The Pharma Innovation Journal 2023; 12(5): 2129-2136

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; 12(5): 2129-2136

www.thepharmajournal.com
Received: 25-02-2023
Accepted: 29-03-2023

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Pathomorphological alterations in cisplatin induced testicular toxicity and its amelioration by gallic acid

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Abstract
The study was aimed to investigate the efficacy of a phytochemical gallic acid (GA) in preventing the pathomorphological alterations induced by cisplatin (CP) in testicular tissue of Wistar albino rats. One hundred and eight Wistar albino rats were equally divided into six groups. Group I served as normal control, Group II received single dose of intraperitoneal injection of CP at 7.5 mg/kg bw, Group III received GA at 75 mg/kg bw for 45 days, Group IV was treated with GA daily for 15 days prior to CP injection and discontinued post CP injection, Group V received CP injection and concurrently received GA for 45 days post CP injection and Group VI was treated with GA for 15 days prior to CP injection and continued for 45 days post CP injection. The testis samples collected on 7th, 14th, 28th and 45th day of post CP injection were subjected for histopathological examination to study the sequential pathomorphological changes. CP administration produced moderate congestion and interstitial oedema, severe seminiferous tubular atrophy, tubular cell degeneration and necrosis. The Gallic acid supplemented groups showed significant improvement in CP induced pathological changes. The pre + concurrent GA supplementation (Group VI) produced much earlier improvement in CP induced pathological changes than only pre and only concurrent GA supplementation. It was concluded that, GA supplementation have protective role against CP induced testicular toxicity.

Keywords: Gallic acid, cisplatin, testicular toxicity, sequential pathomorphology

Introduction
Cancer, a leading cause of death worldwide is accounting for nearly 10 million deaths in 2020, or nearly one in six deaths. Breast, lung, colon, rectum and prostate cancers are the most prevalent types of cancer. Early detection, appropriate cancer treatment and patient care ensures high chance of recovery in many cancer types (WHO, 2022) [31]. Cisplatin (*cis*-diamminedichloroplatinum) is one among the most widely employed cytotoxic chemotherapeutic drugs due to its broader efficacy in treatment of various types of malignancies used in human and veterinary medicine. The mechanism of antineoplastic activity is primarily attributed to its DNA damaging activity by the formation of DNA adducts and cross links, there by cell cycle arrest and finally triggering apoptosis (Barabas *et al*., 2008) [6]. The second mechanism includes generation of reactive oxygen species (ROS) following interaction with DNA. Higher concentration of ROS can result in both apoptosis and necrosis in cancer cells (Tanaka-Kagawa *et al*., 1999 and Ozben, 2007) [30, 23]. The therapeutic efficacy of CP for certain cancer types is remarkably high like in testicular cancer and non-small cell lung carcinoma. As a result, for over a half century, CP and related platinum derivatives have served as backbone in chemotherapy of cancer (Aldossary, 2019 and Rajendrakumar, 2019) [2, 27] and is prescribed in nearly 50% of all tumour chemotherapies (Galanski *et al*., 2005 and Perse, 2021) [14, 25]. Despite cisplatin's effectiveness in treating cancer, organ damage and emergence of chemo resistance are its major drawbacks (Dasari and Tchounwou, 2014) [8]. CP treatment is associated with nephrotoxicity, hepatotoxicity, cardiotoxicity, spermiotoxicity, gastrointestinal toxicity, myelosuppression and ototoxicity (Aldossary, 2019) [2]. Cisplatin induced testicular toxicity is of concern as it clinically associated with infertility. Many studies have indicated CP has profound deleterious effect on reproductive system in male and female rats and humans. (El-Amir *et al.*, 2019; Famurewa *et al.*, 2020 and Moradi *et al.*, 2021) [10, 11, 19]. Hence protection of reproductive health of patients undergoing chemotherapy is of prime concern.

The molecular mechanism of CP induced testicular toxicity is still unclear. However, earlier investigations have proposed mitochondrial oxidative stress, DNA damage, generation of

ROS, inflammation and apoptosis of cells in CP toxicity (Ozkok *et al.,* 2014; Dasari and Tchounwou, 2014 and Almaghrabi *et al.,* 2015) [24, 8, 3]. Recently, phytochemicals having high anti-inflammatory and antioxidant properties are suggested as combined therapy to prevent toxicity associated with chemotherapy. Gallic acid (GA) is one such phytochemical and has attracted the attention of many researchers by virtue of its excellent anti-inflammatory, antioxidant, antimutagenic and anticarcinogenic properties (Dehghani *et al.,* 2020) [9]. Gallic acid is ubiquitously found in plant kingdom in plants, fruits and vegetables. Gallic acid is abundant in processed beverages such as red wine and green tea. Many studies have documented its anti-oxidant, anti-inflammatory, anti-microbial, anti-diabetic, anti-tyrosinase, antimutagenic and anti-cancer activities (Choubey *et al.,* 2018) [7]. Also, Gallic acid is a relatively safe phytochemical and a subacute study demonstrated the absence of cumulative toxicity (Rajalakshmi *et al.,* 2001) [26]. Thus, the aim of the present study to investigate the pathomorphological alterations induced by CP in testicular tissue and ameliorative role of GA in CP induced testicular toxicity.

Materials and Methods

Cisplatin (Cisplat®), was procured from Zydus Celexa, India and gallic acid was procured from Sigma Aldrich Company, China.

Animals and experimental design: Normal adult male Wistar albino rats weighing approximately 160-180 g were procured from commercial animal facility, Bengaluru. Rats were maintained under standard laboratory settings and fed with *ad-libitum* standard commercial rat pelleted feed and clean drinking water. The rats were allowed to acclimatize for a period of 15 days in experimental animal facility. The duration of experiment was for a period of 45 days and prior permission was obtained from the Institutional Animal Ethics Committee (IAEC) for the conduct of experiment. The rats were divided, based on the body weight into six groups with eighteen rats in each group and treated as follow:

Group I (Normal control): Rats injected with 0.5ml sterile normal saline intraperitoneally on Day 1 and gavaged distilled water daily for 45 days.

Group II (CP control): Rats administered with cisplatin at 7.5 mg/kg bw intraperitoneally as a single dose on Day 1.

Group III (GA control): Rats supplemented with gallic acid at the dose rate of 75 mg/kg bw daily for 45 days.

Group IV (GA Pre-treatment group): Rats supplemented with gallic acid for a period of 15 days prior to inducing toxicity by cisplatin on Day 1.

Group V (GA Concurrent group): Rats administered with cisplatin injection on Day 1 and concurrently treated with gallic acid for 45 days post cisplatin injection.

Group VI (GA Pre +concurrent group): Rats treated with gallic acid for 15 days prior to inducing toxicity by cisplatin on Day 1 and continued gallic acid supplementation for 45 days post cisplatin injection.

Histopathology

To study the progressive histopathological effects of the treatments given to different groups, four rats from each group were sacrificed humanely under ketamine hydrochloride and xylazine anaesthesia on 7th, 14th, 28th day and the remaining rats on 45th day of post CP injection. Such sacrificed animals were subjected for detailed post mortem examination and representative testicular tissue samples were collected in 10 per cent neutral buffered formalin and the tissues were processed by the routine paraffin embedding technique and sections of 4 µ thickness were cut using a microtome and subjected to routine hematoxylin and eosin (H&E) staining for the pathomorphological evaluation.

Results and Discussion

The testicular tissue sections of all the treatment groups collected on 7th, 14th, 28th and 45th day of post CP injection were subjected to detailed histopathologic examination.

Group I and Group III: The testis of Group I and Group III rats showed apparently normal microscopical architecture throughout the study period. (Figure 1)

Group II: Microscopically, appreciable morphological changes were observed in the testis of CP treated rats on 7th day of post CP injection. There was moderate degree of congestion, severe interstitial oedema and seminiferous tubules showed loss of normal architecture (Figure 2). Some of the tubules showed atrophy and total absence of cellular components with retainment of only sertoli cells (Figure 2 and Figure 3) while some tubules revealed collection of fallen off degenerating and necrotic spermatogonia, primary spermatocytes and spermatids into the lumen as eosinophilic structureless necrotic material (Figure 4). Such degenerating and necrotic cells appeared swollen with highly vacuolated cytoplasm and pyknotic, karyorrhectic and karyolytic nucleus. There were also cells with apoptotic morphology characterized by cell shrinkage and condensation of nucleus. Occasional tubules also consisted of syncytial cells or giant cells in the lumen which were abnormally large sized with eosinophilic cytoplasm and multinucleation (Figures 5 and 6). On 14th day of post CP injection, there was persistence of lesions observed on 7th day however, only a small number of tubules showed atrophy, loss of normal architecture with presence of highly vacuolated necrotic cells, occasional syncytiated giant cells and necrotic eosinophilic cellular debris in the lumen along with mild congestion and interstitial oedema (Figures 7 and 8). On 28th day of post CP injection, there was significant improvement in testicular architecture, lesions such as mild congestion and mild interstitial oedema and occasional tubules were still showing CP induced damages such as atrophy, sparse cellularity and loss of spermatid arrangement (Figure 9) were evident. On 45th day of post CP injection, there was significant improvement in the testicular architecture and was comparable to architecture of normal control. There was compact arrangement of seminiferous tubules and sperm production was evident (Figure 10).

Group IV (GA pre-treatment group): The morphological changes observed in the Group IV were similar but less severe compared to those observed in Group II on 7th day post CP injection. There was mild congestion and moderate interstitial

oedema. Few seminiferous tubules showed atrophy, irregular shape and disorganization of cellular layers. Some atrophied seminiferous tubules showed complete loss of germinal epithelium while retaining only sertoli cells (Figure 11). On 14th day post CP injection, improvement in the testicular architecture was observed compared to that of 7th day. However, mild CP induced injury persisted which included mild congestion, occasional focal interstitial oedema, atrophy with loss of constituent cells in occasional tubules. On 28th day, the testicular architecture appeared almost normal in morphology. However, there was mild focal interstitial oedema and occasional tubules showing disruption of cellular arrangement and atrophy (Figure 12). On 45th day the testicular architecture appeared almost normal in morphology along with rare tubules with persistence of lesions (Figure 13).

Group V-GA concurrent treatment group

The lesions in the testis of Group V rats were similar to those of Group II rats on 7th day post CP injection however, with reduced severity. There was mild congestion and moderate interstitial oedema, atrophy of seminiferous tubules which retained only sertoli cells with loss of germinal cells, primary spermatocytes and spermatids (Figure 14). Majority of the tubules were of normal architecture. On 14th day post CP injection there was improvement in testicular architecture with most of the tubules showing normal morphology. However, there was mild congestion, mild interstitial oedema and rare tubules with CP injury (Figure 15). On 28th and 45th day also the testicular architecture appeared normal with rarely affected tubules (Figures 16 and 17).

Group VI: On 7th day post CP injection, in the GA pre+concurrent treatment group rats the testicular architecture appeared almost normal with compact arrangement of seminiferous tubules and regular arrangement of constituent cells with sperm production, in comparison with that of Group IV and Group V. However, there was mild congestion and occasional presence of mild CP induced injury in rare tubules (Figure 18). On 28th and 45th day, the testicular architecture appeared almost normal in morphology and function (Figures 19 and 20).

Discussion

In the present investigation sequential pathomorphological alterations in the testis of CP treated rats and protective role of GA in alleviation of these alterations were studied simultaneously. The Cisplatin, a most widely used antineoplastic drug in the treatment of solid tumours produced histopathological changes in the testis of rats such as moderate congestion, interstitial oedema, seminiferous tubular atrophy, varied degrees of degeneration, necrosis and apoptosis of spermatogonia, primary spermatocytes and spermatids cells with presence of eosinophilic structureless necrotic material in the lumen. Similar morphological changes in the testis in CP toxicity have reported by El-Amir *et al.* (2019) [10]; Fouad *et al*. (2019) [13]; Yadav (2019) [32] and Altindag and Meydan (2021) [4] who observed histological damage with loss of spermatogenesis. However, progressive reduction in the severity of these lesions from 14th day of post CP injection with significant improvement in testicular architecture on 28th and 45th day of post CP injection were observed in the present study. The improvement observed in the testicular morphology in Group II rats during 28th and 45th day post CP injection could be related to the cessation of cisplatin effect with its elimination from the body.

Cisplatin induced testicular toxicity could be attributed to CP induced oxidative stress in testicular tissue as demonstrated by many researchers (Amin and Hamza, 2006; El-Amir *et al.* 2019; Fouad *et al*. 2019; Yadav, 2019; Famurewa *et al.*, 2020 and Altindag and Meydan, 2021) [5, 10, 13, 32, 11, 4]. Kohsaka *et al.* (2020) [18] observed that CP treatment increased germ-cell apoptosis and histological damage and resulted in disorganized spermatogenesis accompanied by a significant increase in oxidative stress. Cisplatin alkylates DNA via guanine bases in order to form intra-strand DNA crosslinks, which interfere with DNA repair mechanisms, thereby inducing apoptosis by activation of p53 and cell cycle arrest. Also, CP generates excess amounts of ROS which might have caused lipid peroxidation in testis causing necrosis of the cells.

In the GA supplemented groups, such as Group IV and Group V, testis revealed changes similar to those of Group II on 7th day post CP injection however with less severity. Also, there was progressive improvement in the testicular architecture from 14th day with attainment of normalcy by 45th day. However, occasional tubules showing CP induced damage persisted. Group VI animals revealed only mild CP induced lesions in rare tubules and showed almost normal testicular architecture with compact arrangement of seminiferous tubules and regular arrangement of constituent cells with sperm production in comparison with that of Group IV and Group V from 7th day itself. On 14th, 28th and 45th day the testicular architecture appeared almost normal in morphology and function. With above observations, among all the GA treatment groups the Group VI showed better attenuation of lesions by suppressing CP induced alterations from the beginning of experiment portraying better protection. The protective role of GA on male reproductive system has been demonstrated by several earlier workers against various chemical induced toxicity (Oyagbemi *et al.*, 2016 [22] against cyclophosphamide induced male reproductive toxicity; Novin *et al.*, 2020 [20] against cyclophosphamide induced testicular toxicity; Olukole *et al.*, 2020 [21] against chronic exposure to bisphenol A; Altindag and Meydan, 2021 [4] against cisplatin induced testicular toxicity and Jalili *et al*., 2021 [19] against nicotine-induced testicular toxicity in mice).

Cisplatin induced gonadal toxicity could be attributed to DNA damage, oxidative stress and lipid peroxidation. Studies have also shown that ROS-induced oxidative stress results in oxidative damage to the macromolecule such as DNA, proteins and key enzymes important for testicular steroidogenesis and spermatogenesis (Gupta *et al.*, 2004) [16]. The toxicopathological effects of CP on testicular tissue and spermatogenesis were evident in light microscopy and these pathomorphological changes were ameliorated in experimental rats receiving GA in the present study.

Gallic acid is a plant-derived polyphenolic compound with high antioxidant potential. Its powerful antioxidant property is attributed to its inherent hydrogen-donating capability to free radicals and getting itself oxidized to a stable quinine moiety (Singh *et al.*, 2014) [29]. The protective effect of GA could be due to its free radical scavenging property, inhibition of lipid peroxidation, through increase in antioxidant defence system and sparing effect on glutathione. Cisplatin can cause an increase in the expression of NF-κB, cytokines (IL-1β, TNF-

α, IL-6), NO levels and iNOS activity in the testis and decrease GSH and antioxidant enzymes (Famurewa *et al.* 2020) [11] and abundant evidences suggests that antioxidant agents like gallic acid promote the anti-inflammatory effect by inhibiting the production of NF-κB and while up regulating *Nrf2* in different tissues (Gao *et al*., 2022) [15]. Many studies have demonstrated antioxidant effect of gallic acid is through upregulation of Nuclear factor erythroid 2-related factor 2 (*Nrf2*) (Feng *et al*., 2018) [12]; Zhou *et al*., 2019 and Sanjay *et al*., 2021) [33, 28]. Gallic acid significantly upregulated gene expression of GCLC (Glutamate-Cysteine Ligase Catalytic subunit), Prdx6 (Peroxiredoxin 6), Superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxidase (GPx) by increasing Nrf2-p (activated Nrf2) levels and gene expression levels with an improvement in antioxidant activity of SOD, CAT, GPx and GSH and subsequent reduction in total oxidant levels (Sanjay *et al.* (2021) [28]. These findings suggest that gallic acid can alleviate cisplatin induced oxidative stress and can protect testicular tissue of rats through its inherent antioxidant and free radical scavenging potential.

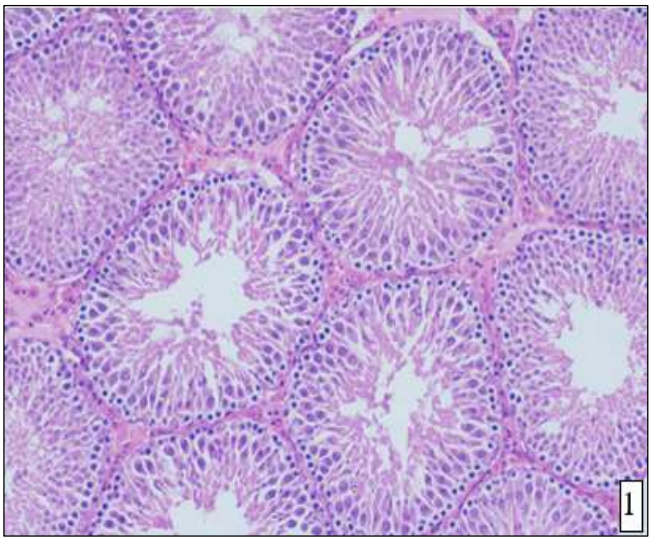

Fig 1: Section of testis from Group I showing normal architecture on 7th day. (H&E X100)

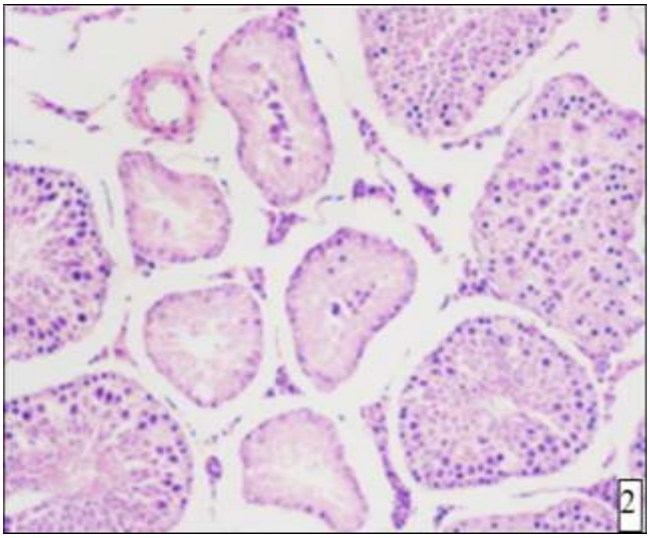

Fig 2: Section of testis from a Group II showing atrophic seminiferous tubules with total loss of cellular contents on 7th day. (H&E X100)

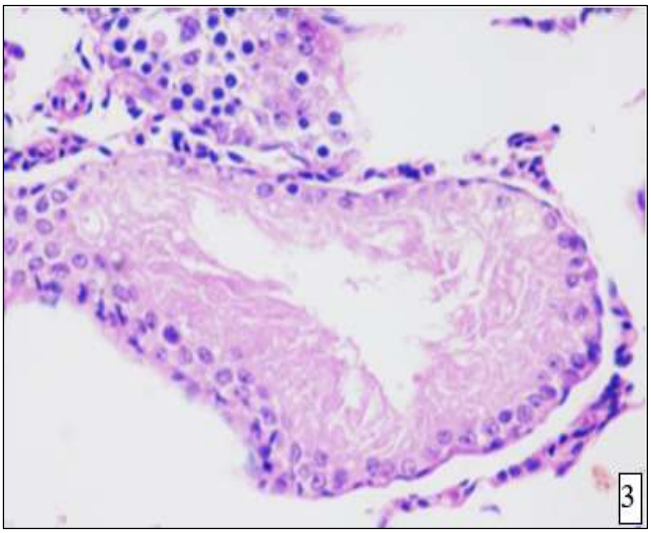

Fig 3: Section of testis from a Group II showing atrophic seminiferous tubule with loss of germinal epithelium and retained Sertoli cells at the periphery on 7th day. (H&E X200)

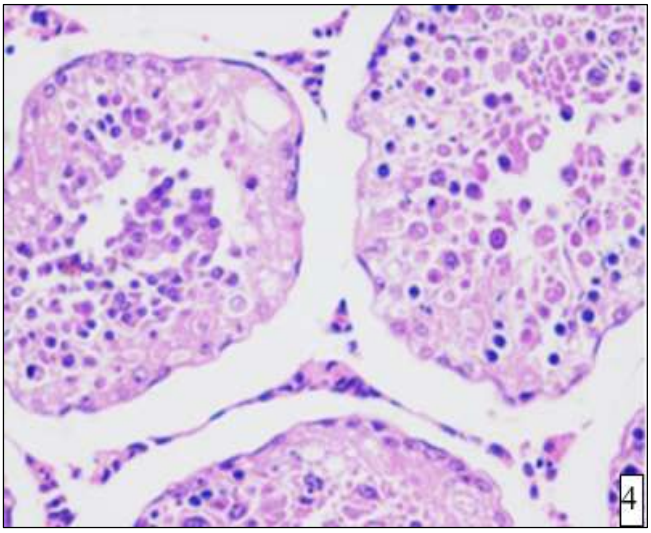

Fig 4: Section of testis from a Group II showing atrophic seminiferous tubule with loss of normal architecture consisting of degenerating and necrotic constituent cells on 7th day. (H&E X200)

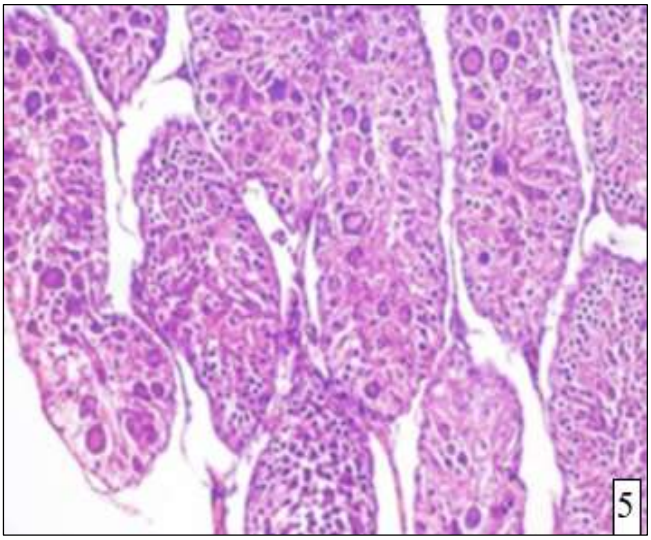

Fig 5: Section of testis from a Group II showing numerous affected tubules consisting of degenerating, necrotic cells along with multiple gaint cells on 7th day. (H&E X100)

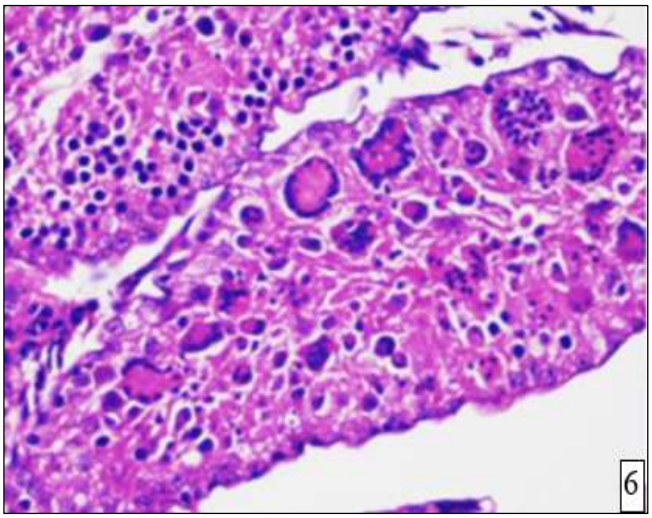

Fig 6: Section of testis from a Group II showing numerous multinucleated giant cells in the seminiferous tubules along with degenerating and necrotic tubular cells on 7th day. (H&E X200)

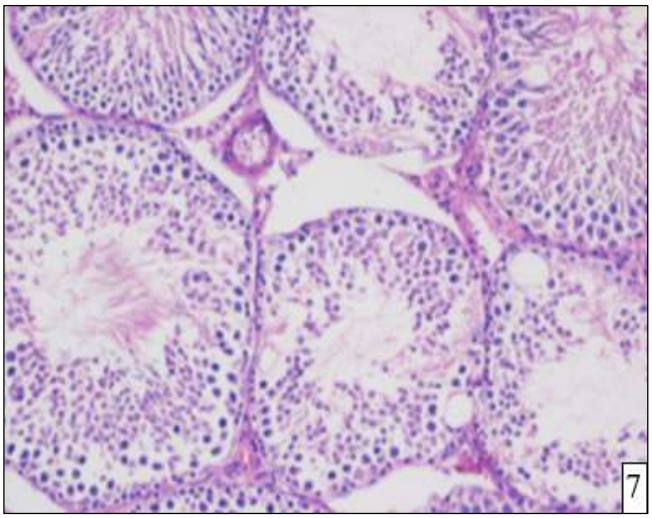

Fig 7: Section of testis from a Group II showing disruption of the normal architecture of seminiferous tubular cell layers with the presence of degenerating and necrotic cells on the 14th day. (H&E X100)

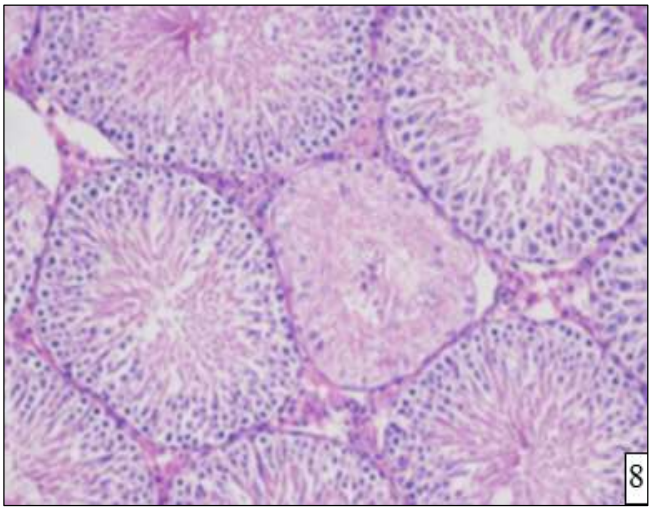

Fig 8: Section of testis from a Group II showing a tubule with complete loss of germinal epithelium and retention of occasional sertoli cells on 14th day. Note: Adjacent normal appearing seminiferous tubules (H&E X100)

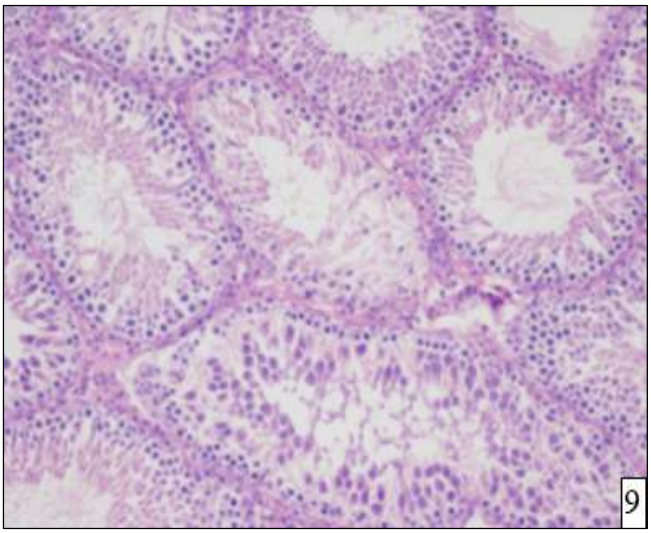

Fig 9: Section of testis from a Group II showing compact arrangement of seminiferous tubules however with presence of degenerating and necrotic cells on 28th day. (H&E X100)

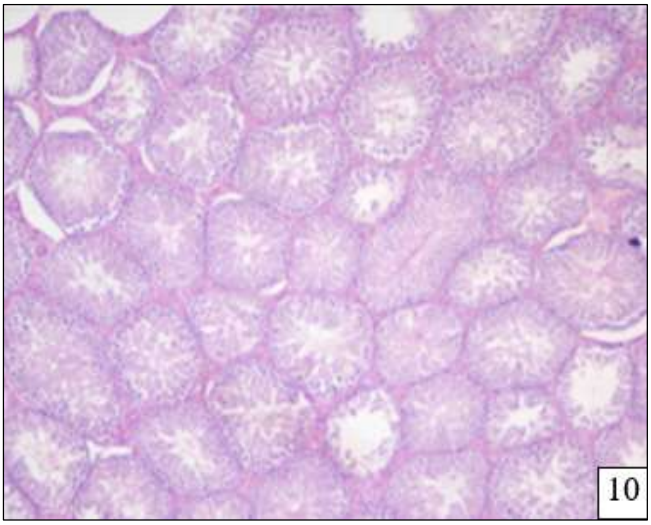

Fig 10: Section of testis from a Group II showing compactly arranged apparently normal appearing seminiferous tubules with only occasional tubules showing loss of tubular cells on 45th day. (H&E X 40)

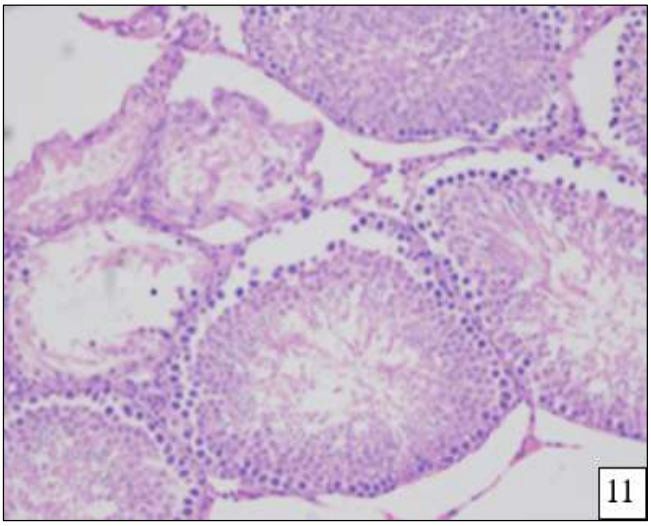

Fig 11: Section of testis from Group IV showing atrophic seminiferous tubules with loss of germinal epithelium and retained sertoli cells on 7th day. (H&E X100)

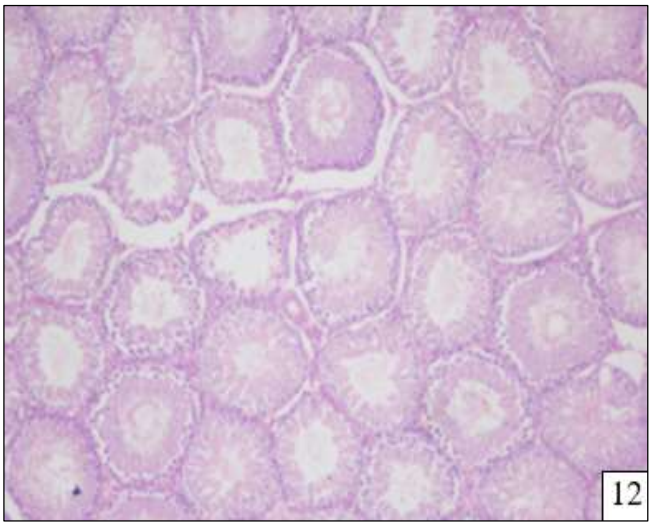

Fig 12: Section of testis from Group IV showing normal compact arrangement of tubules with occasional tubules showing degeneration on 28th day. (H&E X40)

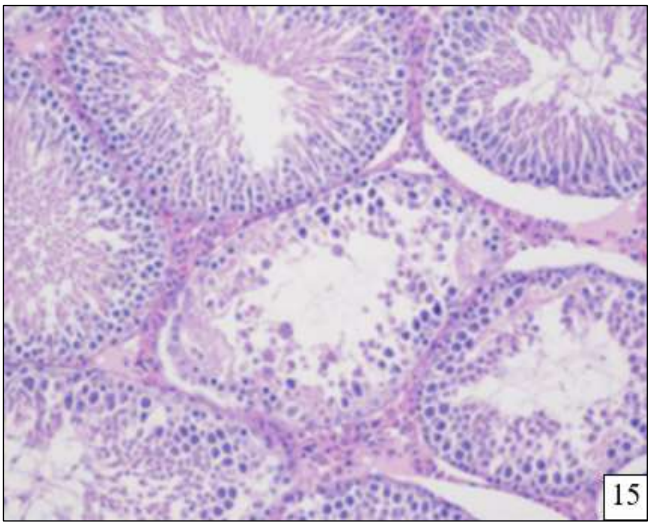

Fig 15: Section of testis from Group V showing degeneration, necrosis and loss of cells in a few seminiferous tubules on 14th day. (H&E X200)

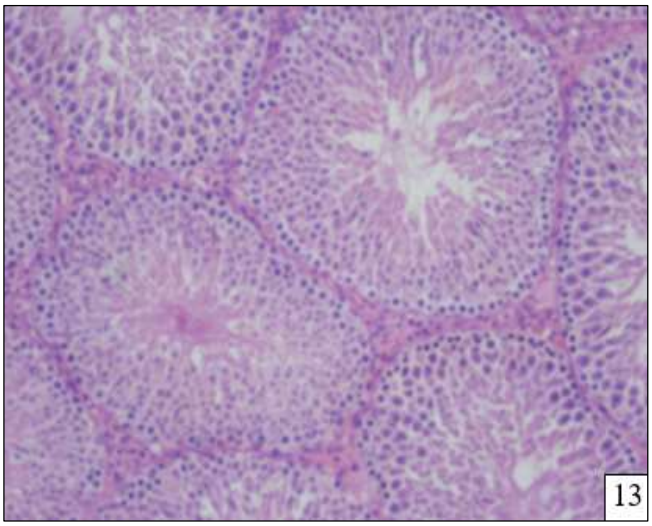

Fig 13: Section of testis from Group IV showing normal compact arrangement of tubules with normal cellularity on 45th day. (H&E X200)

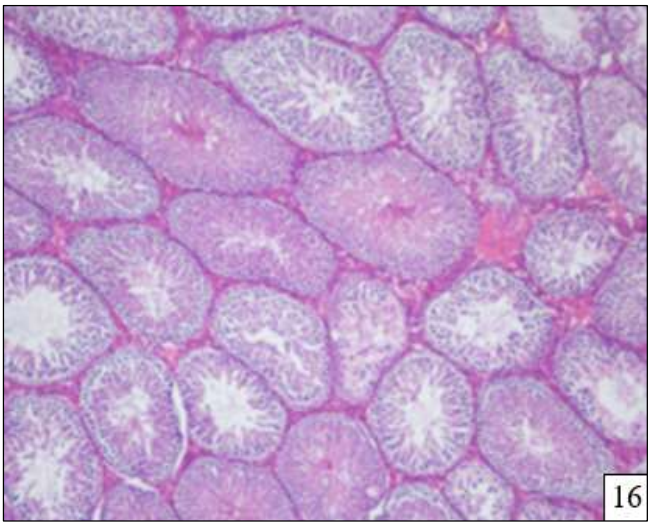

Fig 16: Section of testis from Group V showing compact arrangement of seminiferous tubules on 28th day with mild interstitial oedema. (H&E X40)

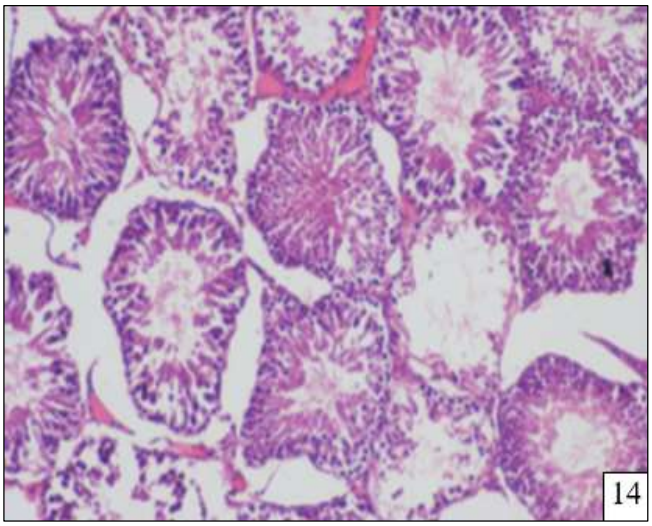

Fig 14: Section of testis from Group V showing degeneration, necrosis and total loss of cells in seminiferous tubules on 7th day. (H&E X100)

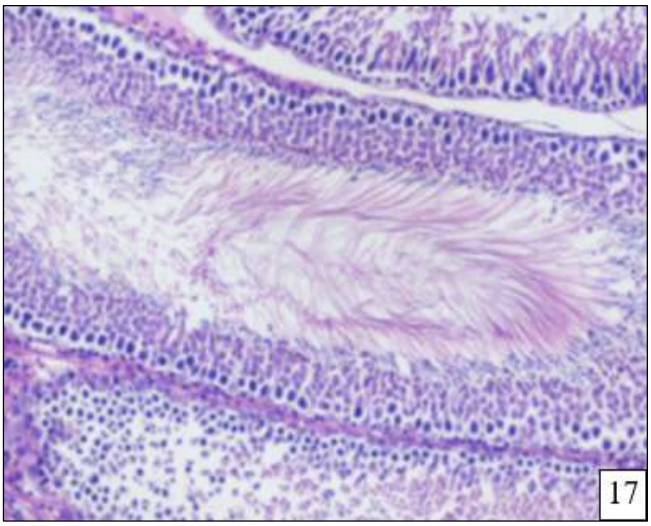

Fig 17: Section of testis from Group V showing almost normal appearing seminiferous tubule on 45th day with spermigenesis. (H&E X200)

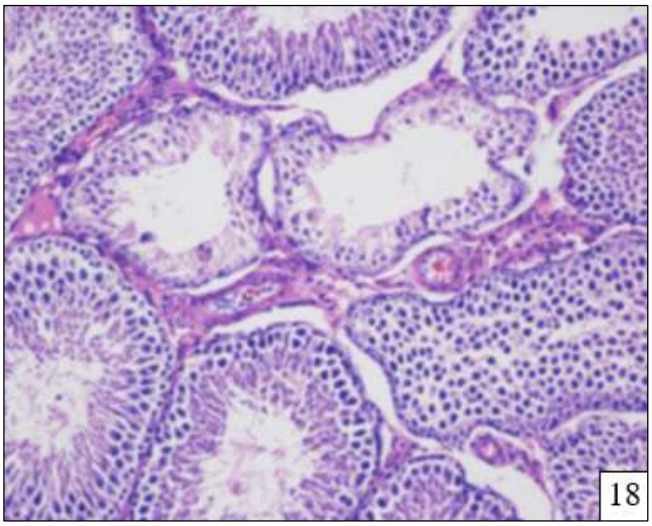

Fig 18: Section of testis from Group VI showing atrophy, degeneration and necrosis of seminiferous tubules with loss of constituent cells on 7th day. (H&E X100)

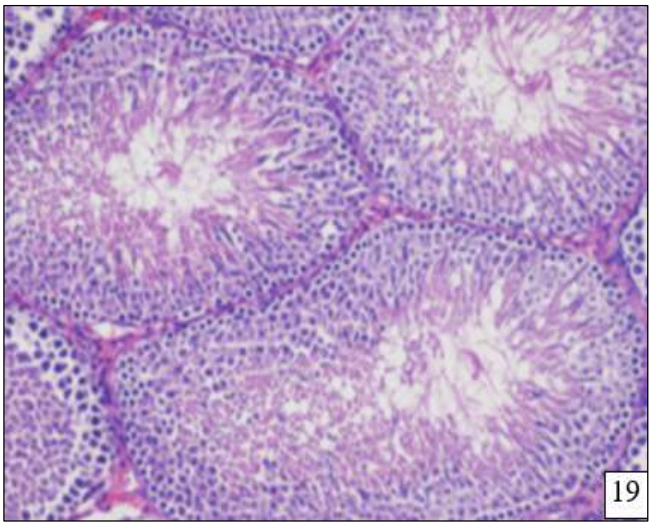

Fig 19: Section of testis from Group VI showing compact arrangement of semeniferous tubules with evidence of sperm production on 28th day. (H&E X100)

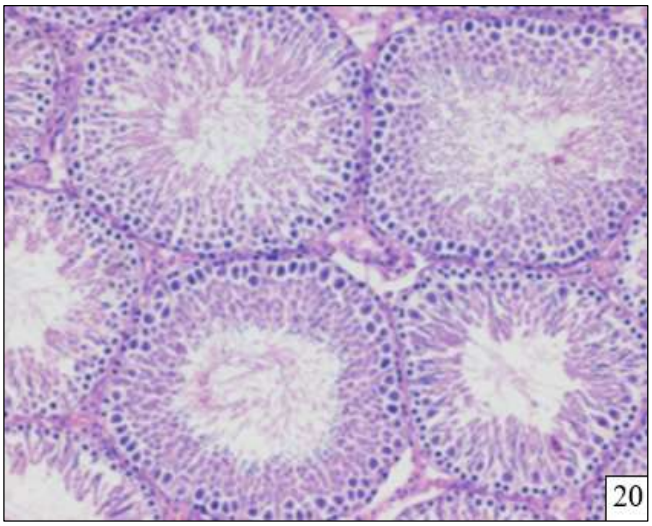

Fig 20: Section of testis from Group VI showing compact arrangement of semeniferous tubules with evidence of sperm production on 45th day. (H&E X100)

Conclusion

The study highlighted the beneficial effects of gallic acid supplementation at 75 mg/kg bw in ameliorating the CP induced pathomorphological alterations in testis of Wistar albino rats and possible protective role of GA against CP induced testicular toxicity.

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