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Screening the estrogenic potency of nonylphenol in rats: *in vivo* and *in silico* approaches

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Abstract

The aim of the present study is evaluation of the effect of intraperitoneal administration of nonylphenol (NP) on sex hormones in male rats and *in silico* analysis for interaction between rat estrogen receptor and nonylphenol. Healthy adult male albino rats were divided into 4 groups with 8 animals each. Animals in first group served as control, second, third and fourth groups were administered with NP at doses of 1, 10 and 100 µg/kg bw every alternative day for 55 days. After the treatment, animals were sacrificed and collected blood and separated the serum. The circulatory levels of testosterone, FSH and LH were estimated in serum. Testosterone was significantly ($p < 0.05$) decreased in a dose dependent manner whereas the levels of FSH and LH were significantly ($p < 0.05$) increased in dose dependent manner when compared with controls. In *in silico* analysis, estradiol showed the strongest binding affinity with -9.62 kcal.mol^{-1} energy with estrogen receptor. NP fit within the binding domain of estrogen receptor, thereby showed the moderate binding capacity with −6.18 kcal mol−1 affinity, and hence it can be regarded as estrogenic effectors. Hence the present study revealed that intraperitoneal administration of NP alters the concentrations of testosterone, FSH and LH; docking studies revealed that NP binds to estrogen receptor.

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1. Introduction

During recent past, evidences have accumulated that exposure to environmental pollutants with estrogenic activity causes reproductive disorders in humans and wildlife (Uguz et al., 2009). It has been claimed that a range of environmental pollutants with different structural formula adversely affect male fertility (Toppari et al., 1996). Although the exact mechanism(s) are not well defined, it is believed that several environmental chemicals at least in part interfere with endocrine mediated steroidogenesis and spermatogenesis thereby affects male fertility. Such chemicals with endocrine disrupting activities are known as endocrine disrupting chemicals (EDCs) (Prins and Birch, 1995; Calafat et al., 2005; Hu et al., 2014). It has also been shown that a range of EDCs exert estrogenic activities and such pollutants are called xenoestrogens. Consequent to structural similarity with estrogens, they bind with the estrogen receptor and mimic the effect of estrogen hormone or interfere with estrogen mediated processes (Roy et al., 2009).

Nonylphenol (NP) is one of the derivative products of alkylphenol polyethoxylate which is

widely used in the production of plastics, textiles, and in household applications such as detergents, paints, pesticides and cosmetics (Nimrod and Benson, 1996). Previously, it has been shown that NP has ability to agonize or mimic endogenous estrogens (Lee et al., 2013) thereby exerts xenoestrogenic effects (Watanabe et al., 2004) which eventually leads to altered reproductive and developmental abnormalities (Naoki et al., 2006). The estrogenic property of NP has been reported in *in vitro* and *in vivo* assay systems (Jobling et al., 1996; Moffat et al., 2001). Several studies reported that exposure to nonylphenol can induce abnormalities in adult male reproductive system in rats (Chitra et al., 2002; Lukacova et al., 2012; Ganga et al., 2014), but some multigenerational studies reported moderate effects of NP on male reproduction (Chapin et al., 1999; Odum et al., 2000; Nagao et al., 2001).

Hormonal regulation of male reproduction in mammals is well established. Gonadotropin releasing hormone (GnRH) secreted by the hypothalamus elicits the release of gonadotropins, FSH and LH from the pituitary gland (De Krester, 1979) which in turn acts on testicular Sertoli cells to stimulate spermatogenesis and Leydig cells to stimulate steroidogenesis. Testosterone is classically known as male hormone, which is critical for proper functions of male reproductive tract. In fact, the structural and functional integrity of reproductive organs require testosterone. FSH and testosterone are able to stimulate all phases of spermatogenesis (O'Dennell et al., 1994). Thus, it seems apparent that disturbances in cascade of hypothalamo-pituitary-testicular axis adversely affect male reproductive functions and questioning the fertility outcome. Several animal studies reported the exposure to endocrine disruptors cause alterations in levels of steroid and gonadotropin hormones, which leads to damage of male reproductive performance (see reviews of Knez, 2013; Kedirvel et al, 2013). Administration of nonylphenol also causes decreased testosterone level in plasma of experimental animals (Han et al., 2004; Wu et al., 2010a & b). However, literature on levels of steroids and gonadotropin hormones by nonylphenol is quite inadequate. In view of this, the present study was undertaken to evaluate the effects of intraperitoneal administration of different doses of nonylphenol at low concentrations on the reproductive hormonal changes in adult male rats. The present study also determines the interaction between rat estrogen receptor and nonylphenol using molecular docking studies to know the estrogenic activity of nonylphenol.

1.2 Objective of Research

To evaluate the estrogenic activity of nonylphenol in adult male rats, through *in vivo* and *in silico* studies.

1.3. Justification of Research

Earlier studies reported that oral exposure of nonylphenol at high concentrations produced various negative effects on male reproductive performance, but literature on intraperitoneal exposure at low dose effects of nonylphenol is quite inadequate on levels of steroids and gonadotropin hormones. There are limited *in silico* studies on interaction between nonylphenol with rat estrogen also. Thus, the present study was undertaken to evaluate the influence of nonylphenol at low doses by intraperitoneal exposure on male rats and assessment of induced alterations in reproductive hormone levels and determination of the binding efficiency of nonylphenol with estrogen receptor of rat using molecular docking studies.

1.4 Research Problem

Elucidation of estrogenic activity of nonylphenol by assessment of levels of reproductive hormones such as testosterone, follicle stimulating and leutinizing hormones in rats through *in vivo* and determination of the binding activity of nonylphenol with estrogen receptor of rat using *in silico* studies.

2. Materials and Methods

2.1 Chemicals

Nonylphenol (CAS NO 46018, purity>98%) was purchased from Sigma-Aldrich, USA. All other reagents were of analytical grade and purchased from local commercial sources.

2.2 Experimental animals

Healthy male rats with 160 ± 2gm body weight were purchased from authorized vendor (M/S Raghavendra Enterprises, Bangalore, India). Animals were housed in polypropylene cages (18" x 10" x 8") lined with sterilized paddy husk, and provided filtered tap water and rat food (purchased from HLL Animal Feed, Bangalore, India) *ad libitum* in an air-conditioned environment (24 ± 2°C) with a 12-hour light and 12-hour dark cycle. All the animals were maintained at the animal facility available at Department of Zoology, Sri Venkateswara University, Tirupati. The experiments were carried out in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India (CPCSEA, 2003).

2.3 Experimental Design
Adult male rats were randomly divided into 4 groups of eight each. Animals in the group I served as control and injected with 100 µl DMSO. Animals in the second, third and fourth groups were administered with 1, 10 and 100 µg/kg bw nonylphenol respectively as i.p. injections, on every alternative day adequate to 55 days.

2.4 Necropsy
The animals were fasted overnight, weighed and killed by cervical dislocation. Blood was collected from the heart using a heparinized syringe from each rat. The serum was separated by centrifugation at 2,000 g for 15 min after overnight storage at 4°C and stored at - 20°C until all of the samples were collected for the hormonal analysis.

2.5 Assay of serum hormone levels
The testosterone, FSH and LH were assayed by CLIA test Kits based on the principle of a Sandwich-CLIA. The micro Sandwich-CLIA plates provided in kits have been pre-coated with an antibody specific to Rat testosterone, FSH and LH respectively. Standards or samples are added to the appropriate micro CLIA plate wells and combined with the specific antibody. Then, a biotinylated detection antibody specific for Rat testosterone, FSH and LH and Horseradish Peroxidase (HRP) conjugate is added to each micro plate well, successively and incubated. The free components are washed away. The substrate solution is added to each well. Only those wells that contain Rat testosterone, FSH and LH respectively, biotinylated detection antibody and HRP conjugate will appear fluorescence. The Relative light unit (RLU) value is measured spectrophotometrically by the Chemiluminescence immunoassay analyzer. The RLU value is positively associated with the concentration of Rat testosterone, FSH and LH. Finally the concentration of Rat serum testosterone, FSH and LH are calculated, in the samples, by comparing the RLU value of the samples to the standard curve.

All the serum samples were stored in a -80°C freezer and had no thaws, prior to assay for hormone levels. Testosterone, FSH and LH were detected using competitive CLIA. Each sample was run in duplicate and 10% of total samples were retested randomly. The intra and inter-assay coefficients of variation were less than 10% for these assays.

2.6 Calculation of specific activity
The mean light intensity (RLU), for each set of controls and treated samples, were recorded. The mean RLU standard curve plotted. RLU values on the Y-axis, and concentrations on the X-axis. The mean RLU values for each group were determined, corresponding to the concentration of Testosterone, FSH and LH in ng/ml from the standard curve.

2.7 Statistical analysis of data
The data were statistically analyzed using one way analysis of variance (ANOVA) followed by Dunnet's multiple comparison test using Student Version 16.0, SPSS Inc., Chertsey, U.K. The data were presented as mean ± SD and differences were considered to be significant at $p < 0.05$.

2.8 Protein preparation
Crystal structure of estrogen receptor beta complex with estradiol of the *Rattus norvegicus* was obtained from the protein data bank (2J7X) (Pike, 2006) and imported into Maestro 9.6. Crystallized water molecules were removed from the complex structure and 2J7X was optimized for docking using the protein preparation and refinement utility provided by Schrödinger LLC. Partial atomic charges were assigned according to the optimized potential liquid simulations for all atoms (OPLS-AA) force field. Minimizations were performed until the average root mean square deviation of the non-hydrogen atoms reached 0.3Å. A grid (10 x 10 x 10 Å) was generated around the centroid of the estradiol ligand within 4 Å.

2.9 Ligand preparation
The nonylphenol and estradiol compounds were obtained from PubChem database (https://pubchem.ncbi.nlm.nih.gov/) and prepared using LigPrep v2.8. LigPrep follows OPLS- AA force fields for energy minimization.

2.10 Computational docking
Extra precision (XP) flexible ligand docking was carried out in Glide of Schrödinger Maestro v9.6 (Friesner et al., 2004) within which penalties were applied to non-cis/trans amide bonds. Vander Waals (vdW) scaling factor and partial charge cutoff was selected to be 0.80 and 0.15, respectively for nonylphenol and estradiol atoms. Final scoring was performed on energy- minimized poses and displayed as Glide score. The best docked pose with lowest XP G score was recorded for both the ligands.

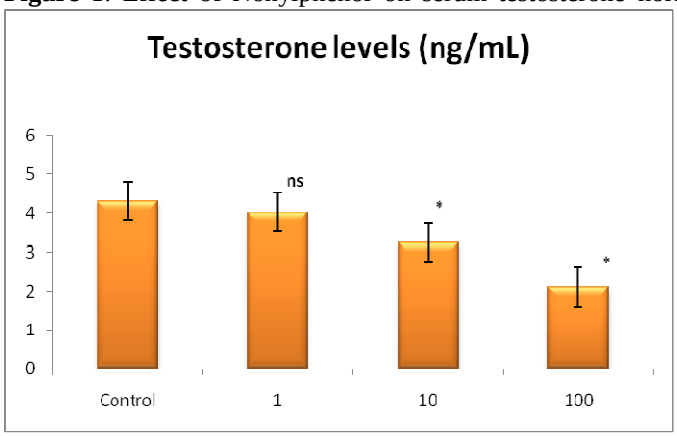
3. Results

3.1 Serum hormone levels
Testosterone

The average circulatory level of the serum testosterone in control rats was 4.31±0.68. The circulatory levels of the serum testosterone in 1,

10 and 100 µg/kg bw nonylphenol exposed rats were 4.02 ± 0.52, 3.25 ± 0.56 and 2.11 ± 0.42, respectively. There was no significant change in 1µg, whereas 10 and 100 µg/kg bw nonylphenol showed a significant ($p<0.05$) decrease in the serum testosterone levels, when compared to the control rats (Figure 1).

Figure 1: Effect of Nonylphenol on serum testosterone hormone levels in adult male rats

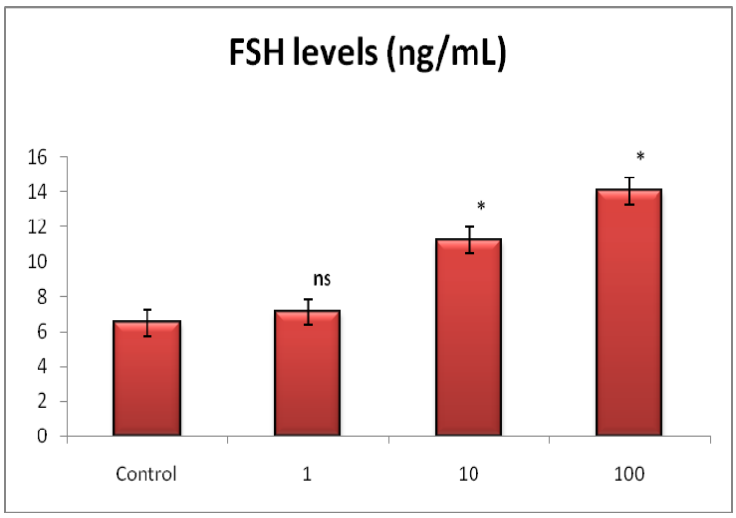


Bars are mean ± SD for 8 animals per group.
Mean values with * are significantly differ from control at $p<0.05$; ns = Not significant

FSH

The mean FSH level in serum of control rats was 9.53±2.61. The mean serum FSH levels in 1, 10 and 100 µg/kg bw nonylphenol exposed rats were 10.31 ±1.04, 14.82 ± 2.21 and 19.41± 1.02, respectively. There was no significant ($p<0.05$) change in 1µ g, whereas 10 and 100 µg/kg BW Nonylphenol showed a significant increase in serum FSH levels, when compared to the control rats (Figure 2).

Figure 2: Effect of Nonylphenol on serum follicle stimulating hormone (FSH) hormone levels in adult male rats.

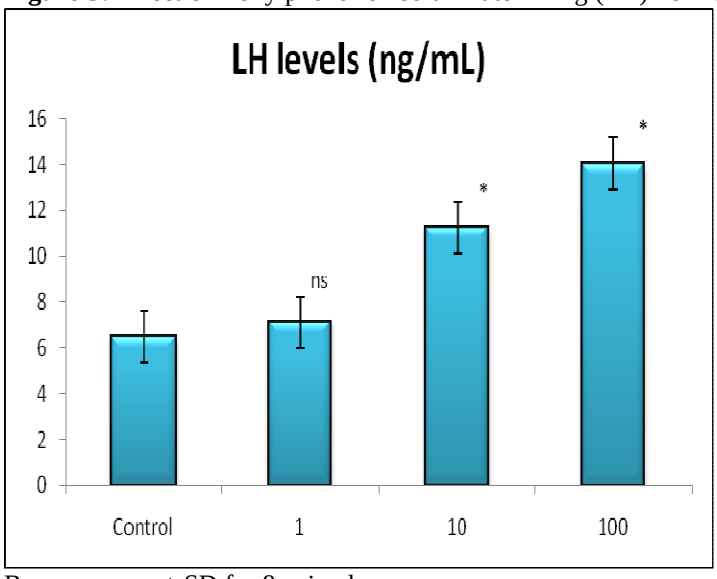


Bars are mean ± SD for 8 animals per group.
Mean values with * are significantly differ from control at $p<0.05$; ns = Not significant

LH

The mean LH level in serum of control rats was 6.55 ± 2.24, whereas the mean levels of LH in serum of 1, 10 and 100 µg/kg bw nonylphenol exposed rats were 7.12 ± 1.07, 11.23 ± 1.41and 14.03 ±1.77, respectively. There was no significant ($p<0.05$) change in 1µg, whereas 10 and 100 µg/kg bw nonylphenol exposure have a significant increase in serum LH levels, when compared to that of the control rats (Figure 3).

Figure 3: Effect of Nonylphenol on serum luteinizing (LH) hormone levels in adult male rats.



Bars are mean ± SD for 8 animals per group.
Mean values with *are significantly differ from control at p<0.05; ns = Not significant

3.2 Structural analysis of rat ER LBD

In the present study, *in silico* docking studies were performed using the crystal structure of estrogen receptor beta complex with estradiol (2J7X), which is believed to be the endogenous ligand for estrogen by using Schrodinger Maestro 9.6 Nonylphenol and estradiol interacts with estrogen receptor at the same binding site while nonylphenol has lower binding affinity than estradiol (Figure 4A & B). The estradiol showed lowest XP G score and good binding interactions with the 2J7X when compared to nonylphenol with respective to experimental binding site. The endogenous estradiol showed the strongest binding to ER with affinity energy of -9.62 kcal mol^{-1}. The nonylphenol exhibited moderate binding affinities to ER with-6.18kcal mol^{-1}. Estradiol formed two hydrogen bonds with His430, Thr254 with 3.08, 3.11A^{o}bond lengths respectively (Table: 1); π- π interaction with Phe311, Glu260 and Arg301 and good vander Waals interactions with Met250, Leu253, Leu256, Ala257, Met291, Met295, Leu298, Ile328, Ile331, Phe332, Leu294, Gly427, Leu431 and Met434 (Figure 5A). In docking interactions nonylphenol also formed in two hydrogen bonds with His430, Thr254 with 3.68, 3.15A^{o} bond lengths respectively (Table 1); π- π interaction with Phe311, Glu260 and Arg301 and good vander Waals contact with residues of Met250, Leu253, Leu256, Ala257, Trp290, Met291, Leu294, Met295, Leu298, Leu309, Ile328, Ile331, Phe332, Leu335, Gly427, Leu431 and Leu446 (Figure 5B). The binding site residue, Phe311 of estrogen receptor was involved in π- π interaction with both estradiol and nonylphenol. Similarly, residues present around 4Å region of binding site such as Met250, Leu253, Thr254, Leu256, Ala257, Met291, Met295, Leu298, Arg301, Ile328, Ile331, Phe332, Gly427, His430 and Leu431 were observed to be involved in good vander Waals contacts with both the ligands.

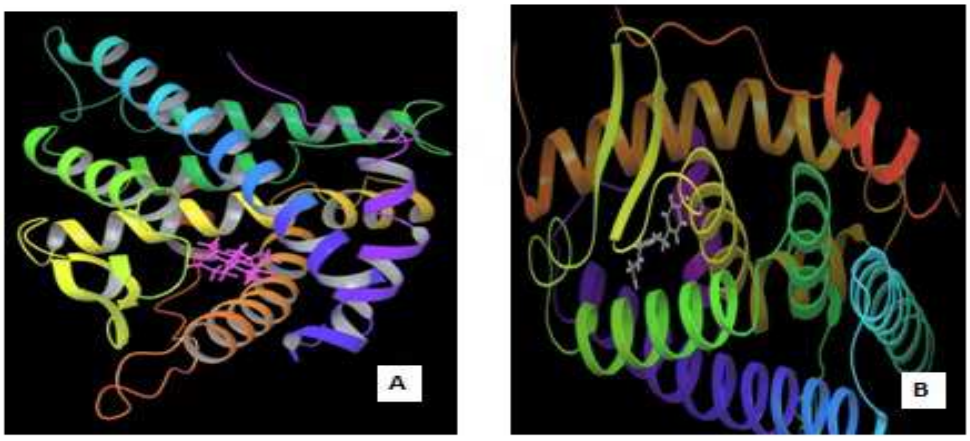
Table 1: Docking energies and number of hydrogen (H) bonds with their ligands docked against ER

Receptor- Ligand	Number of H- bonds	Amino acids involved in H-bonding	Bond distance(A^{0})	Docking affinity (kcal/mol)
ER-Estradiol	2	1.His-430----------3.08 2.Thr-254----------3.11		-9.62
ER-Nonylphenol	2	1.His-430----------3.68 2.Thr-254----------3.15		-6.18

Figure 4: Docking of Estradiol and Nonylphenol binding to Estrogen receptor. Position of Estradiol and 4-nonylphenol

A. Molecular docking of ER with estradiol

B. Molecular docking of ER with nonylphenol



4. Discussion

Male reproductive abnormalities are increasing day- by-day at an alarming rate. These reproductive disorders are at least in part attributed to endocrine disruptors which have ability to inhibit the action of hormone by blocking hormone receptors or mimicking as endogenous hormones and/or both. Finally, perturbs the normal functioning of the endocrine system and eventually causes adverse effects on hormone-dependent functions including reproduction system (Carlsen et al., 1992). Among a range of endocrine disruptors, nonylphenol can induce alterations in spermatogenesis and hormonal system of many organisms (Lukacova et al., 2012; Hu et al., 2014). Many studies attributed that the chemical structure of nonylphenol having phenolic component which enables NP to agonize/mimic endogenous estrogens (Lee et al., 2013) resulting in induced reproductive and developmental abnormalities (Naoki et al., 2006; Ganga et al., 2014). Moreover, nonylphenol can accumulate to high concentrations in lipids and membranes from which they can be slowly released to provide a low, persistent level of compound in blood. This clearly indicates the lipophilic nature of NP allows in stimulating certain estrogenic-like responses (Katzenellenbogen, 1995).

Testosterone, FSH and LH are prime regulators of germ cell development. The quantitative production of spermatozoa generally requires the presence of testosterone, FSH and LH. FSH acts directly on the seminiferous tubules and plays a key role at initiation of spermatogenesis and maturation of spermatozoa (Anderson et al., 1997) and LH stimulates spermatogenesis indirectly via testosterone. Abnormal spermatogenesis is often associated with altered serum gonadotropins and testosterone. The results of present study indicated that exposure of rats to nonylphenol significantly decreases the serum testosterone levels. The results are in agreement with earlier findings; Han et al. (2004) reported that oral treatment with high concentrations of nonylphenol (250 mg/kg/day for 50 days) caused significant decrease in the concentration of testosterone in plasma. Moreover, it has also been shown that, intravenous administration of nonylphenol at 100 µg/kg bw to mice impairs leydig cells and inhibits hCG- induced testosterone release and thereby disturbs the homeostasis of the reproductive hormones (Wu et al., 2010a&b). The reduction in the serum level of testosterone could probably be due to the toxic effect of nonylphenol on testicular steroidogenic machinery and also could be attributed to its endocrine disrupting property.

The data also revealed elevated levels of FSH and LH in rats exposed to nonylphenol. The increase in serum LH levels could be due to diminished responsiveness of Leydig cells to synthesize LH and/or direct inhibition of estrogen synthesis in rats exposed to nonylphenol. The increased levels of serum FSH levels observed in the present study might indicate an impairment of spermatogenesis in experimental rats and reflects the germ cell loss or damage to Sertoli cells, thereby affecting the feedback regulation of FSH secretion (Van Thiel et al., 1972). All the testicular cells are regulated by testosterone, FSH and LH and their cross-talks are crucial for normal functioning of steroidogenesis and spermatogenesis (Chimento et al.,2014). In the present study lowered levels of serum testosterone

with elevated levels of FSH and LH in experimental rats also indicate compromised estrogen production (Weniger, 1988, 1990; Pelliniemi et al., 1993; Tsai-Morris et al., 1986; Atanassova et al., 2001). Earlier studies used *in vivo* and *in vitro* assays for evaluation of the estrogenic potential of different endocrine disruptors.

In previous studies *in vitro* methods such as MVLN assay, E-screen and YES-assays were used to screen estrogenic potency of nonylphenol and its isomers. Whereas in the present study Glide (grid-based ligand docking with energetics) docking program was used to screen estrogenic activity of nonylphenol. Consequent to structural similarities, nonylphenol showed binding activity and interactions with estrogen receptor. The endogenous estradiol showed the strongest binding to ER with affinity energy of -9.62 kcal mol^{-1} whereas nonylphenol exhibited moderate binding affinities to ER with -6.18 kcal mol^{-1}, at the same binding position. Earlier studies reported that nonylphenol exhibits less potency of binding than 17β-estradiol with estrogen receptor (Lutz and Kloas, 1999; White et al., 1994). Though its biological activity is weak, but the potential estrogenic effect leads to an adverse impact on male fertility (Chapin et al., 1999; Lee et al., 1999; Kerdivel et al., 2013). In the present study also administration of nonylphenol showed changes in the levels of reproductive hormones. The alterations in hormonal levels in the present study may be nonylphenol mimicking action of estrogen or it may interfere with estrogen pathway. Though, *in silico* studies provides clear evidence that NP binds estrogen receptor and shares similar binding sites as compared to natural ligand, estradiol, further, cell-based studies are required to authenticate its mode of action, because, steroid based hormones including estrogens exerts their actions in a non-genomic pattern thereby triggers signal transduction mechanisms.

Research Highlights

Intraperitoneal administration of nonylphenol at low doses decreased serum testosterone and increased FSH and LH in rats.
NP binds with estrogen receptor of rat similar to estradiol with moderate energy as revealed by *in silico* studies.

Limitations

Literature on nonylphenol administration through intraperitoneal at low doses on reproductive hormonal changes and interaction of nonylphenol with rat estrogen receptor is limited. Hence the present study was aimed to further explore the reproductive toxicity of nonylphenol and to complement the existing research data using rat model.

Recommendations

In view of the fact that nonylphenol may mimic like estrogen or interfere with estrogen pathway, long time exposure to nonylphenol at low doses suppresses reproduction in adult rats, it is hereby advised to not to use continuously alkylphenol such as nonylphenol.

Competing Interests

Authors declare that no competing interest exists in the publication of this manuscript.

Authors' Contribution and Competing Interests

PSR, BK, AU and SBS conceived the idea, participated in its design, supervised the work, evaluated the data and coordinated the study. UKG and MM also participated in designing the study, performed the hormone analysis and docking. All the authors drafted the manuscript for publication and authors read and approved the final manuscript. The authors declare that they have no competing interests.

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