, Bereta, Stelmaszynska. 1995; Park, Schuller-Levis, Jia, Quinn. 1997; Barua, Liu, Quinn. 2001), prevents elevation of the nitric oxide and prostaglandin E2 (Park, Quinn, Schuller-Levis. 2000; Chorazy-Massalska et al., 2004; Kim et al., 2007), decreases the activity of matrix metalloproteinases and initiates apoptosis of the leukocytes terminating the acute inflammation (Klamt and Shacter, 2005).

2.4.3.2.2. Effects of taurine on the sperm quality

There are some studies reporting that taurine is synthesized by male reproductive organs particularly in Leydig cells of the testes, the cellular source of testosterone in males, as well as the cremaster muscle, efferent ducts, and peritubular myoid cells surrounding seminiferous tubules (Li, Ling, Fan, Zhang, Cui. 2006).

Taurine has been defined as the major free amino acid in the sperm cells and seminal fluid (Aaronson, Iman, Walsh, Kurhanewicz, Turek. 2010). It is

well known that oxidative stress plays an important role also in the diseases including the destruction of male rat reproductive system. because of its antioxidant activity, taurine has been reported to play a key role in cytoprotection and decrease of apoptosis (Das, Sil. 2012).

It is well known that oxidative stress is related to mitochondrial dysfunction and most beneficial effects of taurine are related to its antioxidant capacity (Marcinkiewicz, Kontny. 2012). In spermatozoa, taurine level were found to be correlated with sperm quality, probably by protection against lipid peroxidation through antioxidant activity of taurine and also through facilitating the maturation of spermatozoa via the capacitation, motility, and the acrosomal reaction (Das, Ghosh, Manna, Sinha, Sil. 2009; Yang et al. 2010).

Taurine have been reported to be present in spermatozoa and seminal fluid of numerous species and have beneficial effects on sperm characteristics in mammals (Holmes, Goodman, Hurst, Shihabi, Jarrow. 1992; van der Horst, Grooten. 1966). Taurine have significant roles in the maintenance and stimulation of sperm motility and stimulation of capacitation and acrosome reactions in vivo and in vitro (Meizel, Lui, Working, Mrsny. 1980; Mrsny, Waxman, Meizel. 1979). Taurine also inhibited the lipid peroxidation in rabbit spermatozoa and prevented the loss of motility (Alvarez, Storey. 1983).

The present results show that taurine can significantly increase the motility of sperm in adult rats but had no clear effect on the other parameters of semen quality. In aged rats, although taurine can increase the count and the motility of sperm, and the count of live sperm, no significant effect was found on the rate of sperm with intact acrosome. The results indicate that taurine can improve the semen quality in male animals but particularly in aged male animals.

Although it has been found that aging results in a significant decrease in serum and testis taurine content (Wallace, Dawson. 1990; Coleman, Barthold, Osbaldiston, Foster, Jonas. 1977), taurine administration to aged male rats can improve the male reproductive function presumably via the stimulation of

testosterone secretion, promotion of testis homeostasis, and antioxidation (Jiancheng et al. 2010).

In the light of all this information, in this study, we aimed to investigate the potential effects of taurine administered at increasing doses (75, 150 and 300 mg/kg/day) in the prevention and/or treatment of testicular injury due to CP administration in rats.

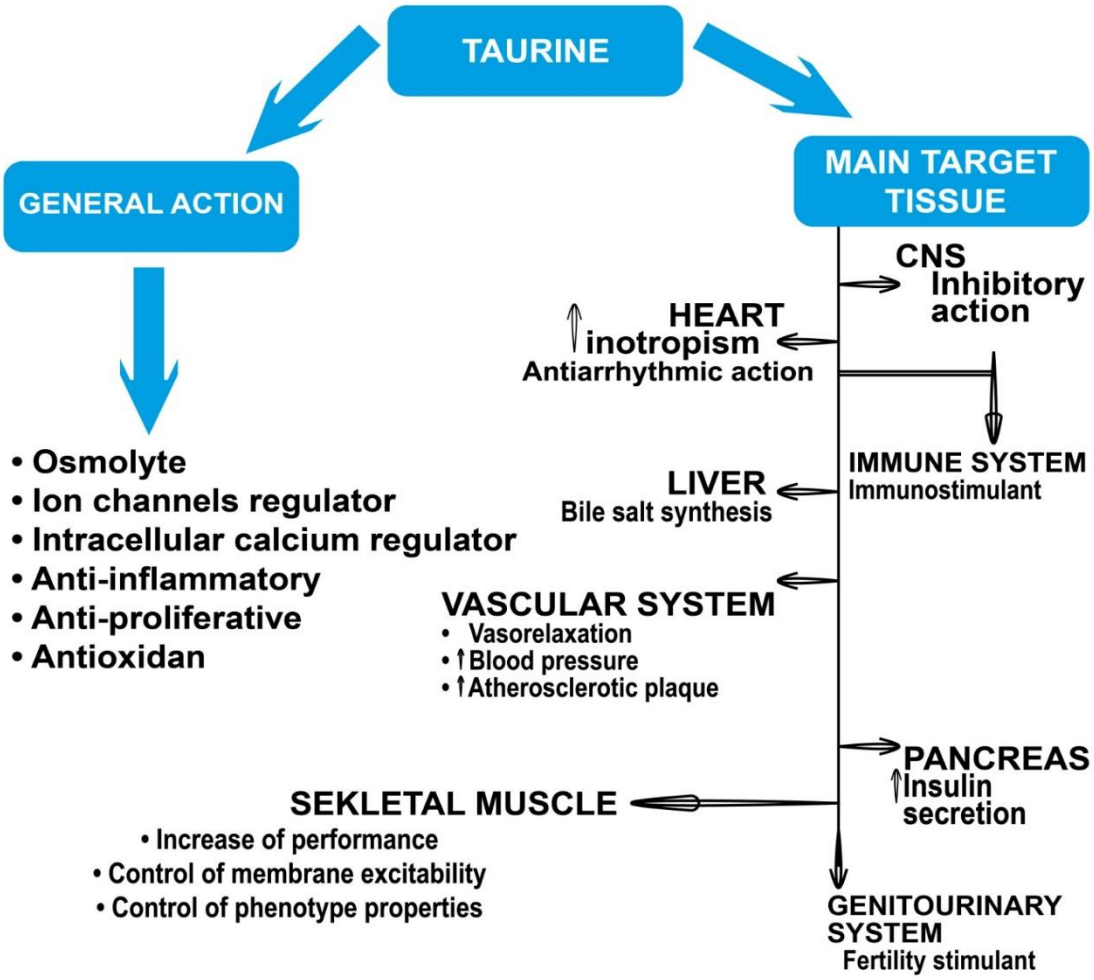


Fig 2.10. Taurine Basic Effects

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Animals

Adult Sprague-Dawley male rats weighing approximately 200-300 g were used in the experiments. The rats were taken to the laboratory one week before the beginning of the experiment to accommodate the working environment and were housed in well ventilated rooms at 24 ± 1 ° C for 12 hours dark 12 hours light cycle. During the adaptation period and experiment period, they were fed with standard animal feed and tap water. All experiments carried out during the study were conducted after approval of the Animal Experimentation Ethics Committee of Eskisehir Osmangazi University (File Registration No.2018-464-1).

3.1.2. Devices Used

Elisa reader	BiotekPowerWave XS, USA
Precision Scales	Ohaus, USA
Homogenizer	Heidolp, Germany
Incubator	Lab. Companion S1-600, USA
Refrigerated centrifuge	Eppendorf(5810R), Germany
Magnetic Stirrer	DAIHAN, South Korea
Vortex	Heidolp REAX, Germany
Pipette set	Brand, Germany
Automatic pipette	Eppendorf, Germany

3.1.3. Chemicals and Kits

Interleukin 2(IL-2) ELİSA KİT (Rat)	Shanghai Yl biotech, CHINA
Interleukin 6(IL-6) ELİSA KİT (Rat)	Shanghai Yl biotech, CHINA
TAS (Total antioxidant status)	Rel Assay Diagnostic, Turkey
TOS (Total oxidant status)	Rel Assay Diagnostic, Turkey

Taurine	Sigma Aldrich, Germany
Ketamine (Ketalar®)	Pfizer, Turkey
Xylazine (Rompun®)	Bayer, Turkey
PBS tablets	BIOMATIK, Canada

3.2. Method

3.2.1. Test protocol

Experimental groups consisted of a total of 40 male rats in 5 groups consisting of 8 animals in each group. TAU75, TAU150 and TAU300 groups were treated orally with taurine at doses of 75 mg / kg, 150 mg / kg and 300 mg / kg for 14 days solved in saline, respectively. The control and the CP groups were treated with saline orally for 14 days.

Group 1; Rats were treated orally with saline for 7 days and intraperitoneal saline was administered on the 8th day and then rats were treated orally with saline until the 14th day (n: 8) (CONTROL)

Group 2; Rats were orally treated with saline for 7 days, 200 mg / kg intraperitoneal CP was administered on the 8th day and then rats were treated again with oral saline until the 14th day (n: 8) (CP)

Group 3; 75 mg / kg taurine dissolved in saline was administered for 7 days orally, 200 mg / kg intraperitoneal CP was administered on the 8th day and rats were treated with 75 mg/kg oral taurine until the 14th day (n: 8) (TAU75)

Group 4; 150 mg / kg taurine dissolved in saline was administered for 7 days orally, 200 mg / kg intraperitoneal CP was administered on the 8th day and rats were treated with 150 mg/kg oral taurine until the 14th day (n: 8) (TAU 150)

Group 5; 300 mg / kg taurine dissolved in saline was administered for 7 days orally, 200 mg / kg intraperitoneal CP was administered on the 8th day and rats were treated with 300 mg/kg oral taurine until the 14th day (n: 8) (TAU 300)

Day/Group	Control	CP	TAU75	TAU150	TAU300
1-7. day	SF (oral)	SF (oral)	Taurine 75 mg/kg (oral)	Taurine 150 mg/kg (oral)	Taurine 300 mg/kg (oral)
8. day	SF (i.p)	CP (i.p)	CP (i.p)	CP (i.p)	CP (i.p)
8-14. day	SF (oral)	SF (oral)	Taurine 75 mg/kg (oral)	Taurine 150 mg/kg (oral)	Taurine 300 mg/kg (oral)

Table 3.1. Animal groups and experimental model

At the end of the experimental protocol and 24 hours after the last dose of taurine or saline, sampling was performed according to the following protocol.

3.2.2. Sample collection

1. At the end of the 14-day experimental period, rats from experimental groups were anesthetized with 60 mg / kg-ketamine and 5 mg kg-xylazine intraperitoneally and then euthanized by cervical dislocation.

2. Immediately thereafter, both testes of the rats were removed. Right testis was separated for histological examination by placing it in a plastic lidded specimen cup containing phosphate buffer solution (NaCl: 8 g, KCl: 0.2 g, KH2PO4: 0.2 g, Na2HPO4: 1.14 g in 1L distilled water).

3. The left testis, which was cleaned from blood and other impurities, was kept on filter paper for a certain period and the excess moisture was removed and then the testis weight was determined and recorded.

4. Approximately 100 mg of tissue sample taken from left testis was placed in a V-bottom capped tube containing phosphate buffer solution at 100 mg / ml (1:10 weight / volume) to prepare testis homogenate and stored at -20 ° C until used.

5. Samples taken from -20 ° C were thawed at room temperature and then homogenized in semi-liquid, semi-ice form with the help of homogenizer and then placed in a cooler centrifuge device and centrifuged at 15000rpm for 15 minutes at + 4 ° C.

6. After centrifugation, the supernatants of the samples were removed by pipette and transferred to another V-bottom capped tube and stored at + 4 ° C until used for cytokines and oxidative stress ELISA kits.

3.2.3. Biochemical analysis

3.2.3.1. Oxidative stress assessment

1. TAS Measurement: Total antioxidant status (TAS) of the samples was measured using Rel Assay commercial kits (Rel Assay Kit Diagnostics, Turkey). Trolox, a water-soluble analog of vitamin E, was used as a calibrator.

2. TOS Measurement: Total oxidant status (TOS) levels of the samples were measured using Rel Assay commercial kits (Rel Assay Kit Diagnostics, Turkey). Hydrogen peroxide was used as a calibrator. The results are expressed in µmol H2O2 equiv / lt.

Oxidative Stress Index: When calculating OSI, which is expressed as a percentage of the ratio of TOS levels to TAS levels, the following formula was used, and the results were expressed as “arbitrary unit” (AU) (Erel 2005).

$$OS\dot{I} = \frac{\text{TOS, µmol H2O2 equiv./lt}}{\text{TAS, mmol Trolox equiv./lt x 10}}$$

3.2.3.2. Evaluation of cytokine levels

Commercially available IL-2 (Shanghai YI biotech Co. Ltd. Rat Interleukin 2 ELISA Kit, CHINA) and IL-6 (Shanghai YI biotech Co. Ltd. RatInterleukin6 ELISA Kit, CHINA) ELISA kits were used to assess cytokine levels in homogenates from testis tissue and tested according to the test procedure specified by the manufacturer.

3.2.3.3. Histological evaluation

The extracted testes were taken into 10% neutral buffered formaldehyde and kept for 24 hours. The testes were then divided into transverse slices and the fixation continued for an additional 24 hours. Slices from the upper, middle and lower parts of the testes were washed with tap water and then dehydrated by passing through 70%, 80%, 90% and 96% ethanol series, respectively. It was held twice in xylol to make it light-permeable. After the liquid was passed through the paraffin series, paraffin blocks were obtained. Hematoxylin-eosin staining technique was used for general histopathological evaluation and caspase 3 and Bcl-2 immunohistochemistry were performed to assess apoptosis status. Images representing the findings of the examination performed under a binocular microscope were taken with a digital camera. (Süllü et al .2006).

After immunochemical staining for caspase-3 apoptotic index (AI) representing the presence of apoptosis in the testicular tissue was calculated by dividing the percentage of apoptotic cells by the total percentage of normal cells in the sample. On the other hand, bcl-2 immunoreactivity was scored as follows:

- 0-5% immunoreactivity
+ 5-25% immunoreactivity
++ 26-50% immunoreactivity
+++ 51-75% immunoreactivity
++++ 76-100% immunoreactivity

3.2.3.4. Statistical analysis

All data analyzes were performed with SPSS 21.0 package programs.

ANOVA One-way Analysis of Variance test was applied to the variables consisting of independent groups and showing normal distribution. The variables that were not normally distributed were analyzed by **Kruskal-Wallis One-way Analysis of Variance** on Ranks test. The probability values of $p < 0.05$ were considered significant.

4. RESULTS

4.1. Morphometric measurements

Body and testis weight determined at the beginning and end of the study protocol are shown in Table 4.1.

	Control	CP	CP+TAU75	CP+TAU150	CP+TAU300
Body weight at the beginning (g)	217.50 ± 13.46	205.00 ± 17.61	232.50 ± 9.58	231.88 ± 13.99	158.75 ± 14.21
Body weight at the end (g)	331.25 ± 25.06	207.14± 17.74^{a***}	249.71 ± 17.03	294.88 ± 20.67^{b*}	192.50 ± 13.50
Left Testis weight (mg)	1643.13±57.13	1483.00 ± 75.57	1565.29±81.73	1579.13 ± 51.57	1489.00 ± 73.77
Testis weight / body weight	5.09 ± 0.26	7.31 ± 0.31 ^{a***}	6.35 ± 0.29	5.47 ± 0.30 ^{b*}	7.91 ± 0.53

Table 4.1. Body and testis weight of the animals (^{a} compared to control and ^{b} compared to CP; *p<0.05 and ***p<0.001)

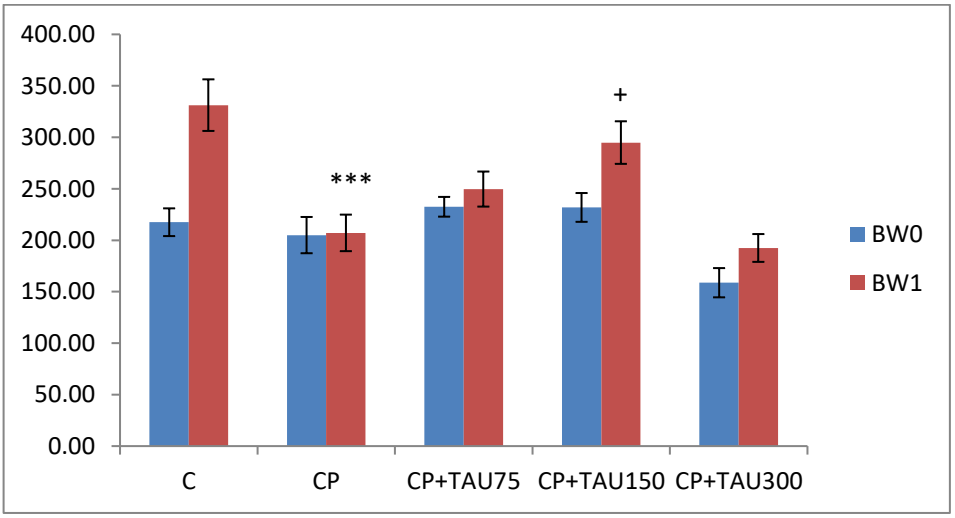


Figure 4.1. Body weight at the beginning (BW0) and at the end (BW1) of the study (g) Kruskal-Wallis analysis applied to (BW0) ANOVA analysis applied to (BW1) ***, p<0.001 compared to control; +, p<0.05 compared to CP

Body weight at the beginning (BW0) and at the end (BW1) of the study protocol are shown in Figure 4.1 There was no difference between the groups in terms of BW0 ($p>0.05$). However, BW1 was significantly different in CP group compared to the controls ($p<0.001$) and in TAU150 group compared to CP group ($p<0.05$).

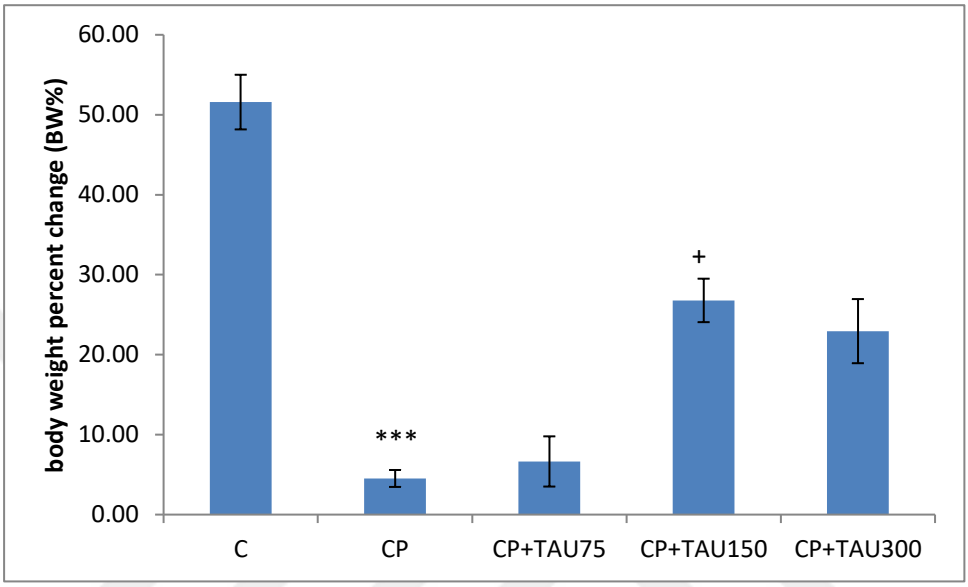


Figure 4.2. The percent change observed in body weight at the end of the study (BW%)
Kruskal-Wallis analysis applied to (BW%)
***, $p<0.001$ compared to control; +, $p<0.05$ compared to CP

At the end of the study protocol, the percent change seen in body weight was significantly lower in CP group compared to the control ($p<0.001$). Although taurine administered at the doses of 75 and 300 mg/kg/day resulted in no change, BW% was significantly higher in animals treated with CP and 150 mg/kg/day taurine compared to those treated with only CP ($p<0.05$) (Figure 4.2)

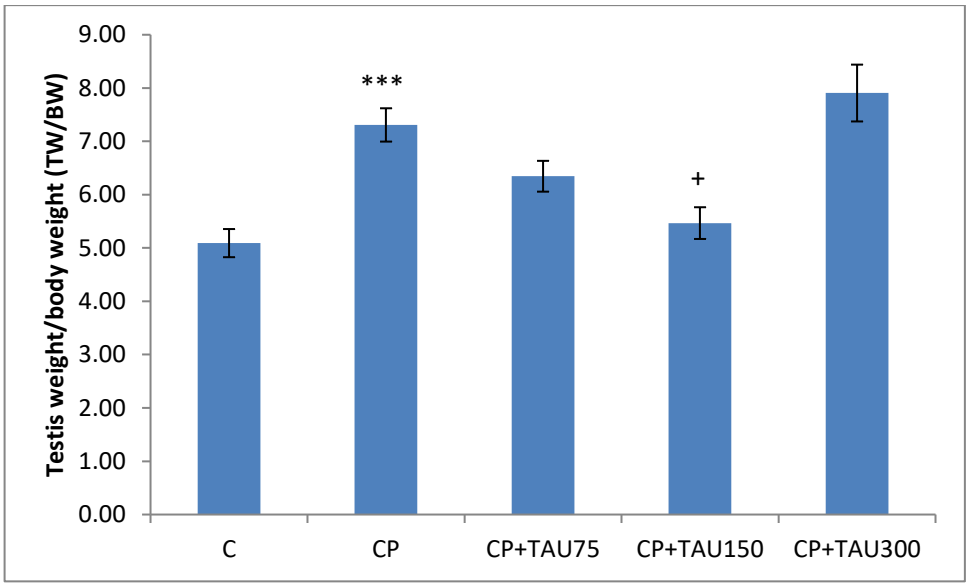


Figure 4.3. The ratio of testis weight to body weight at the end of the study (TW/BW)

ANOVA analysis applied to (TW/BW)

***, p<0.001 compared to control; +, p<0.05 compared to CP

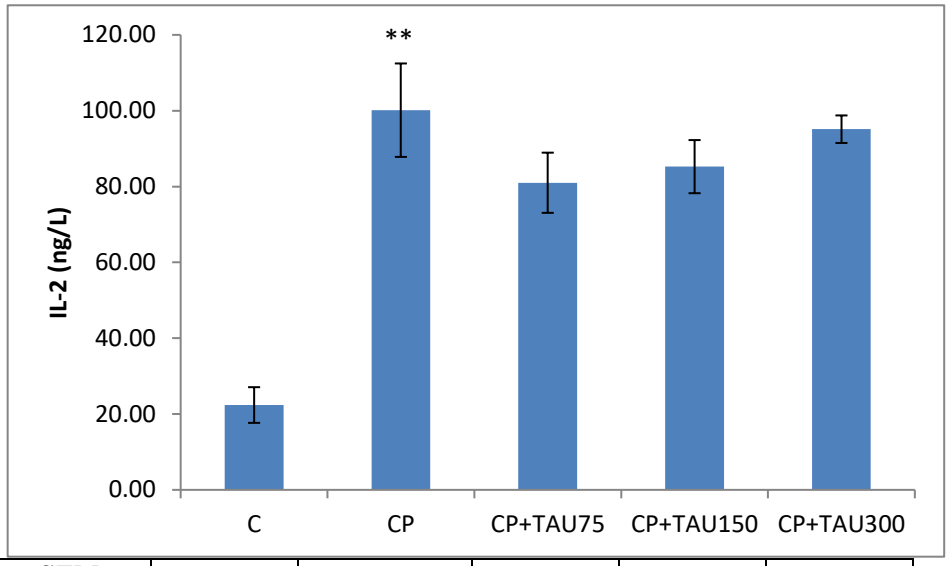
In terms of the ratio of testicular weight to body weight, it was significantly higher in CP group compared to the control group (p<0.001) and significantly lower in TAU150 group compared to the CP group (p<0.05) (Figure 4.3).

4.2. Biochemical findings

	Control	CP	CP+TAU75	CP+TAU150	CP+TAU300
OSI (AU)	2.07 ± 0.21	2.70 ± 0.13	2.01 ± 0.10	1.96 ± 0.27	2.41 ± 0.21
IL-2 (ng/L)	22.38 ± 4.71	100.14 ± 12.33 [a]**	81.00± 7.93	85.25 ± 7.01	95.13 ± 3.64
IL-6 (ng/L)	0.65 ± 0.34	0.89 ± 0.28	1.07 ± 0,27	1.10 ± 0.30	0.62 ± 0.27

Table 4.2. Oxidative stress status and cytokine levels in the groups.

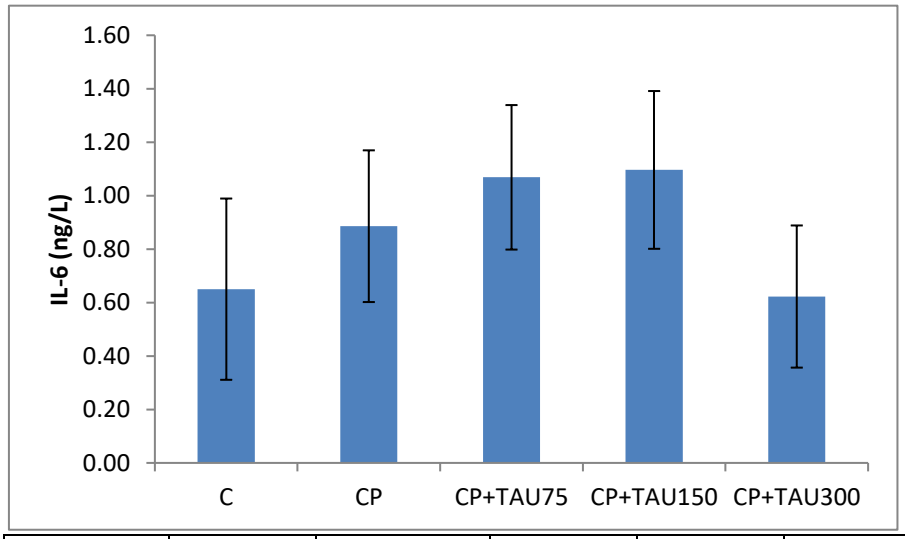
([a] compared to control **, p<0.01)



SEM	4.71	12.33	7.93	7.01	3.64

Figure 4.4. IL-2 levels in testis homogenate (ng/L) results are given as mean ± sem Kruskal-Wallis analysis applied to (IL-2) **, p<0.01 compared to control

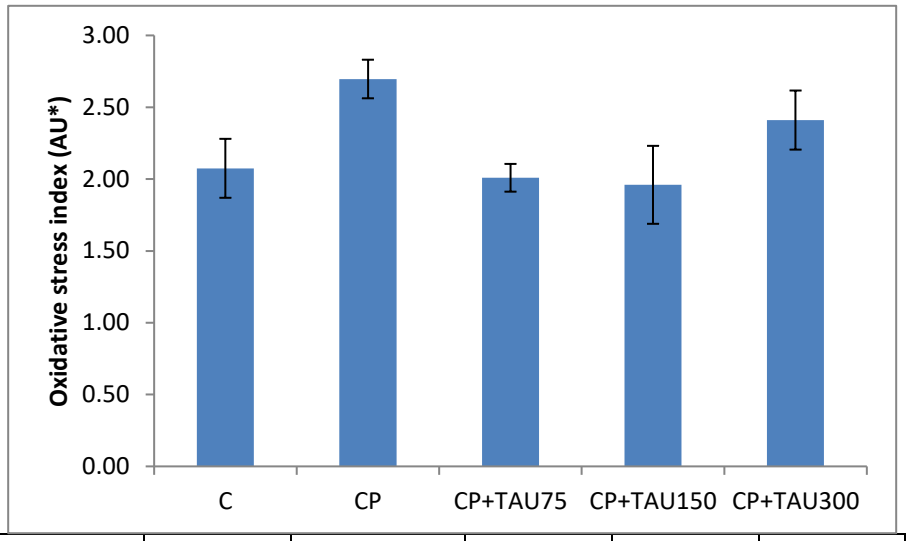
IL-2 level measured by ELISA method in testis homogenate was found to be increased significantly in CP-treated animals compared to the control animals (p<0.01). Taurine groups showed no significant effect on testis IL-2 levels (p>0.05).



SEM	0.34	0.28	0.27	0.30	0.27

Figure 4.5. IL-6 levels in testis homogenate (ng/L) results are given as mean ± sem Kruskal-Wallis analysis applied to (IL-6)

Although IL-6 level was slightly higher in CP group compared to the control group and slightly lower in TAU300 group compared to the CP group, there were no significant difference in IL-6 level between the groups (p>0.05).



SEM	0.21	0.13	0.10	0.27	0.21

Figure 4.6. Oxidative stress index (OSI) estimated in testis homogenate
results are given as mean ± sem (AU*: arbitrary unit)
ANOVA analysis applied to (OSI)

OSI, indicating the status of oxidative and antioxidative systems in testicular tissue was slightly higher in CP-treated animals compared to the control animals and slightly lower in all three Taurine-treated groups compared to the CP group, but there were no statistically significant difference ($p>0.05$) (Figure 4.6).

4.3. Histological Results

4.3.1. General histopathological assessment

Although there was a normal histological appearance in the testicular sections of control animals, CP group showed intense hemorrhage and edema in the interstitial area and seminiferous tubules in which the germinal cells were peeled off into the lumen, undulating in the peritubular tissue, occasional vacuolization in the germinal epithelium and a decrease in the thickness of the germinal epithelium. In testicular tissue of animals from TAU75 group, the hemorrhage and edema found in the interstitial area of CP group was improved with decreased vacuolization and peritubular undulation compared to the CP group. On the other hand, TAU150 and TAU300 groups showed similarity with CP group in terms of vacuolization, and hemorrhage and edema in the interstitial area with decreased germ cell count peeled off into seminiferous tubule lumen (Figure 4.7).

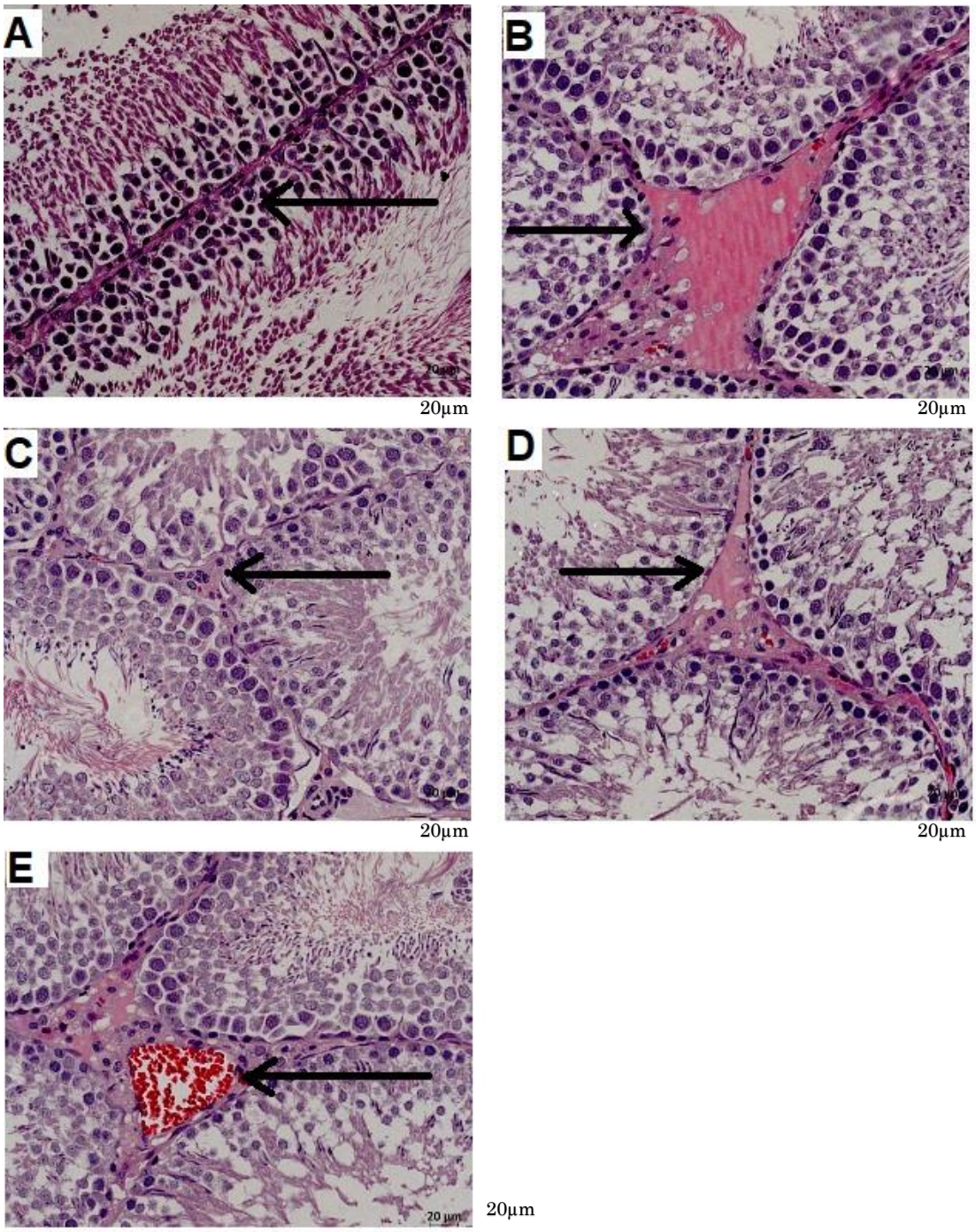


Figure 4.7. Hematoxylin & Eosin staining in testis preparations (40x).

A: Control group showed seminiferous tubules with normal histological structure and interstitial area as shown with arrow.
B: CP group showed intense hemorrhage and edema in the interstitial area and seminiferous tubules as shown with arrow.
C: TAU75 group, the hemorrhage and edema were improved with decreased vacuolization and peritubular undulation compared to the CP group as shown with arrow.
D: TAU150 group showed similarity with CP group in terms of vacuolization, and hemorrhage and edema in the interstitial area as shown with arrow.
E: TAU300 group showed similarity with CP group in terms of vacuolization, and hemorrhage and edema in the interstitial area with decreased germ cell count peeled off into seminiferous tubule lumen as shown with arrow.

Germinal epithelial height being 89.43±1.27 μm in control group was significantly to 78.08±1.01 μm in CP group ($p<0.001$) and significantly increased to 86.69±1.07, 84.28±1.53 and 84.86±1.49 μm in Tau75, TAU150 and TAU300 groups respectively ($p<0.001$, $p<0.05$ and $p<0.01$, respectively) (Figure 4.8).

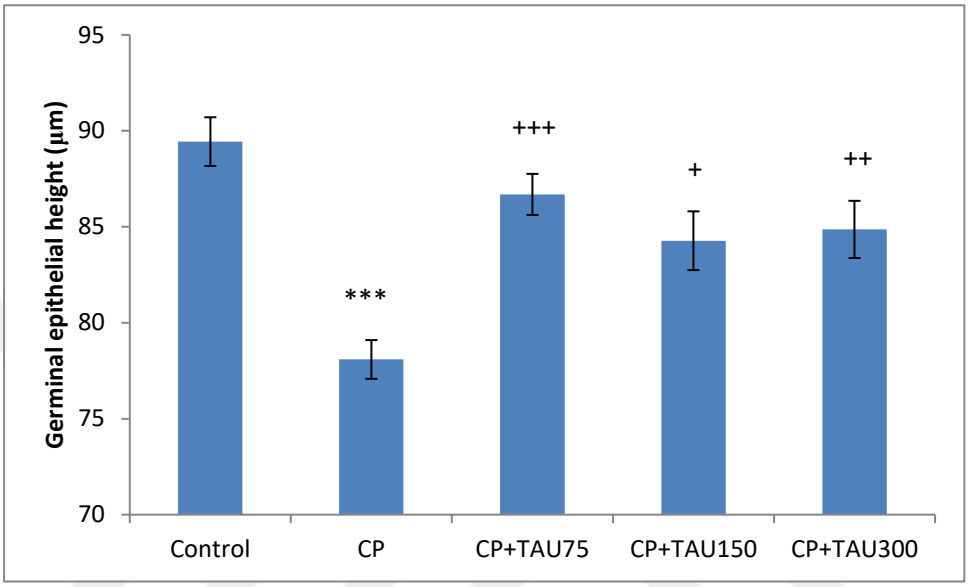


Figure 4.8. Germinal epithelial height; results are given as mean ± sem

***, $p<0.001$ compared to control; +, $p<0.05$ ++, $p<0.01$ and +++$p<0.001$ compared to CP respectively.

ANOVA analysis applied to Germinal epithelial height

4.3.2. Apoptosis assessment

In the testis preparations immunochemically stained for caspase-3, the apoptotic index being 8.11±1.84 in the control group was significantly increased to 26.40±1.33 in CP group ($p<0.001$) and significantly decreased to 17.31±0.70 in TAU75 group ($p<0.001$). Although there was no statistical significance, AI was slightly decreased in TAU150 and TAU300 groups compared to the CP-treated group (21.87±1.11 and 22.55±0.88, respectively) (Figure 4.9) (Figure 4.10).

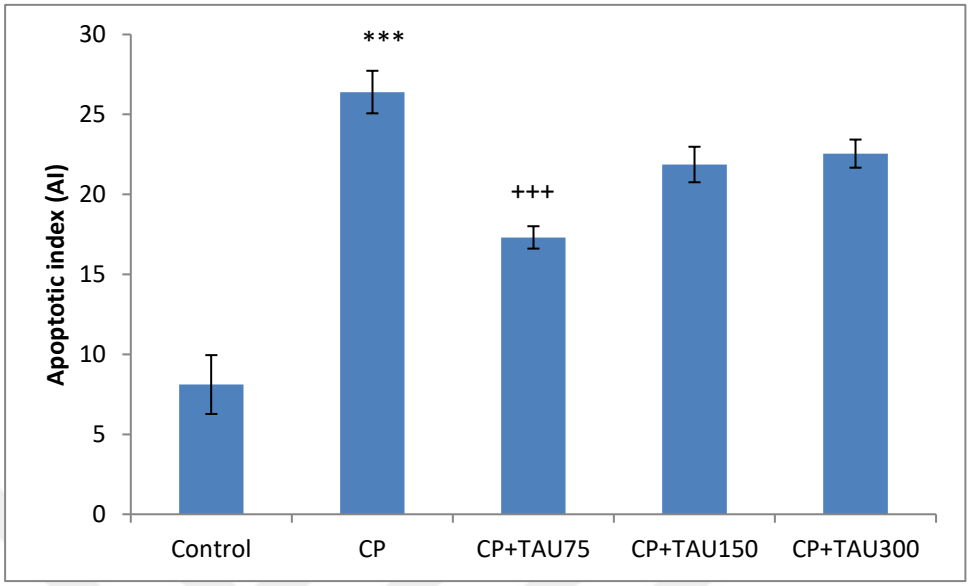


Figure 4.9. Apoptotic index (AI); results are given as mean ± sem

***, p<0.001 compared to control; +++p<0.001 compared to CP

ANOVA analysis applied to (AI)

In testicular preparations immunohistochemically stained for bcl-2, the immunoreactivity was significantly decreased in CP group compared to control group ($p<0.001$). On the other hand, TAU75 and TAU150 groups showed significantly increased immunoreactivity for bcl-2 compared to the CP group ($p<0.05$ for both). (Figure 4.11).

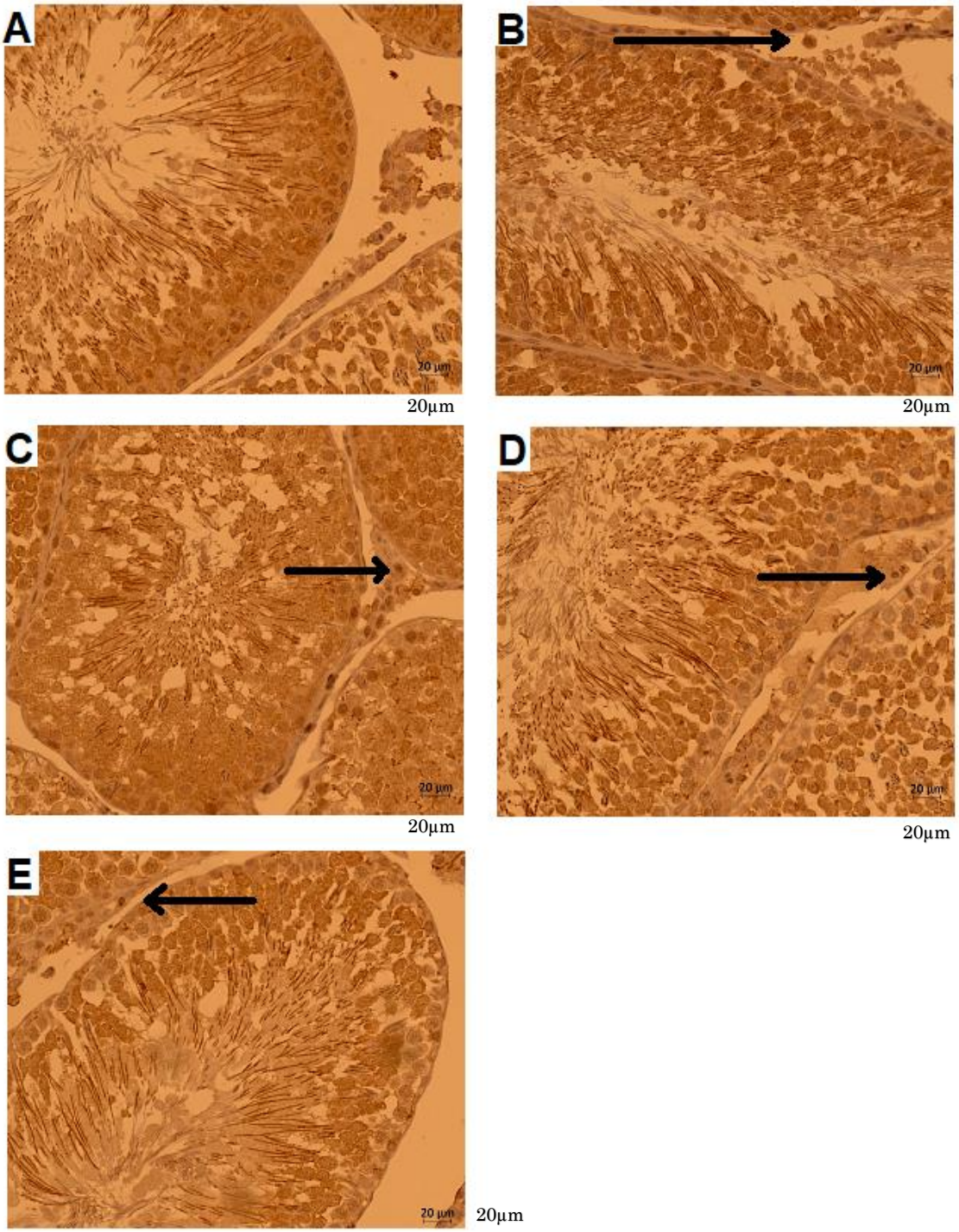


Figure 4.10. Caspase-3 immune reaction of rat testis sections (40x).

A: The control group apoptotic index has been 8.11±1.84.
B: CP group was significantly increased to 26.40±1.33 ($p<0.001$).
C: TAU75 group was significantly decreased to 17.31±0.70 ($p<0.001$).
D: TAU150 group AI was slightly decreased to 21.87±1.11 with no statistical significance compared to the CP-treated group.
E: TAU300 group AI was slightly decreased to 22.55±0.88 with no statistical significance compared to the CP-treated group.

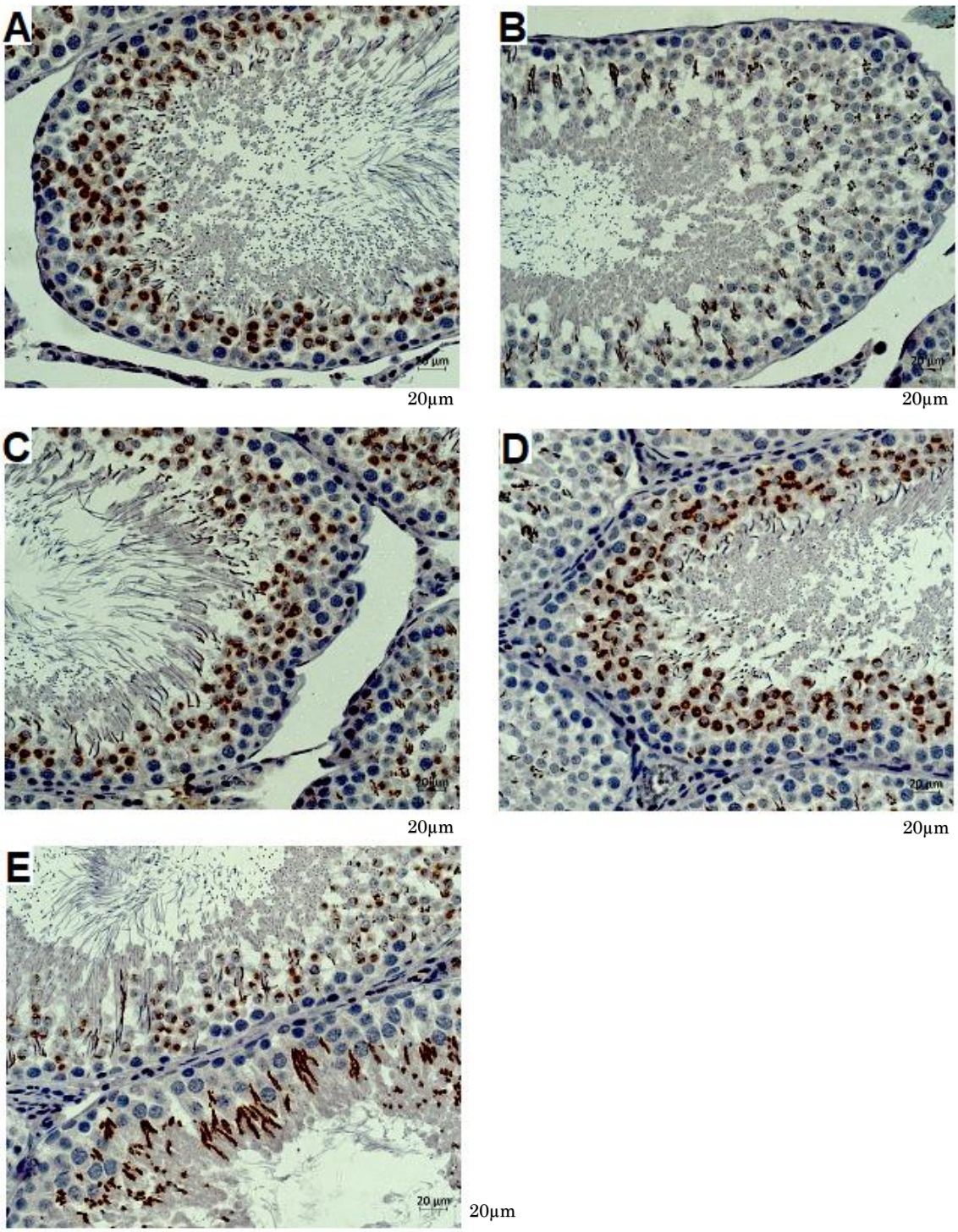


Figure 4.11. Immunohistochemically stained for Bcl-2 (40x)**.**

A: Significant positive reaction in the control group.
B: The immunoreactivity was significantly decreased in CP group compared to control group ($p<0.001$).
C: TAU75 group showed significantly increased immunoreactivity for bcl-2 compared to the CP group ($p<0.05$).
D: TAU150 group showed significantly increased immunoreactivity for bcl-2 compared to the CP group ($p<0.05$).
E: TAU 300 showed little increased immunoreactivity for bcl-2 compared to the CP group with no significantly

5. Discussion

The present study investigated the potential of taurine supplement in the prevention and/or treatment of CP-induced gonadotoxicity in male rats. These potential beneficial effects of taurine on CP-induced testicular injury were evaluated in terms of oxidative stress, inflammation, histological changes and apoptosis. Although we found slight but insignificant changes in oxidative stress and cytokine levels, taurine treatment improved the testicular degeneration and decreased the apoptosis induced by CP.

After oral taurine administration for 14 days at doses of 75 mg / kg, 150 mg / kg and 300 mg / kg and single intraperitoneal injection of 200 mg / kg CP on Day 7, body weights measured at the beginning (BW0) and at the end (BW1) of the study protocol were evaluated (Figure 4.1, Table 4.1). Although BW0 was slightly lower in CP and Tau300 groups compared to all other animal groups, there was no significant difference between the groups ($p>0.05$). On the other hand, BW1 was significantly lower in CP group than the controls ($p<0.001$). Body weight loss is the most common side effect of alkylating agents including CP which has been associated with poor clinical outcomes and intolerance to chemotherapy (Ross et al. 2004). Similarly, administration of CP to animals has led to a significant decrease in body weight in most of the previous studies (Salimnejad, Soleimani, Mohammad Nejad, Roshangar. 2018). This effect of CP may be related to its direct effect on energy metabolism, however there are also some authors suggesting that CP may have an antiproliferative effect on adipocyte progenitors (Myers et al., 2017). Because BW0 was not identical across the experimental groups, we used the percent change seen in body weight (BW%) to evaluate the beneficial potential of taurine on body weight loss. In that respect, CP resulted in a significant decrease in BW% ($p<0.001$) and although all taurine doses lead to an increase in BW%, only 150 mg/kg taurine produced a significant increase in BW% compared to the CP group ($p<0.05$) (Figure 4.2).

Murakami S. found that taurine is effective in decreasing body weight of obese animals and in suppressing inflammatory responses. (Murakami. 2015) Likewise, another study found that taurine supplementation significantly decreased the body weight and authors have concluded that taurine produces a beneficial effect on lipid metabolism and may have an important role in cardiovascular disease prevention in overweight or obese subjects (Zhang et al. 2004). The lack of a significant increase in our study in BW% in groups of TAU75 and TAU300 can also be attributed the body weight decreasing effect of taurine.

In order to evaluate the beneficial effect of different doses of taurine on testicular damage induced by CP, we estimated the ratio of testicular weight to body weight and found a significant increase in CP group compared to the controls ($p<0.001$) and a significant decrease in TAU150 group compared to the CP group ($p<0.05$) (Figure 4.3). There are many previous studies about the effect of CP on testicular weight reporting a significant decrease (Abd EI Tawab, Shahin, AbdelMohsen, 2014; Selvakumar, Prahalathan, Mythili, Varalakshmi, 2004; Salimnejad, et al. 2018), a significant increase (Motawi, Sadik, Refaat, 2010) or no significant change (Oyagbemi, et al. 2016; Xie et al. 2018). The decrease seen in testicular weight has been linked to decreased sperm production and the histological impairments found in testicular tissue including apoptosis, degeneration and parencymal atrophy (Abd EI Tawab, Shahin, AbdelMohsen, 2014; Selvakumar, et al. 2004; Salimnejad, et al. 2018).

In our study, using a single dose of 200 mg/kg of CP may have resulted in inconsistent findings reported in previous studies using higher or longer CP treatment protocols. Because the CP exposure was probably lesser in our study, the toxicity of CP was limited to the body weight with no effect on testicular weight, leading to an increase in TW/BW because of decreased BW1 and unchanged testicular weight. It is also possible that the testicular weight was not changed because of the use of adult animals in the present study. As suggested by Xie et al reporting no significant change in testicular weight of adult animals treated with a single dose of 100 mg/kg CP, a significant change

might be observed in prepubertal animals with use of same dose of CP (Xie et al, 2018).

In the present study, taurine administration was associated with slightly lower oxidative stress in all taurine-treated groups compared to the CP group, but with no statistically significant difference ($p>0.05$) (Figure 4.6). A major mechanism for CP-induced tissue injury is the generation of free radicals or by decrease in the antioxidant enzymes activities (Motawi et al., 2010). CP-induced toxicity of reproductive system can be attributed to its metabolite acrolein, which can lead to apoptosis or necrosis resulting from impaired redox balance, deficient antioxidant system and increased oxidative stress (Arumugam, Sivakumar Thanislass, Devaraj, 1997; Mythili, Sudharsan, Selvakumar, Varalakshmi, 2004).

Increased level of free radicals in the cell can induce peroxidation of lipids present in the cellular membranes, which in turn compromises the cellular integrity. Although present study found a slight but not significant increase in OSI index, there are many previous studies showing increased oxidative stress and decreased antioxidant capacity in rats treated with various doses of CP with different treatment regimens (Motawi, Sadik, Refaat, 2010, Abd EI Tawab, Shahin, AbdelMohsen, 2014, Salimnejad, Rad, Nejad, Roshangar, 2018; Onaolapo, Oladipo, Onaolapo, 2017; Anan, Zidan, Abd EL-Baset, Ali, 2018). Under normal conditions, the production and removal of ROS is in balance within the cell and impairment of this balance leads to cellular injury (Vladimir-Knezevic, et al. 2014). Moreover, increased oxidative stress was shown to result in ROS-induced damage to the macromolecules such as DNA, proteins and key enzymes important for testicular steroidogenesis and spermatogenesis (Sen, Mukherjee, Ray, Raha. 2003).

The antioxidant defense system comprising of the enzymatic and non-enzymatic antioxidants confer protection on biological tissues, including testes by their direct involvement in the removal of free radicals (Adedara and Farombi, 2010). Superoxide dismutase is the first defense system against oxygen-derived radicals and induces the dismutation of superoxide radicals to

H_2O_2. On the other hand, catalase removes H_2O_2 metabolically from the cell, thereby further reducing the H_2O_2 and, in turn, hydroxyl radical generation (Adedara and Farombi, 2012). Although taurine has been reported to have both in vitro and in vivo antioxidant activity (Jong et al., 2012; Schaffer, Jong, Ito, Azuma. 2014), we found no significant decrease in OSI of animals treated with three different doses of taurine. The reason for the insignificant changes found in OSI in the present study may be the insufficiency of the treatment duration with taurine.

Sperm is susceptible to oxidative damage and normal enzymatic protection from partially reduced oxygen intermediates is lacking. Taurine is found in seminal fluid and sperm in millimolar quantities and protects against oxidative damage by decreasing the formation of malondialdehyde from unsaturated membrane lipids (Huxtable. 1992). In fact, the protective effect of taurine on male sexual function is complicated. Besides the antioxidant activity, the possible other mechanisms involve sperm cell osmoregulation. Because oxidative damage results in osmoregulatory disturbances, loss of cell constituents, and cell death, taurine maintaining the osmoregulation protects the cell from excessive stretching (Huxtable. 1992).

Cytokines are extracellular proteins or glycoproteins and were found to play many pathophysiological roles in several diseases. The level of many cytokines such as tumor necrosis factor-alpha (TNF-α) interleukin (IL)-1 and interferon-gamma (INF-γ) are found to change dramatically in the process of CP-induced inflammatory events (Egido et al., 1993, Vielhauer and Mayadas, 2007). In our study, IL-2 and IL-6 levels measured in testis homogenate were found to be increased significantly and insignificantly respectively in CP-treated animals compared to the control animals ($p<0.01$. Although, Xu et al reported decreased IL-2 levels in serum from mice treated CP, they used a 28-day treatment protocol in contrast to the single intraperitoneal injection of CP in our study (Xu and Zhang, 2015). In accordance to our study, both Girard et al (2011) and Dantas et al (2010) have reported increased IL-6 levels in rats treated with CP. In a study by Smaldone et al. (2009), the bladder and urinary

levels of various cytokines were measured in rats treated with CP. The authors have reported that the tissue levels for the cytokines other than IL-2 were significantly increased in CP-treated animals compared to the control animals (Smaldone, 2009).

Cytokines play important roles in the immune system they are included in the preservation of homeostasis by coordination of lymphoid cells, inflammatory cells, and hematopoietic cells (Schooltink, Rose-John. 2002). The network of the cytokine is complicated and different cytokines usually react together and affect the synthesis of other cytokines. CD4+ T cells can differentiate into several kinds of T cells that secrete different cytokines. Th1 cells secrete IL-2, IL-12, IFN-γ, and TNF, which promote cell-mediated immune responses. In contrast, Th2 cells secrete IL-4, IL-5, IL-6, and IL-10, which promote humoral or allergic responses (Constant. 1997). The acute inflammation induced by CP in our study might resulted in different responses in different cytokines playing a complex role in this process.

On the other hand, in the study by Marcinkiewicz et al., TauCl therapy did not affect the production of IL-2, (Marcinkiewicz et al. 1999) in accordance with our experimental taurine groups showing no significant effect on testicular IL-2 levels ($p>0.05$) A series of studies performed on RA patients, show that TauCl inhibits many pathological functions of their proliferation (Kontny, et al.1999), IL-6 and IL-8 production (Kontny et al.2000) and COX-2-mediated generation of PGE2 (Kontny, Rudnicka, Kowalczewski, Marcinkiewicz, Maslinski, 2003).

In the present study, IL-6 level was slightly lower in TAU300 group compared to the CP group with no significant difference in IL-6 level between the groups ($p>0.05$) similar to previously reported findings (Chorazy, Kontny, Marcinkiewicz, Maslinski. 2002). supporting the suggestion that TauCl at low concentrations, which increase TNF-a synthesis, may favor the initiation of protective inflammation, while at a higher concentration (400 mM) it may control the further amplification of the process and gradually reduce pro-inflammatory cytokine synthesis. TauCl by reducing the TNF-a production can

limit the level of the Th1 response. This thesis accords with the decreased production of IL-2 by PHA-activated non-adherent leukocytes upon TauCl treatment reported by Park (Park, Jia, Quinn, Schullerlevis. 2002). These authors have shown that TauCl, at a concentration of 400 mM, suppressed superoxide anion, IL-6 and IL-8 production in activated healthy human peripheral blood PMNs.

In our study, testicular sections of the CP group showed intense hemorrhage and edema in the interstitial area and seminiferous tubules in which the germinal cells were peeled off into the lumen, undulating in the peritubular tissue, occasional vacuolization in the germinal epithelium and a decrease in the thickness of the germinal epithelium. CP is known to be toxic to the male reproductive system, with studies on rodents demonstrating reduced testicular weight, and various deleterious histological changes in the testes and epididymis (Anderson, Bishop, Garner, Ostrosky-Wegman, & Selby, 1995; Kaur, Sangha, & Bilaspuri, 1997).

In testicular tissue of animals from TAU75 group, the hemorrhage and edema found in the interstitial area of CP group was improved with decreased vacuolization and peritubular undulation compared to the CP group. On the other hand, TAU150 and TAU300 groups showed similarity with CP group in terms of vacuolization, and hemorrhage and edema in the interstitial area with decreased germ cell count peeled off into seminiferous tubule lumen. Germinal epithelial height in TAU75, TAU150 and TAU300 groups was found to be similar to that of control group. CP is known to induce many histological alterations in the testis with increased damaged in higher doses of CP (Sakr, Shalaby, Beder. 2017). The histological findings in the present study is similar to that found in previous studies using CP and several antioxidant and/or anti-inflammatory agents (Sakr, et al. 2017, Elangovan, Chiou, Tzeng, Chu. 2006; Tripathi, Jena. 2008). In a study by El Seedy et al, CP was found to induce marked sperm abnormalities which can be attributed to the interference of CP with different stages of spermatogenetic cells (El-Seedy, Taha, El-Seehy, Maklouf. 2005). The decreased seminiferous tubule diameter and germinal

epithelial height might also result from the inhibitory effect of CP on spermatogenesis (Bustos-Obregon, Carvallo, Hartley-Belmar, Sarabia, Ponce. 2007). The positive effect of taurine on testis and sperm maturation might resulted in the reduction and/or improvement of CP-induced testicular toxicity.

Taurine has been reported to play a key role in cytoprotection and decrease the apoptosis (Das J, Sil PC 2012). It is well known that oxidative stress is related to mitochondrial dysfunction and most beneficial effects of taurine are related to its antioxidant capacity (Marcinkiewicz, Kontny 2012).

It is well known that oxidative stress initiates the apoptotic cascade (Shaker, Abboud, Assad, Hadi, 2018). ROS produced during oxidative stress activates the pro-apoptotic proteins and results in the release of cytochrome C via voltage-gated anion channels (Korsmeyer, Wei, Saito, Weiler, Oh, Schlesinger, 2000). Healthy cells contain anti-apoptotic genes such as Bcl-2 in their mitochondria. Bax, a pro-apoptotic protein that can migrate into mitochondria inhibits the anti-apoptotic Bcl-2 gene and results in testicular injury (Abd EI Tawab, Shahin, AbdelMohsen, 2014; Schwartz & Waxman, 2001). Moreover, pro-apoptotic proteins result in migration of cytochrome C from the outer mitochondrial membrane to out of mitochondria and lead to conjugation with apoptotic protease activating factor-1 (Apaf-1) forming the complexes called as apoptosome (Green & Reed,1998). Apoptosome attaches to procaspase-9 to be activated to the form of caspase-9, which in turn activates the cascade from caspase-3, -7 and -12 (Li & Yuan, 2008). The activation of caspase-3 mediated pathways results in the germ cell apoptosis (Ryan, O'Callaghan, O'Brien, 2004; Kim, Ghosh, Weil, Zirkin, 2001; Potnuri, Allakonda, Lahkar, 2018).

Accordingly, in the testis preparations of our study immunochemically stained for caspase-3, the apoptotic index was significantly increased in CP group compared to the control group, supporting the suggestion that CP induces a stage-specific apoptosis in spermatogonia and spermatocytes (Cai, Hales, Robaire, 1997). On the other hand, the apoptotic index significantly

decreased in TAU75 group which is in accordance with the previous studies showing that taurine inhibits apoptosis in various cells (Wang, Huang, Huang. 2015). On the other hand, although there was no statistical significance in TAU150 and TAU300 groups compared to the CP-treated group, AI was slightly decreased in these two groups. Moreover, bcl-2 immunoreactivity was significantly decreased in CP group compared to control group and significantly increased with the addition of 75 mg/kg or 150 mg/kg taurine to the treatment. Although many previous studies showed anti-apototic effects for taurine in animals treated with various chemotherapeutic agents, in spite of similar doses of oral taurine used in these studies, treatment period was approximately 28 days (Azab, Kamel, Ismail, El Din Hosni, El Fatah. 2019, Das, Ghosh, Manna, Sil. 2012). Therefore, it would be not surprising to see a more pronounced and dose-dependent anti-apoptotic effect if we had used taurine for longer treatment duration.

6.CONCLUSION AND RECOMMENDATIONS

The results of present study suggest that CP resulted in a significant decrease in body weight gain representing the general toxicity of the drug and also resulted in histological deterioration and increased apoptosis in testis representing the organ specific toxicity of the drug. The testicular injury induced by CP was evaluated in terms of oxidative stress, inflammation and apoptosis with a significant inflammatory and apoptotic response, and an insignificant oxidative stress. Taurine treatment at the doses of 75 mg/kg, 150 mg/kg and 300 mg/kg resulted in improvement in body weight gain, oxidative stress, inflammation and apoptosis whether significant or not. The improvement seen was more pronounced for antiapoptotic effect of taurine in the testis of CP-treated animals.

With the expanding literature on potential beneficial effect of taurine on the cytotoxicity of several chemotherapeutic agents, our results show for the first time, in our knowledge, that taurine may prevent and/or treat the testicular toxicity in male rats treated with a single intraperitoneal injection of CP. We hope that these results on animals can be expanded to humans after prospective controlled clinical studies carried on prepubertal and/or adult males using CP for any reason to overcome the infertility associated with CP use.

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