Antiapoptotic effects of resveratrol ameliorate perfluorooctanoic acid-induced inhibition of testosterone biosynthesis in TM3 Leydig cells

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Abstract

The perfluorooctanoate (PFOA) deteriorates testicular steroidogenesis. However, the exact mechanism of action remains to be clarified. To address this question, the effect of PFOA on mouse Leydig cells was investigated. The TM3 cells were cultured in vitro (obtained from NCCS, Pune) and exposed to PFOA at concentrations of 10 µg/ml, 50µg/ml and 100 µg/ml over a period of 24 h. The effects of PFOA on cell proliferation and apoptosis were analyzed via flow cytometry and caspase-3 levels in TM3 cells were determined by calorimetric assay. The results indicate that PFOA treatment at all concentrations significantly decreased the live cells as compared to the control cells.

Further, the levels of caspase-3 were significantly elevated in PFOA treated TM3 cells in a dose-dependent manner as compared to the controls. Resveratrol treatment on the other hand showed ameliorative effects on testosterone biosynthesis, associated with reduced apoptosis rate in PFOA treated cells. Significant elevation of StAR mRNA levels was noticed in TM3 Leydig cells treated with both PFOA and resveratrol at selected concentrations. In silico analysis also revealed that the binding affinity of PFOA with ratStAR was greater than that of cholesterol-ratStAR protein. Taken together, our data suggest that PFOA-induced TM3 cell toxic effect may involve caspase-3 mediated death receptor signaling pathway and resveratrol with its antioxidant and steroidogenic effects restoring TM3 Leydig cells against PFOA-induced toxicity.

Keywords: Perfluorooctanoic acid, testosterone, TM3 leydig cells.

Introduction

Endocrine disruptors are the chemicals that mimic or block the action of endocrine factors and male reproductive tract functions occur in an endocrine-dependent manner[31]. Thus, even a minute change in the hormones leads to deterioration of male fertility efficacy[33]. Perfluorooctanoic acid (PFOA) is a synthetic fluoro surfactant which is widely used in manufacturing of clothes, food packages, firefighting foams, carpets and footwear products[5]. PFOA comprised of terminal carboxylate group and also a perfluorinated 7 carbon alkyl chain. Due to the presence of carbon to fluorine bond, PFOA is stable and non-biodegradable and hence, categorized under persistent chemical (half-life: 3.5 years in humans)[27].

Experimental studies have shown that the exposure to PFOA causes altered liver metabolism, liver enlargement and also interferes with pancreatic functions[21]. With respect to male reproduction, PFOA deteriorates testicular functions such as spermatogenesis and steroidogenesis[38]. Further, it has been shown that PFOA exposure leads to disturbances at the level of blood-testis barrier *in vivo* and *in vitro*[25]. Most strikingly, accumulation of PFOA in the seminal plasma of humans has been reported[5].

Furthermore, *in utero* exposure to PFOA leads to its accumulation in umbilical cord blood[26] and even fetal organs of humans and rodents. Even the occurrence of PFOA in breast milk has also been found[11]. *In utero* exposure to PFOA showed altered production of reproductive hormones and deteriorated sperm quality in men at their adulthood[19].

Published reports have shown that men may have higher concentration of perfluorinated compounds in their serum than women and most strikingly, younger individuals may have higher levels of perfluorinated compounds than older men[10]. Hence, it is conceivable that the young men may possess very high exposure levels and could be at risk for potential negative consequences of PFOA. Though the potential reproductive and developmental toxicity of PFOA have been demonstrated, the exact mechanisms of PFOA suppressed inhibition of testosterone are not yet well understood[22].

Antioxidant therapy has been shown in numerous studies to improve testicular function when exposed to a wide range of substances including pharmaceuticals and environmental toxins with endocrine disruptive properties[3]. Homeostasis between the generation of free radicals and endogenous antioxidants is essential for normal cellular/tissue physiological functions and therefore, an imbalance between the excess generation of free radicals and endogenous antioxidants, a condition known as oxidative stress, may cause free radical attack at the level of vital components of the cells. DNA, lipids and proteins thereby alter normal physiological events[23]. Thus, antioxidant therapy is one of the preferrable choices to protect the testicular functions against chemical/toxic insult induced oxidative injury.

Resveratrol (RES) is one of the antioxidants present in peanuts, fruits like blueberry, blackberry, grapes and red wine. The higher quantities of RES are present in roots of an herb, *Polygonum cuspidatum* (Japan), leaves and roots of *Veratrum grandiflorum* and *V. formosanum* (China) which are widely used in traditional medicine. Studies have shown that the RES when supplemented exogenously, is able to negate the effects of oxidative stress via boosting enzymatic and non-enzymatic antioxidants[1,30]. Hence, the present study has been designed with resveratrol as antioxidant against PFOA.

Material and Methods

Chemicals: Perfluorooctanoic acid (PFOA; CAS no. 335-67-1; Purity: 95%) and dimethyl sulfoxide were obtained from Sigma Chemicals; BD Cell viability kit (dyes to stain cells) was procured from BD Biosciences, USA, Caspase-3/CPP32 Colorimetric assay kit was obtained from Biovision, USA. All other chemicals, reagents and media chemicals used in this study were obtained from Merck (India) and Gibco (Bethasda, USA). Dulbecco's modified Eagle medium (DMEM)/nutrient mixture (Ham's) F-12 with HEPES, fetal bovine serum (FBS), were obtained from Gibco (Bethesda, USA).

Cell lines: The normal mouse Leydig cell line TM3 was obtained from Cell Bank (No. 1440/2021-22), National Centre for Cell Science (NCCS), Pune, India. After arrival, TM3 cells were maintained and propagated in Ham'F12 medium with various supplements as suggested by NCCS. The cells were then transferred to 25 mL culture flasks and further incubated in a humidified incubator (Thermo Scientifics) with 5% CO_2 at 37 °C. The medium was replaced after 12 h. Cells were treated with 0.25% trypsin and passaged at a ratio of 1:2 when reaching 75% confluence.

The experiments were performed at TUVRheinland, Visakapatnam, India (http://www.tuv.com), a laboratory with International Standard ISO/IEC 17025:2017 for biomaterial testing facility, medical, non-medical devices and EMC testing Services by the National Accreditation Board for Testing and Calibration Laboratories (NABL).

Cell treatment: Cells in logarithmic phase were used in all experiments (75 % confluency) and TM3 cells were seeded at a density of 8.1 $\times 10^3$ cells/well in a 96-well plate (200 µL/well). The concentrations of PFOA (10 µg/ml, 50 µg/ml and 100 µg/ml) selected in this study were based on Eggert et al[13] and the concentration of resveratrol (RES: 25 µg/ml and 50 µg/ml) was based on Berge et al[7]. Solutions of resveratrol and PFOA were prepared in DMSO and during the experiments, the final concentration of DMSO was 0.1%. The control culture contained 1 µl of DMSO.

In the current study, four cell treatments were performed: PFOA with three concentrations, resveratrol with two concentrations, PFOA (100 µg/ml) plus RES (25 µg/ml) and PFOA (100 µg/ml) plus RES (50 µg/ml). All the experiments were performed in replicates of three and repeated at least four times with similar results.

Cell viability: Cell viability was assessed through MTT {3-[4,5-dimethylthiazol-2-yl)-2,5-diphenylterazoliumbromide} technique. Briefly, the TM3 cell lines after completion of the experimental period (24 hr) were subjected to centrifugation and the pellets were washed three times with PBS followed by the addition of DMEM/F12 (150 µL) medium without serum and 20% MTT (20 µL). After stipulated incubation time at 37 °C for 4 h, the medium was gently removed without disturbing the plate and 150 µL of DMSO was added to the plates. The plates were slowly shaken at low speed over a period of 10 min at room temperature.

The absorbance was immediately measured on a microplate reader (Model: Epoch microtitre plate) at 490 nm. Celll viability was expressed as the relative formazan formation in PFOA treated, RES treated and PFOA plus RES treated samples after normalization against the background absorbance (absorbance corresponding to the well containing culture medium and MTT but without cells).

Caspase 3 levels: In the current study, caspase-3/CPP32 colorimetric assay kit (BioVision) provides a simple and convenient means for assaying the activity of caspases that initiates apoptosis in mammalian cells which occurs via recognition of the sequence DEVD. The principle of the assay was based on spectrophotometric detection of the chromophore p-nitroaniline (pNA) after cleavage from the labeled substrate DEVD-pNA. The pNA light emission can be quantified using a spectrophotometer or a microtiter plate (Model: Epoch microtitre plate) reader at 405 nm. Comparison of the absorbance of pNA from an apoptotic sample with an uninduced control allows determination of the fold increase in CPP32 activity. The methodology was followed in accordance to the manufacturer's instructions. Briefly, the untreated and treated cells were pelleted (1 to 5 $\times 10^6$ cells) and suspended in cell lysis buffer (chilled) and incubated on ice for 10 minutes followed by centrifugation (10,000 x g).

The cytosolic supernatant was used for caspase activity. For caspase activity, 100 µg protein to 50 µl cell lysis buffer was used. To the cytosolic part, 50 µl of 2X reaction buffer (containing 10 mM DTT) and 5 µl of the 4 mM DEVD-pNA substrate (200 µM final conc.) were also added and then incubated at 37°C for1-2 hour. After experimental duration, the samples were read at 400- or 405-nm in a microtiter plate reader, or spectrophotometer using a 100-µl micro-quartz cuvette (Sigma). The fold increase in CPP32 activity can be determined by comparing these results with the level of the uninduced control. The background readings from cell lysates and buffers were subtracted from the readings of both induced and the uninduced samples before calculating fold increase in CPP32 activity.

One-unit caspase 3 activity is equivalent to the concentration of enzyme required to cleave 1.0 nM of the Ac-DEVD-pNA per hour (colorimetric substrate) at 37°C under saturated substrate concentrations.

Flow cytometry analysis: After exposure to different doses of PFOA, PFOA plus resveratrol and resveratrol alone for 24 h, cells were trypsinized, harvested and washed with PBS. The cell density used for the flow cytometry was 5×10^5 cells/mL from each group. Apoptotic cells were then detected by using BD™ Cell viability kit (BD Bioscienes, USA) according to the manufacturer's instructions. Cells with compromised cell membranes (dead cells) allow propidium iodide (PI) while thiozole orange stains all the cells. The fluorescent signal from TO in viable cells allows their enumeration even when debris in the cell preparation contaminates a scatter gate around the cells. Thus, the combination of these two dyes provides a robust method for discriminating live and dead cells. Flow cytometric analysis was performed by using BD FACSTM brand flow cytometer equipped with 488-nm laser excitation and BD CellQuestTM software.

Testosterone assay *in vitro*: In brief, the cell culture media from each well (control and treated samples) was collected individually and centrifuged to obtain the supernatant. The resultant supernatant was used for the testosterone assay (Diametra, Italy) as per the manufacturer's instructions. All the analysis was performed in triplicate and the mean value was considered as the testosterone concentration in treated and untreated cultures. The concentrations were determined based on the standard curve plotted provided with the standard concentrations of the kit. The values were expressed as ng/ml.

RT-qPCR studies: Steroidogenic acute regulatory protein and caspase 3 mRNA levels were studied in this study during experimental conditions using RT-qPCR studies. In brief, total RNA was isolated using the trizol plus purification system (Invitrogen, Carlsbad, USA) and the purity of RNA was analysed spectrophotometrically (Jasco v-750; Mary's Court Easton, MD 2160) and by agarose gel electrophoresis. The concentration of isolated RNA was quantified using NanoDrop-2000 spectrophotometer (Thermo-fisher scientific). The cDNA synthesis of first strand was performed according to the manufacturer's protocol of iscript™ cDNA synthesis kit (Biorad, India) using 1µg of total RNA.

The cDNA synthesized was used to express the mRNA levels of selected genes through RT-qPCR (quantitative real time PCR; Applied Biosystems). In the present study, GAPDH: glyceraldehyde phosphate dehydrogenase was selected as the house gene. The forward (F: 5'-3') and reverse (R: 5'-3') primers used for quantitative real time PCR studies were as follows: *StAR*: F: cgtcggagctctctacttgg; R: cccaaggccttttgcatagc; product size:145 bp), *casp3* (F: tggactgcggtattgagaca; R: gcgcaaagtgactggatgaa; product size: 160 bp); and *GAPDH* (F: ggtcggagtgaacggatt; R: ctcgctcctggaagatgg; product size: 227 bp). The primers were selected based on our previous studies[28]. RT-qPCR assay was carried out using SYRB™ green master mix, 2µl cDNA, 0.5µl of each primer (50nm) and RNase\DNase-free H_2O up to 20µl.

Prior to RT-qPCR analysis, the efficiency of primers was determined and found to be >90%. The reaction parameters were as follows: 95° C for 10 min followed by 40 cycles at 95° C for 15 s, 60 °C (*Star*) for 30 s and 72°C for 5 min. The reaction parameters for casp3 were as follows: 95° C for 10 min followed by 40 cycles at 95° C for 15 s, 56 °C for 30 secs and 72°C for 30 s. DNA quantification was performed using the standard curves prepared from the cDNA reaction products after serial dilutions. All samples were run in triplicate including a negative control. The mean Ct values were determined from the triplicates. The obtained Ct values were used for quantification of normalized expression according to the $2^{-\Delta\Delta Ct}$ method using reference gene *GAPDH***.** The data was expressed as relative mRNA expression after normalization for each sample in PFOA and the control groups.

***In silico* studies:** Molecular docking analysis of testicular proteins such as steroidogenic acute regulatory protein (StAR) and androgen receptor (AR) was performed against their respective ligands and was also docked against PFOA to predict the binding affinity and interactive amino acids of these proteins. The amino acid sequences of StAR (UniProt sequence Id: P97826) and AR (UniProt sequence Id: P15207) from rats were collected from uniprot database (https://www.uniprot.org/). In order to select the suitable templates, the amino acid sequences of rat proteins were subjected to BLASTp against PDB database. Based on the percent identity, human StAR 3P0L was selected as suitable template for rat StAR and the PDB structure 2NW4 was directly selected as template for rat AR.

For homology modelling studies, the rat StAR amino acid sequence was BLAST searched to identify resolved PDB structure of StAR protein. BLAST results revealed that the human StAR protein with PDB ID 3P0L showed > 80% identity to that of rat StAR amino acid sequence. Homology modelling of rat StAR protein was performed using Modeller9v.16[32] using the human StAR protein template, 3P0L. The resultant protein model was analyzed for its quality and stability using SAVES program which comprised of in-built programs including Ramachandran plot. Further, validation was performed to assess bond length, bond angle, torsion angle deviations, sequence mismatches, missing atoms or residues, fold prediction accuracy, incorrectly positioned water molecules, chirality errors, extra atoms or residues. The same residues were 100% conserved in modelled structure of rat StAR protein and were visualized using discovery studio.

Molecular docking analysis of selected rat proteins was subjected to Autodock (version 4.2.6) tool against their

respective ligands i.e. for StAR and AR, cholesterol and testosterone were selected as ligands. To predict the binding affinity and molecular interactions between the endogenous ligands and PFOA and selected rat proteins, Autodock was performed. The ligands and the proteins were prepared in accordance to the pipeline of autodock tool. Optimization of interactions between receptor-ligand was done by using molecular dynamics software CHARMM and Clean Geometry of ligands with 1000 steps of steepest descent (SD) algorithm[8].

The root mean square deviation (RMSD) value was set at <1.0 Å. Ligand-receptor minimization was performed on the complexes to inhibit van der Waals forces prior scoring and calculating binding energy. During minimization, SD value was set at 5000 steps[12]. After completion of optimization and minimization steps, the binding energy of protein-ligand complexes was calculated from the free energies of the complex. The free energies of individual protein and the ligand were analyzed using CHARMM software. The docked protein was visualized by using Discovery Studio 2.1.

Statistical analysis: The data were expressed as means ± SD and statistical analysis was performed by using SPSS v13.0 (IBM, Armonk, NY, USA). $P<0.05$ and $p<0.01$ were considered significant and extremely significant respectively.

Results

MTT assay: Figure 1 represents the cell viability of un-induced and induced TM3 cells. A dose-dependent reduction in the viability of cells was recorded in PFOA (0, treated cells) whereas PFOA at 100 µg/ml plus RES at 25 µg/ml or 50 µg/ml treatment increased the number of viable TM3 cells as compared to the PFOA treated cells at 100 µg/ml. On the other hand, RES at selected concentrations did not cause death of TM3 cells comparable to un-induced cells.

Caspase 3 levels: Figure 2 shows the activity levels of caspase in TM3 cells under PFOA stress and PFOA plus RES. In TM3 cells, caspase 3 activity was 0.0056, 0.0061 and 0.0071 µM pNa/h/mL for 10, 50 and 100 µg/mL of the PFOA respectively. On the other hand, significant reduction in the caspase 3 activity levels was observed in cells treated with both PFOA and RES at selected concentrations. RES at selected concentrations i.e. 25 and 50 µg/mL treatment did not show any changes in the caspase 3 activity levels as compared to un-induced TM3 cells.

Flow cytometry analysis: The findings have shown that there was an increase in apoptosis rate of TM3 cells as detected by flow cytometric analysis after treatment with PFOA (10, 50 and 100 µg/ml) for 24 hrs. As shown in fig. 3, PFOA treatment significantly reduced viable cells (47.1%; 37.3%; and 30.3% respectively).

However, the apoptosis rates (42.5%; 52.3% and 62.3%, respectively) significantly increased with the increasing concentrations of PFOA as compared with the control group. TM3 cells treated with both PFOA and RES over a period of 24 hrs showed enhancement in the viable cells (PFOA at 100 µg/ml plus RES at 25 µg/ml: 58.3 %; PFOA at 100 µg/ml plus RES at 50 µg/ml: 67.3 %) associated with a reduction in the apoptosis rate (PFOA at 100 µg/ml plus RES at 25 µg/ml: 28.1 %; PFOA at 100 µg/ml plus RES at 50 µg/ml: 19.2 %). RES at concentrations 25 µg/ml and 50 µg/ml showed a cell viability of 90% and 88% respectively.

Testosterone levels: Figure 4 represents changes in the testosterone levels in TM3 Leydig cells treated with DMSO, uninduced, RES treatment, PFOA treatment and both PFOA and RES treatment. PFOA at selected concentrations deteriorated the testosterone levels in TM3 Leydig cells as compared to the controls.

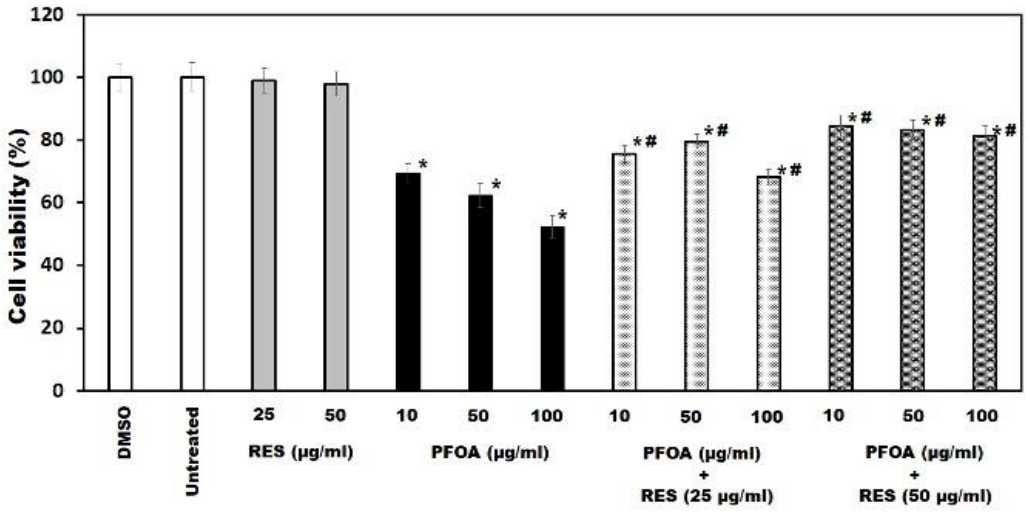

Figure 1: Changes in the cell viability in TM3 Leydig cells from control and experimental groups using MTT assay
Bars represent mean ± S.D. of three individual replicates.
***represents significant over DMSO, Uninduced and resveratrol (RES) treated groups at p<0.001**
#represents significant over perfluorooctanoic acid (PFOA) treated group at p<0.001

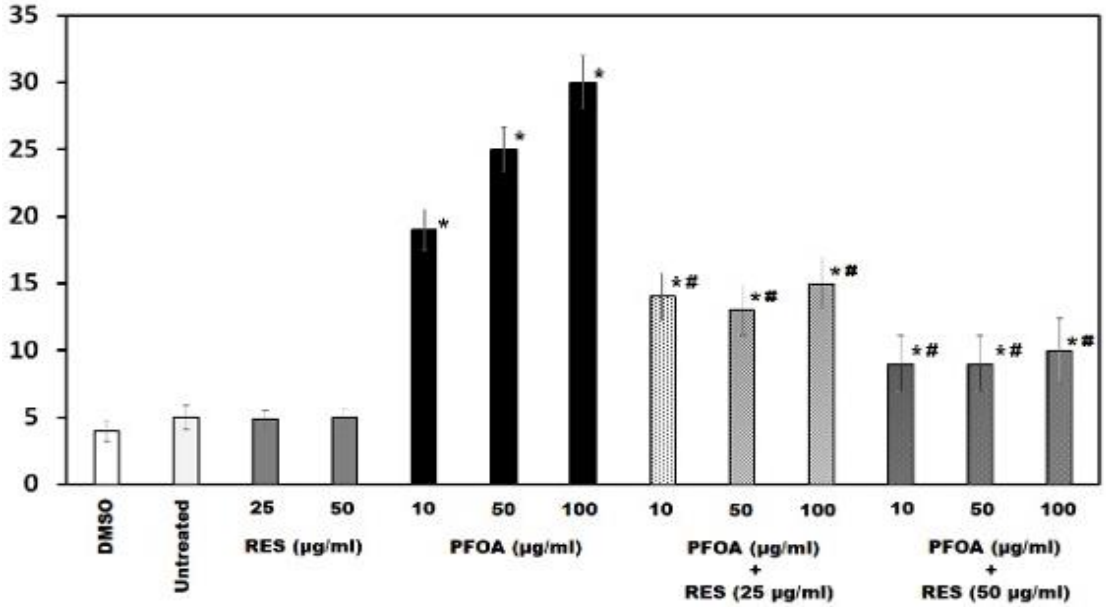

Figure 2: Changes in the activity levels of caspase 3 in the TM3 Leydig cells from control and experimental groups
X axis, Unit/mg protein; One unit is the amount of enzyme that will cleave 1.0 nmol of the colorimetric substrate Ac-DEVD-pNA per hour at 37°C under saturated substrate concentrations.
Bars represent mean ± S.D. of three individual replicates.
***represents significant over DMSO, Uninduced and resveratrol (RES) treated groups at p<0.001**
#represents significant over perfluorooctanoic acid (PFOA) treated group at p<0.001

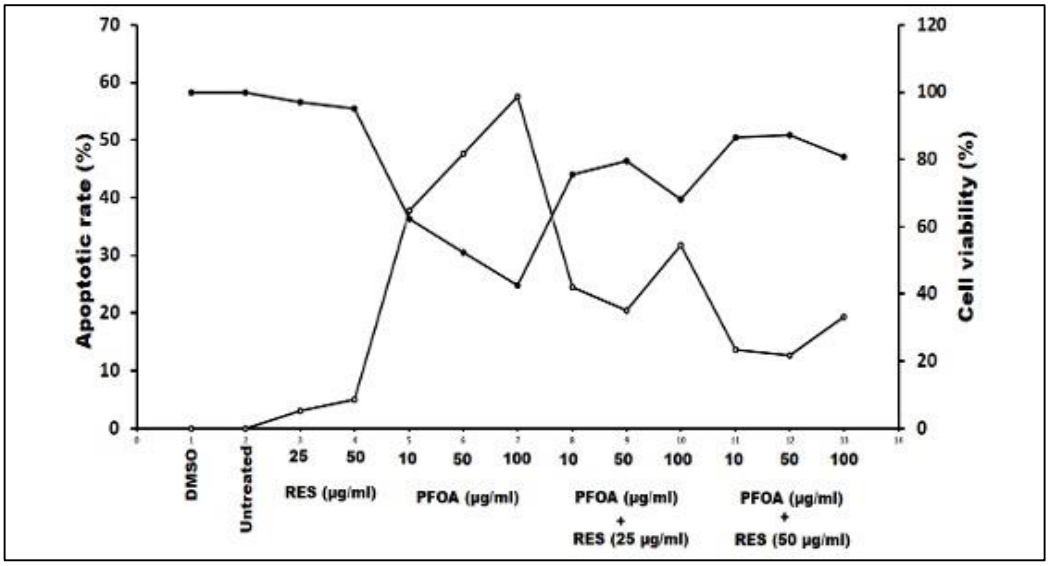

Figure 3: Changes in the apoptotic rate *vs* cell viability of TM3 Leydig cells from control and experimental groups using flow cytometry analysis
Each circle/point represents mean ± S.D. of three individual replicates.
Closed circles, cell viability; Open circles, Apoptotic rate

In contrary, significant elevation was observed in the testosterone levels in TM3 Leydig cells treated with both RES and PFOA. Further no changes in the levels of testosterone were observed in the TM3 Leydig cells treated with RES either at 25 or 50 µg/ml.

***StAR* and *caspase 3* mRNA expression levels:** PFOA (100 µg/ml) showed a significant down regulation of *StAR* mRNA levels as compared to controls. The expression of *caspase 3* mRNA was significantly up regulated in PFOA treated TM3 cells. Whereas, reversal effects of expression of *StAR* mRNA and *caspase 3* mRNA levels were observed in cotreatment of cells with RES (either at 25 or 50 µg/ml) + PFOA (100 µg/ml) (Table 1).

***In silico* analysis:** Steroidogenic acute regulatory protein (StAR) is essential for testosterone biosynthesis. To predict the interactions between the PFOA and StAR, molecular docking analysis was performed (Fig. 4). The binding affinity between the PFOA and StAR was found to be -7.2

k.cal/mol over the binding energy between the StAR and endogenous ligand, cholesterol was found to be -6.0 k.cal/mol. Only alkyl bonds between the cholesterol and StAR protein were observed and the interacting amino acids were Ile 190, Lys52 and Lys 187, whereas conventional hydrogen bonds were noticed between the amino acids of StAR and PFOA interacting amino acids were Arg 116, Ala 106 and Thr 197 and alkyl bond between the Val 85 and PFOA.

In addition to the vander waal forces present in both the interactions i.e. between cholesterol and StAR and PFOA and StAR, additional halogen bonds were also present between the PFOA and StAR protein (Table 2).

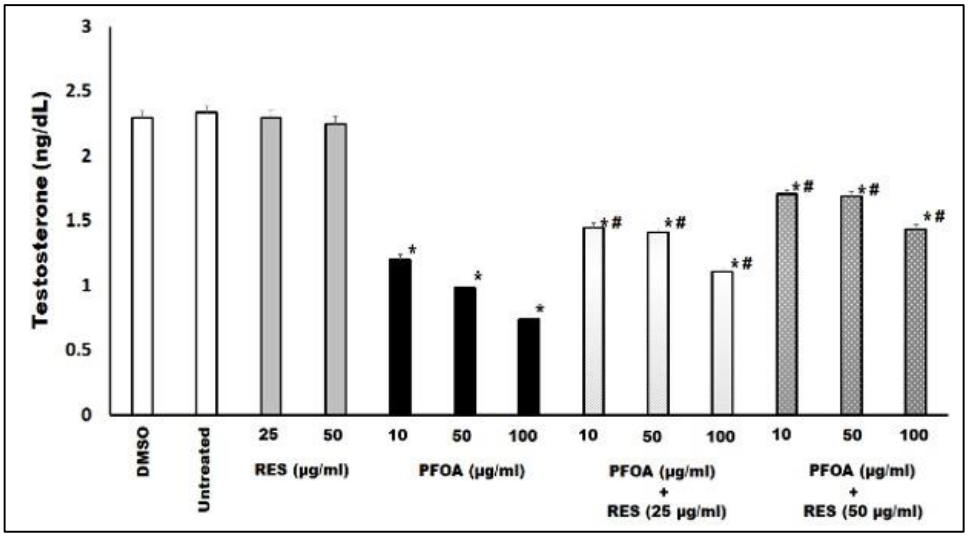

Figure 4: Changes in the levels of testosterone in TM3 Leydig cells from control and experimental groups. Bars represent mean ± S.D. of three individual replicates.
***represents significant over DMSO, Uninduced and resveratrol (RES) treated groups at p<0.001**
#represents significant over perfluorooctanoic acid (PFOA) treated group at p<0.001

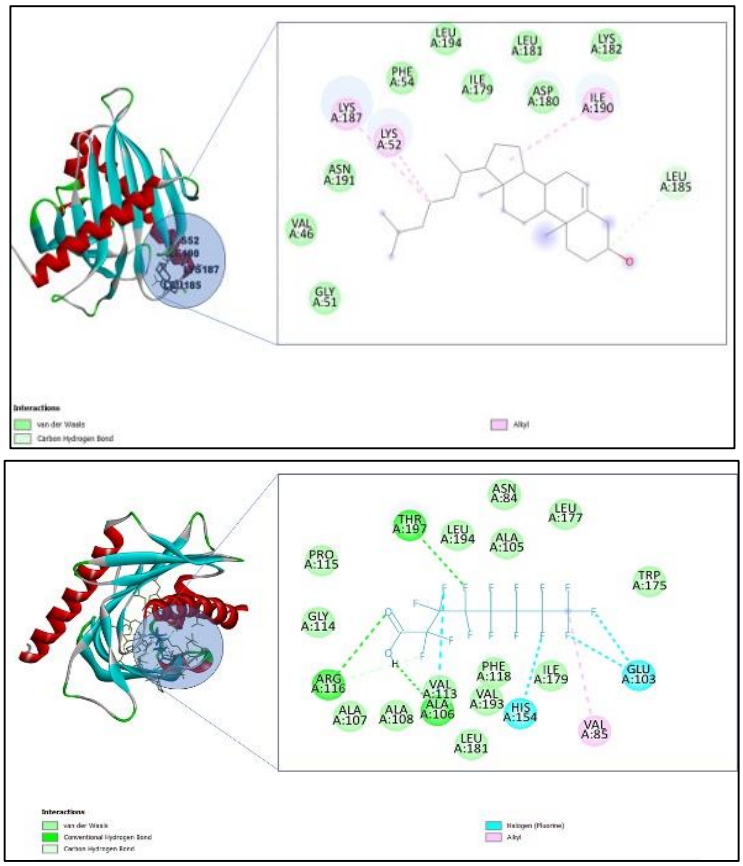

Figure 5: Molecular docking analysis of selected ligands and rat steroidogenic acute regulatory protein (StAR) Upper panel, cholesterol and StAR; Lower panel, Perfluorooctanoic acid and StAR

Table 1
Effect of perfluorooctanoic acid (PFOA) on relative fold change determined by quantitative real time PCR of selected genes in TM3 Leydig cells treated with or without resveratrol (RES)

Groups	Untreated	RES (25 µg/ml)	RES (50 µg/ml)	PFOA (100 µg/ml)	PFOA (100 µg/ml) + RES (25 µg/ml)	PFOA (100 µg/ml) + RES (50 µg/ml)
Genes						
StAR	$1.01^{a} \pm 0.002$	$1.01^{a} \pm 0.004$	$0.97^{a} \pm 0.002$	$-4.34^{b} \pm 0.008$	$-2.41^{c} \pm 0.011$	$-1.74^{d} \pm 0.004$
Caspase3	$1.01^{a} \pm 0.003$	$1.02^{a} \pm 0.003$	$0.98^{a} \pm 0.003$	$5.64^{b} \pm 0.006$	$2.62^{c} \pm 0.009$	$1.81^{d} \pm 0.009$

Values are mean ± S.D. of four individual samples.
All data were normalized with *GAPDH* expression and given as relative to control.
Mean values with different alphabets differ significantly from each other at $p \leq 0.01$.

Table 2
Binding affinities and interacting amino acids between the selected ligands and proteins

Protein	Ligand	Binding affinity (K.cal/mol)	Interacting amino acids	Hydrogen bonds	Alkyl and pi bonds
Rat StAR	Cholesterol	-6.0	Ile 190, Lys 52, Lys 187	-	3
	PFOA	-7.2	**Arg 116, Ala 106 Thr 197** Val 85	3	1

StAR, Steroidogenic acute regulatory protein; AR, Androgen receptor;
PFOA, Perfluorooctanoic acid
Amino acids in bold represent interacting amino acids that form conventional hydrogen bonds with ligands.

Discussion

Testicular steroidogenesis is process through which testosterone biosynthesis occurs in the Leydig cells. Therefore, proper maintenance of Leydig cell population is of prime importance. However, several endocrine disrupting chemicals disrupt testosterone production, but the mechanisms are poorly known. PFOA is one of the endocrine disruptors which is able to interfere with testosterone production[29]. Published reports have shown that the exposure of rats to PFOA deteriorates the testosterone biosynthesis, thereby inhibits spermatogenesis[13,35,37]. To investigate the possible mechanism of PFOA-induced deterioration of testicular steroidogenesis, TM3 Leydig cell lines were selected and they were treated to different concentrations of PFOA.

Therefore, the present study aimed to investigate the underlying mechanisms of PFOA on widely used Leydig cell lines in andrological studies, TM3 (Leydig cell lines derived from 11 – 13d mouse testes) cell lines[36]. The application of TM3 cell lines even extended to predict the effects of environmental toxicants on the Leydig cells[20].

The present findings (via flow cytometric analysis) revealed that the TM3 Leydig cell lines exposed to PFOA showed dose dependent apoptosis in TM3 cell lines. In a previous study, it has been shown that the reduction in the testosterone could be associated with the reduction in the progesterone levels and down regulation of cytochrome P450 cholesterol side-chain cleavage enzyme in cAMP or 22R-hydroxycholesterol stimulated mLTC-1 cells[37].

Though not significant, but studies of Eggert et al[13] have shown that the PFOA treatment provoke a tendency of apoptosis in fetal rat Leydig cells via TUNNEL assays. Further, studies of Zhao et al[38] have shown that the exposure of adult rat Leydig cells to PFOA at concentrations of 17.7 and 53.2 µM inhibited testicular steroidogenic marker enzymes, 3β- and 17β-hydroxysteroid dehydrogenase involved in testosterone biosynthesis. However, our results suggested that the PFOA could mediate apoptosis in the TM3 Leydig cells. These events in turn lead to improper Leydig cell population. We conclude that the PFOA could target a) steroidogenic enzymes of testosterone pathway b) reduced conversion of cholesterol to progesterone c) apoptosis of Leydig cells and d) a, b and c.

StAR protein plays a key role in the transport of cholesterol, a precursor of testosterone across the mitochondrial membranes of Leydig cells. Accordingly, we noticed that the expressions of *StAR* mRNA and *caspase* mRNA in PFOA (100 µg/ml) treated cells were significantly altered. The down regulation of *StAR* mRNA could be suggestive of improper channeling of cholesterol, while the up regulation of *caspase 3 mRNA* could be suggestive of apoptosis of Leydig cells treated with PFOA at higher dose. Molecular docking analysis also revealed that the PFOA occupied the same ligand pocket in StAR protein similar to that of

cholesterol and exhibited three conventional hydrogen bonds Arg 116, Ala 106 and Thr 197, suggesting that PFOA can compete with cholesterol to interact with StAR protein.

Testosterone biosynthesis and spermatogenesis are regulated by testicular cells, the germ cells, Leydig cells and Sertoli cells. The current results indicated that TM3 Leydig cells cultured in the presence of RES at a concentration of 25 µg/ml and 50 µg/ml and PFOA at a concentration of 10 µg/ml, 50 µg/ml and 100µg/ml reduced the number of dead cells as evidenced by flow cytometry analysis. RES-induced antioxidant effects against chemical induced oxidative damage to the Leydig cells and Sertoli cells. RES exhibited cytoprotective role against nicotine[24] and hydrogen peroxide[17] induced Leydig cell damage and PFOS induced Sertoli cell damage[34]. Studies also indicated that treatment of Sertoli cells with RES showed protection against the cytotoxicity-induced by mycotoxin, zearalenone[9]. Sertoli cells and germ cells require adequate energetic balance in terms of lactate metabolism and fatty acid metabolism for normal spermatogenesis. Studies of Gorga et al[16] indicated that the Sertoli cells isolated from 20-day old rats and incubated with RES at concentrations 10 or 50 µM resulted in elevated levels of lactate accompanied by glucose utilization and augmented cytoplasmic fatty acid levels, reflecting possible role of RES in energy metabolism of Sertoli cells. Further, studies of Gorga et al[16] also suggested that RES at concentrations 10 or 50 µM promotes Sertoli cell proliferation.

Conclusion

In summary, the present findings demonstrated that PFOA significantly inhibited proliferation of TM3 cells *in vitro* and induced apoptosis as evidenced by enhanced caspase-3 activity which could be associated with PFOA-induced TM3 cytotoxicity. RES supplementation restored viability of PFOA-treated TM3 cell lines as indicated by percent increase in viable cells associated with reduced activity levels of caspase-3 and the restorative effects could be ascribed to anti-apoptotic property of RES. These events probably lead to the restoration of Leydig cell activity in terms of testosterone biosynthesis in PFOA exposed rats supplemented with RES.

Co-treatment of RES at selected doses (25 or 50 µg/ml) plus PFOA (100 µg/ml) showed significant elevation of *StAR* mRNA and decreased expression of *caspase 3* mRNA levels in TM3 cells associated with increased testosterone levels and reduction in number of dead cells suggesting antiapoptotic and steroidogenic effects of RES.

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