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# Involvement of Pro- and Anti-Apoptotic Markers in Perfluorooctanoic Acid (PFOA) Induced Testicular Disruption in Wistar Rats

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**ABSTRACT**

Perfluorooctanoic Acid (PFOA) induces reproductive toxicity in males by obstructing germ cell differentiation, increasing oxidative stress, and degrading vital intercellular junctions. While extensive research associates PFOA with testicular dysfunction and atrophy, its impact on pro- and anti-apoptotic factors remains less explored. The present study investigates the dose-dependent effects of PFOA on p53, caspase-3, and BCL2 to evaluate their potential impact on testicular growth. Four groups of Wistar male rats were studied: Group II received 2 mg/kg PFOA/day, Group III received 5 mg/kg PFOA/day, and Group IV received 10 mg/kg PFOA/day. Each group consisted of five rats treated for 45 days. A control group (Group I) of equal size was included for parallel comparison. The weights of the main and accessory reproductive organs were recorded. The expression of p53, BCL2, and caspase-3 in testicular tissues was assessed immunohistochemically to determine the impact of PFOA on testicular cell proliferation. The results indicated a sharp, dose-dependent decline in testicular and epididymal weight. The expression of p53 increased substantially in animals administered 10 mg/kg PFOA/day. Similarly, caspase-3 expression significantly increased in a dose-dependent manner, demonstrating a direct association with PFOA exposure. Conversely, BCL2 expression decreased markedly in animals treated with 10 mg/kg PFOA/day. These findings suggest a caspase-3-dependent cleavage of BCL2 and activation of p53 in the germ cells of PFOA-treated rats. In conclusion, the study demonstrates that the loss of testicular and epididymal weight in PFOA-administered rats is attributable to high apoptosis, ultimately leading to testicular atrophy.

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## INTRODUCTION

Perfluorooctanoic acid (PFOA), a member of the perfluoroalkyl substances (PFAS) family comprising approximately 5000 chemicals, is a pervasive environmental contaminant (Pan *et al*., 2018). PFOA is widely utilized in industrial and commercial applications, including food packaging, kitchenware, and water supply infrastructure. Its chemical stability and resistance to degradation result in prolonged environmental persistence, leading to contamination of water, soil, and air. Due to its extended half-life and widespread presence, PFOA is detectable in large human population (Calafat *et al*., 2007).

Extensive research has established associations between PFOA exposure and the development of liver, kidney, testicular, and pancreatic cancers in animal models (Biegel *et al*., 2001; Barry *et al*., 2013; Kamendulis *et al*., 2022). Its adverse effects on the reproductive system have also been widely reported. Previous records suggest that PFOA induces reproductive toxicity in both males and females, including the inhibition of steroid hormones and damage to oocytes and follicles in females (Shi *et al*., 2024). In male, PFOA reduces semen quality and disrupt reproductive hormonal balance (Vested *et al*., 2013). While there is relatively less information on male reproductive toxicity compared to female reproductive adversities in relation to PFOA. Nonetheless, available studies indicate that PFOA has been demonstrated to exert selective apoptotic effects on specific testicular cell populations (Han and Park, 2023; Fujiwara *et al*., 2023).

PFOA has long been recognized for its negative

impact on steroidogenic processes, earning its classification as an endocrine disruptor. A study by Bräuner *et al*. (2021) highlighted a significant association between endocrine disruptors and testicular cancer. The induction of testicular cancer through chemical exposure is not a novel concept; an early study by Damjanov *et al*. (1978) demonstrated that injecting 5 mg of nickel subsulfide could induce testicular sarcomas in rats. The authors of this pioneering work outlined the progression of events in three phases: (1) initial necrosis, (2) testicular atrophy, and (3) the formation of neoplasms or fibrosarcomas. Interestingly, earlier studies have reported a similar pattern of cellular destruction in the testis following PFOA exposure (Lu *et al*., 2016; Liu *et al*., 2015). Since PFOA is known to trigger apoptotic events in the testis, followed by necrosis and atrophy, the present study aims to evaluate the roles of anti- and pro-apoptotic factors in dose-dependent, PFOA-induced testicular toxicity.

## MATERIALS AND METHODS

### Test material

Perfluorooctanoic acid (PFOA), with the chemical formula $CF_3(CF_2)_6COONH_4$ and a purity of over 95%, was procured commercially from Sigma Aldrich (Merck, Germany).

### Animal model and experimental approval

Male Wistar albino rats (*Rattus norvegicus*), aged three months and weighing between 150–200 g, were selected for the study. The healthy and fertile animals were randomly assigned to groups and housed in polypropylene cages measuring 43×27×15 cm. A commercially prepared pellet diet, obtained from Ashirwad Pvt. Ltd., RJ, India, was provided to all animals, along with drinking water made available *ad libitum*. The rats were maintained under a controlled 12-hour light and 12-hour dark cycle to ensure proper day-night conditions. All experimental animals were housed in the departmental animal facility at the Department of Zoology, University of Rajasthan, RJ, India, where they received proper care under the supervision of a veterinary expert.

The handling and treatment of the animals adhered to the guidelines set forth by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, 2010). Approval for the study was obtained in advance from the Institutional Animal Ethics Committee (IAEC). All experimental procedures were conducted in accordance with the Indian National Science Academy (INSA) guidelines for the care and use of animals in scientific research.

### Experiment design

Animals were administered PFOA orally at doses of 2, 5 and 10 mg/kg b.w. daily for 45 consecutive days. The selection of these doses was based on a previous study conducted by Kojo *et al*. (1986). The experimental groups consisted of five animals each, with one serving as the control (Group I). Group II received 2 mg/kg b.w. of PFOA, Group III received 5 mg/kg
b.w. of PFOA, and Group IV received 10 mg/kg b.w. of PFOA. All animals were sacrificed on the 46th day of experiment.

### Testicular and epididymal weight

At the conclusion of the experiment, the animals were euthanized, and the testes and epididymides were carefully dissected and cleared of extraneous tissues. The weights of the left and right testes and epididymides were recorded individually, and the mean values were calculated for representation.

### Immunohistochemical (IHC) assays for apoptotic markers in testicular tissues

Slides containing testicular tissue were rehydrated using graded alcohol, followed by antigen retrieval via microwave heating in 0.01 M citrate buffer (pH 6). The tissues were treated with 3% $H_2O_2$, washed with phosphate-buffered saline, and blocked with a blocking solution (Invitrogen, MD, USA).

The processed slides were incubated for 12 hours with primary antibodies (p53, BCl2, and caspase-3) at a 1:100 dilution (Invitrogen, MD, USA). After incubation, the slides were washed with PBS and treated with Histostain-Plus HRP and a broad-spectrum secondary antibody (60:40 dilution, ThermoFisher Scientific, MA, USA), then left at room temperature for 30 minutes. Following two PBS washes, HRP-Streptavidin (ThermoFisher Scientific, MA, USA) was applied, and the reaction was stopped after 30 minutes at room temperature. Brown coloration was developed using DAB reagent (ThermoFisher Scientific, MA, USA) for 30 seconds and observed under a light microscope.

The slides were counterstained with haematoxylin for 5 minutes, rinsed with distilled water, dehydrated in graded alcohol (50% to absolute), and cleared in xylene. A coverslip was placed over the tissue with a drop of DPX. Immunolabeling was assessed by evaluating the intensity of the blueish-brown coloration in the tissue. The number of IHC positive cells was counted (Image J software, NIH, USA) from 10 randomly selected fields in each testicular cross-section and adjusted to a 1.0 $mm^2$ area.

### Statistical observations

The data for testicular and epididymal weights, as well as IHC positive cells, were presented as the mean± SEM. One-way analysis of variance (ANOVA) was applied for comparison between control and test samples (MINITAB, Pennsylvania, US). Significant variation Significant variations were evaluated based on confidence intervals (CIs) of 95%, 99%, and 99.99%, which are classified as significant, highly significant, and extremely significant, respectively.

## RESULTS

### Reproductive organ weights

The administration of PFOA resulted in a significant decline in the weight of major reproductive organs. Testicular weight decreased by approximately 7% in Group IV compared to the control, while Groups II and III showed a reduction of nearly 4% each. This suggests a substantial impact of PFOA on testicular growth, potentially due to testicular atrophy (Table 1). Interestingly, epididymal weights exhibited a sharper decline, with Group IV showing nearly a 15% reduction, while Groups II and III showed epididymal weight loss of 4% and 6%, respectively (Table 1). Similarly, a marked reduction in the weight of the vas deferens (VD) was observed, with Group IV showing a 14% decline, and Groups II and III exhibiting decreases of 5% and 10%, respectively, compared to the control.

For accessory reproductive organs, including the ventral prostate (VP) and seminal vesicle (SV), changes were more nuanced. A minor 4% decline in SV weight was noted in Group IV compared to the control, while no significant changes were observed in the other test groups (Table 1). In contrast, the weight of the VP showed a notable reduction of 13% and 15% in Groups III and IV, respectively.

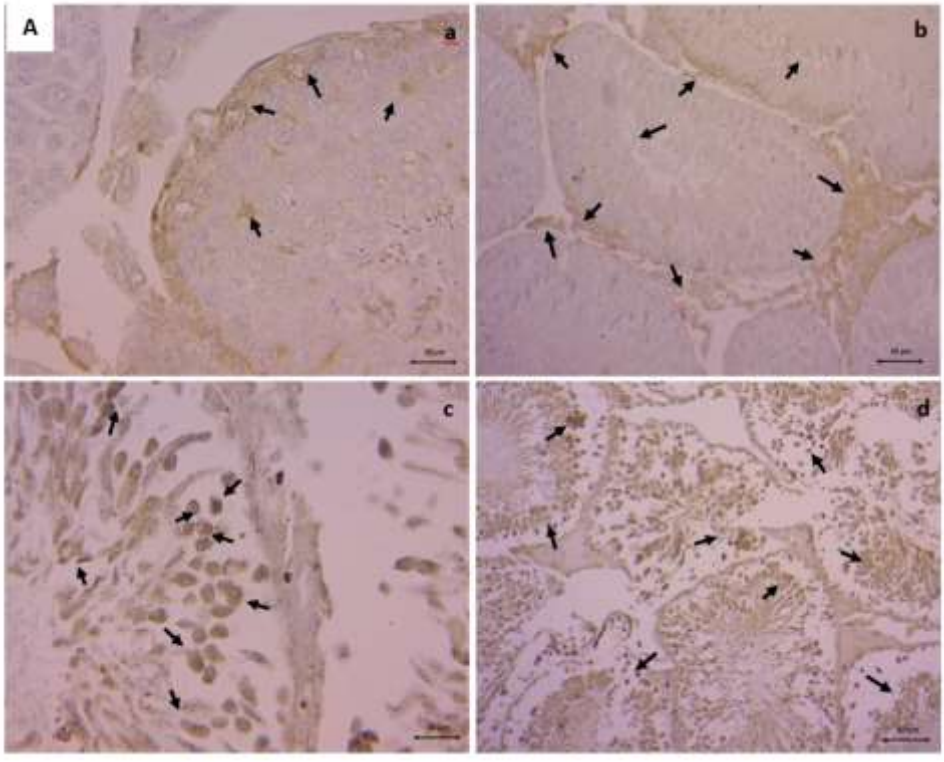
**Table 1: Reproductive organ weight following PFOA treatment for 45 consecutive days respective to administered doses.**

| | Testis (g) | Epididymis (mg) | VD (mg) | VP (mg) | SV (mg) |
|---|---|---|---|---|---|
| Group I | 1.55±0.05 | 73.07±0.95 | 9.18±0.24 | 44.00±1.79 | 226.85±1.78 |
| Group II | 1.50±0.06* | 69.97±1.16* | 8.78±0.26 | 40.92±1.41 | 224.11±2.15 |
| Group III | 1.50±0.05* | 68.20±1.34* | 8.27±0.38* | 38.11±1.39* | 224.15±2.77 |
| Group IV | 1.45±0.05** | 62.45±1.71** | 7.98±0.69* | 37.39±1.14* | 218.70±1.92* |

Level of significance was measured against Group I, *p<0.05 was considered significant, while **p<0.01 considered highly significant. VD: vas deferens; VP: ventral prostate; SV: seminal vesicle.

### Expression of p53 in testis

The expression of p53 in testicular tissues of PFOA-treated animals showed a significant increase in immunostaining compared to the control group (Group I) (Figure 1A). Group II exhibited minimal effects from PFOA treatment (p>0.05) compared to Groups III and IV, which showed a highly significant increase (p<0.001). Positive staining was more prominently observed in spermatogonia and primary spermatocytes. A high number of germ cells exhibited positive p53 expression in the group of animals treated with the highest dose of PFOA, indicating a dose-dependent effect on p53 activity (Figure 1B).



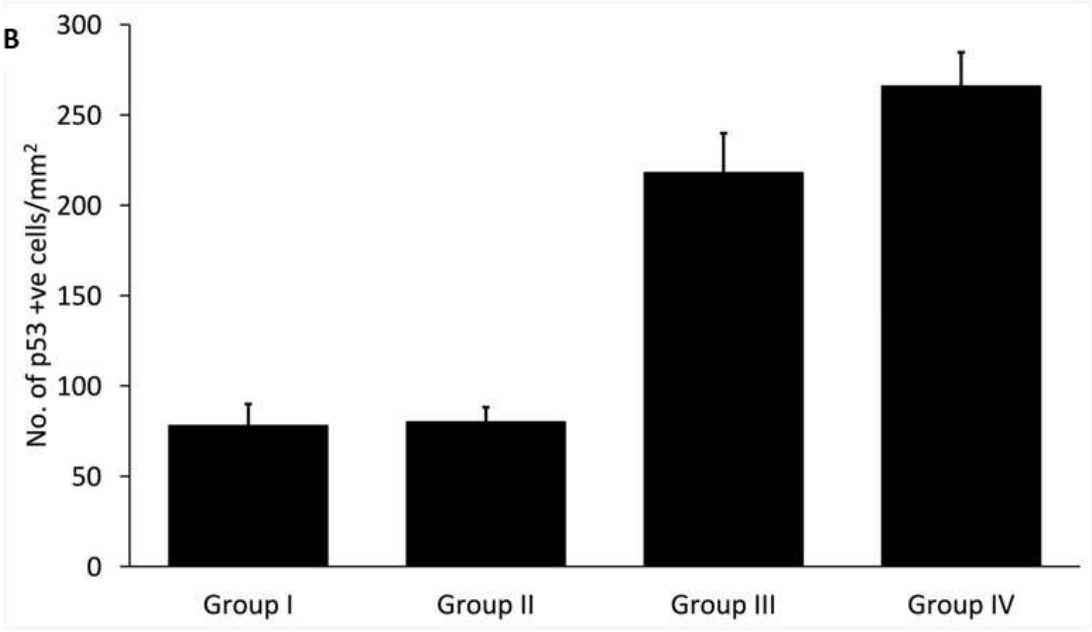
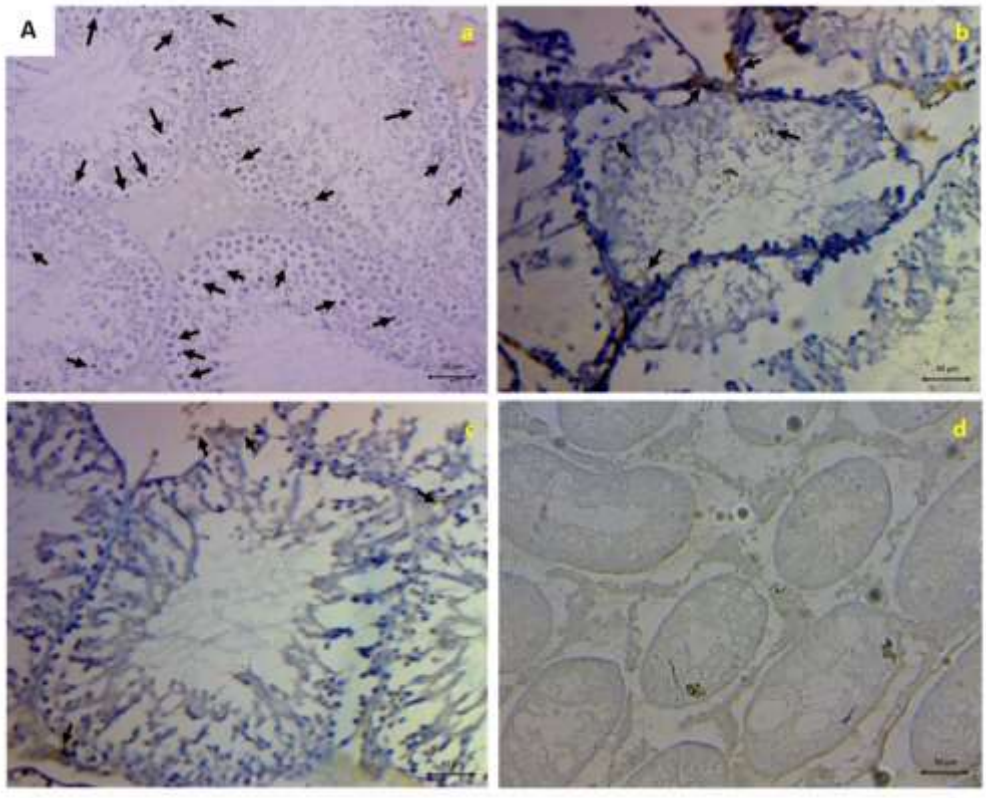



**Figure 1:** A. Expression of p53 was minimum in Group I (a) which was mainly observed in primary spermatocytes and often in spermatogonial cells. Group II (b) also indicated minimum expression of p53 mostly around the interstitial spaces. However, Group III (c) and IV (d) indicated significant increase in number of cells undergone p53 activation. These positive cells were mostly germ cells, including spermatogonia, spermatocytes and spermatids. B. Area found positive for p53 clearly showed extremely significant increase in number of testicular cells in Groups III and IV, indicating largescale apoptotic activation.

**Expression of BCL2 in testis**

The activity of BCL2 in PFOA-treated groups showed a severe depletion, which appeared to be dose-dependent (Figure 1A). The number of germ cells positive for BCL2 expression declined significantly in Groups II ($p<0.01$) and III ($p<0.01$) compared to the control. In Group IV, only a negligible number of cells were positive for BCL2 expression ($p<0.001$), indicating that higher doses were associated with a proportional reduction in BCL2 expression (Figure 2B).



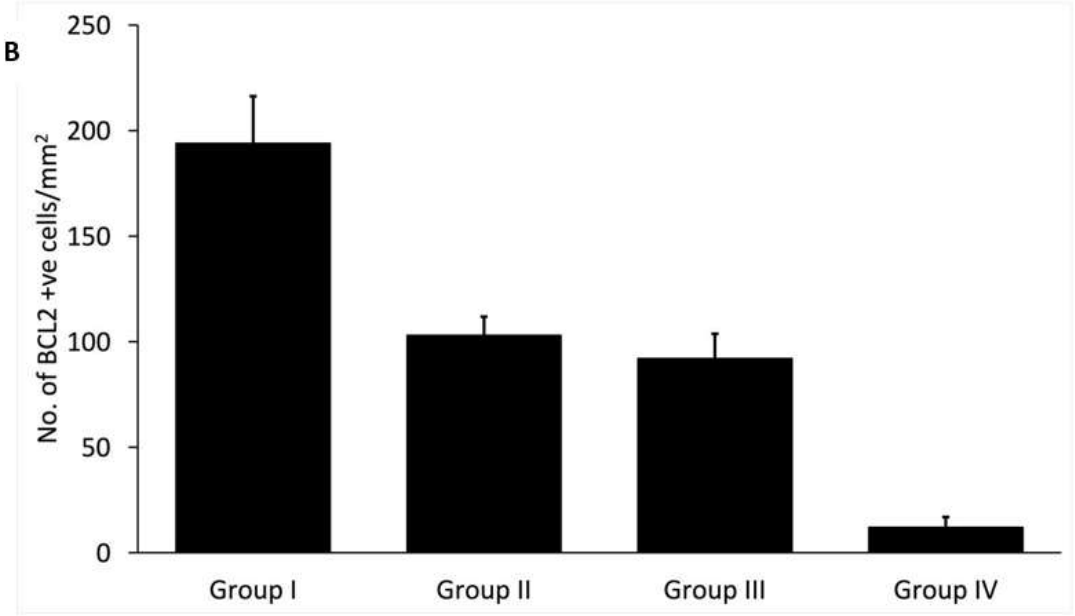
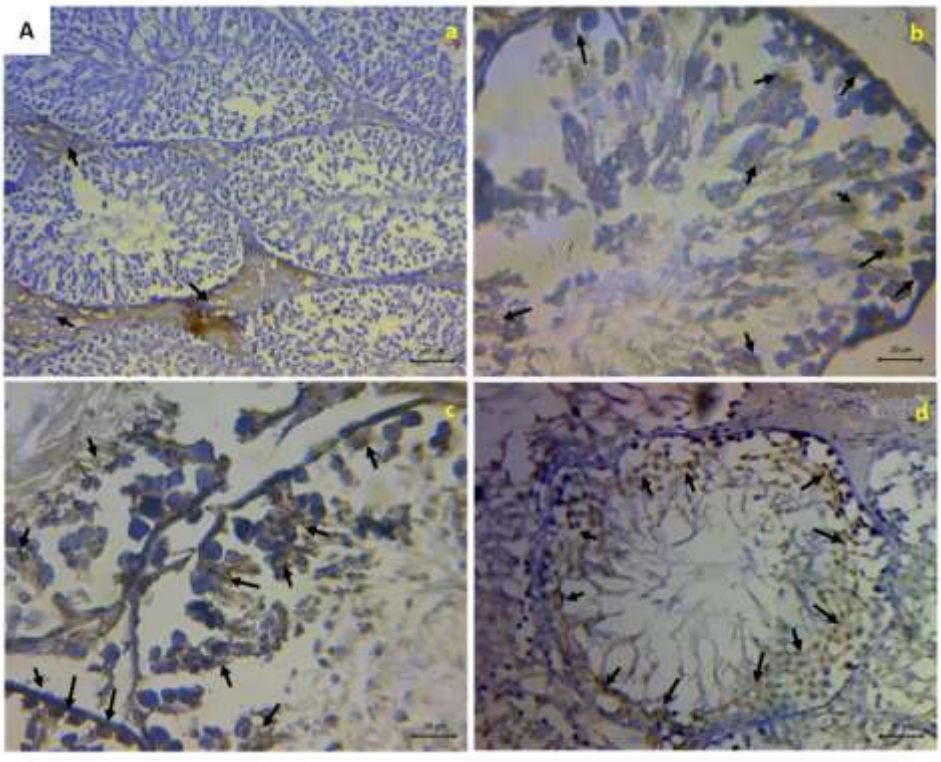



**Figure 2:** A. Immunohistochemical assay localized maximum expression of BCL2 in Group I (a), most expressed germ cells were spermatogonia and primary spermatocytes. Group II (b) and III (c) indicated relatively low expression for BCL2 which was mostly localized in and around interstitial space. Besides some spermatogonial cell also showed positive expression for BCL2. Group IV (d) showed minimum expression of BCL2 some positive staining was observed in around basal lamina and interstitial spaces. B. Area wise distribution of BCL2 positive cells indicated Group I (control) as the most expressed comparing to PFOA exposed test groups, where, Group IV indicated minimum expression of BCL2.

**Expression of Caspase 3 in testis**

Caspase 3 expression in PFOA-treated animals was clearly dose-dependent, as the number of positive germ cells increased with higher doses (Figure 3A). The data indicated that caspase 3 was most prominently activated in primary spermatocytes, followed by spermatogonia. Testicular cells expressing caspase 3 were extremely elevated in Group IV ($p<0.001$) (Figure 3B). Positive expression of caspase 3 in Groups II and III also showed significant elevation ($p<0.01$), though these levels were substantially lower compared to Group IV (Figure 3B).



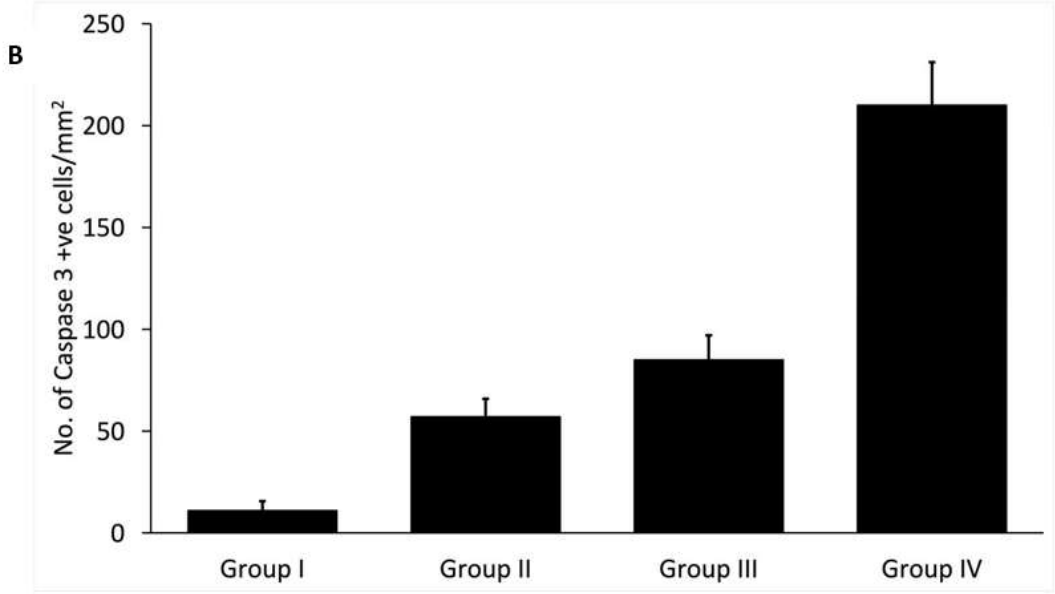



**Figure 3:** The expression of caspase 3 in Group I was limited to some site of interstitial spaces
(a). Group II (b) showed expression of caspase 3 in various testicular cells including spermatogonial cells and spermatocytes. Occasional positive expression was witnessed in Sertoli cells. Group III (c) indicated similar pattern of positive cells to that of the Group II,
however, in this group primary spermatocytes and spermatogonial stem cells were prominently expressed. Group IV (d) revealed extensive positive expression among germ cells. Spermatogonia, spermatocytes, and spermatids were found positive for caspase 3 in this group.
B. Maximum area for caspase 3 positive germ cell were observed in Group IV followed by Group III and II, when compared with control.

**DISCUSSION**

There is substantial evidence clearly establishing the role of per- and polyfluoroalkyl substances (PFAS) in increasing the risk of tumour development across various organs, including the liver, spleen, mammary glands, and pancreas (Hundley *et al*., 2006; Choi *et al*., 2022; Pierozan *et al*., 2018). Its specific impact on the male reproductive system is also well- documented, highlighting targeted toxicity in the testes and disruption of spermatogenesis (Sun *et al*., 2023). The primary mechanisms of toxicity in testicular cells include oxidative stress, apoptosis, autophagy, and functional impairment of critical cell types (Shi *et al*., 2024). While various studies have explored the role of PFAS in tumorigenesis, its specific association with germ cell apoptosis and potential carcinogenesis remains relatively understudied. This study investigates pro-apoptotic and anti-apoptotic markers to elucidate the dose-dependent effects of PFOA exposure on these markers and its association with significant changes in the weights of primary and accessory reproductive organs.

Higher levels of apoptosis are associated with organ weight loss in both animal and plant species (Bonda-Ostaszewska and Włostowski, 2015; Ledda-Clumbano *et al*., 1989). The present study clearly demonstrated a loss of testicular weight in PFOA-treated animals. However, the decline in weight did not exhibit a dose-dependent pattern, suggesting potential cell loss through an indirect mechanism. Previous studies have reported excessive generation of reactive oxygen species in testicular cells (Liu *et al*., 2015; Umar Ijaz *et al*., 2022). A study by Sharma *et al*. (2023) indicated that oxidative stress can induce apoptosis and autophagy in testicular tissues. It can, therefore, be presumed that oxidative stress may have triggered the activation of apoptotic inducers. Interestingly, the present study also revealed substantial weight loss in the epididymis and vas deferens. This finding suggests that PFOA may pose a toxic challenge to epididymal cells. Furthermore, previous research has indicated that loss of testicular function may eventually lead to a reduction in epididymal weight (Vidal and Whitney, 2014). Thus, the reduction in epididymal and vas deferens weight could be attributed to impaired testicular function. Further studies are needed to elucidate the specific effects of PFOA on these organs.

The expression of p53 was notably high in the testicular tissues of animals administered 5 and 10 mg/kg of PFOA. Most p53-positive cells in these two groups were identified as spermatogonia and primary spermatocytes. It is important to note that during spermatogenesis, the removal of germ cells is a normal physiological process, which ultimately eliminates a significant number of mature sperm cells (Dunkel *et al*., 1997). The present study also observed a moderate number of p53-positive cells in the control group. Nonetheless, the number of positive cells

increased substantially in animals exposed to higher doses. This suggests the activation of heightened germ cell deletion in animals treated with 5 and 10 mg/kg of PFOA. Several studies have reported that PFOA exposure leads to a reduction in sperm count (Tarapore *et al*., 2021; Calvert *et al*., 2022).

It is worth noting that apoptosis occasionally occurs during the maturation division of spermatocytes. However, the degree of elimination of spermatocytes is lower compared to spermatogonia (Zakaria *et al*., 2022). The expression of p53 peaks in primary spermatocytes due to the frequency of DNA damage and recombination repair during the meiotic process (Li *et al*., 2024). Based on the localization of p53 expression in spermatocytes, it appears that a large number of these cells underwent apoptosis. PFOA-induced oxidative stress in testicular tissue is hypothesized to be the primary cause of p53 activation in germ cells. Previous studies have noted a dose-dependent increase in oxidative stress caused by PFOA. It appears that the 2 mg/kg dose was better tolerated by the endogenous antioxidative mechanisms, which may have failed under higher doses.

BCL2 is an anti-apoptotic protein that sequesters the activity of caspases and apoptogenic factors (Tsujimoto, 1998). The present study demonstrated a clear reduction in BCL2 expression in germ cells among groups of animals exposed to daily doses of PFOA. Interestingly, a minimal difference was observed between the groups of animals administered 2 mg/kg and 5 mg/kg of PFOA, while a dose of 10 mg/kg significantly decreased BCL2 expression. A study by Liu *et al*. (2015) similarly reported a remarkable decline in testicular weight following the administration of 10 mg/kg of PFOA for 14 consecutive days. Likewise, Eggert et al. (2019) reported that PFOA affects cell growth by inducing a decline in meiotic division and arresting growth at the G2/M phase in the testis. Previous studies have noted that BCL2 can influence the cell cycle by affecting the G2/M and G0/G1 phases (Bonnefoy-Berard *et al*., 2004). These findings suggest that PFOA significantly impacts spermatogenesis by regulating germ cell proliferation through the modulation of BCL2.

Since BCL2 regulates caspases and apoptosome complexes, its dose-dependent decline could potentially affect the activity of caspase 3 in the testicular tissues of PFOA-treated rats. The present study demonstrated a clear elevation of caspase 3 activity in the PFOA-treated groups. Unlike BCL2, the activity of caspase 3 in the exposed groups showed a strict dose dependency and was highly correlated with increasing dose variations. A study by Kirsch *et al*. (1999) reported that caspase 3 is crucial for BCL2 cleavage and the activation of cytochrome c, which further promotes caspase activation and cell death. These findings suggest that the cell death observed in germ cells following PFOA treatment is likely induced through the mitochondrial pathway. However, further studies are required to confirm this claim.

The caspase-positive cells were primarily localized in proliferating cells during the initial stages of spermatogenesis, indicating a significant role of PFOA-induced oxidative stress. Previous studies have reported that spermatogonial cells are highly tolerant to reactive oxygen species due to the presence of Cu/Zn SOD, whereas later-stage germ cells are increasingly vulnerable to oxidative stress (Celino *et al*., 2011). Nevertheless, higher apoptosis rates in spermatogonial cells are not uncommon. For example, a study by Dunkel *et al*. (1997) found that cryptorchid testes exhibit increased levels of apoptosis in spermatogonia. Oxidative stress has been identified as a major factor in cryptorchidism, primarily due to increased testicular temperatures (Peltola *et al*., 1995; Misro *et al*., 2005; Turner and Lysiak, 2008).

Significant alterations in male fertility rates are closely linked to the endogenous balance of oxidative stress in key reproductive organs, including the testis, epididymis, and vas deferens (Lohiya *et al*., 2014). Therefore, it is presumed that PFOA-induced oxidative stress potentially activates caspase 3 in testicular tissues, leading to BCL2 cleavage and the activation of p53, which contributes to a substantial decline in testicular and epididymal weight.

## CONCLUSION

The present study concluded that PFOA exposure induces a typical mitochondrial pathway of apoptosis in testicular tissue. The role of PFOA in the testes is dose-dependent; therefore, the activity of pro- and anti-apoptotic markers depends on the intensity of oxidative stress induced as a consequence. The loss of weight in the main reproductive organs is primarily due to atrophy caused by large-scale apoptosis in testicular tissue. Hence, doses greater than 5 mg/kg of PFOA administered over a prolonged period may induce apoptosis in the testicular tissue of male rats, which is likely to result in reduced fertility rates.

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