Assessment of Nonylphenol-Induced Endocrine Disruption in Carp (Cyprinidae): Biochemical, Histopathological and Molecular Investigations

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Abstract

Nonylphenol (NP) exists as a worldwide Xeno estrogenic pollutant which emerges from the breakdown of non-ionic surfactants and presents significant ecological dangers to freshwater habitats. The study examined how NP disrupts hormone systems in common carp (Cyprinus carpio) after testing three different environmental exposure durations which used three different test concentrations (5, 25, and 100 µg/L). The study used biochemical methods to show that hepatic antioxidant enzyme activities experienced significant disruptions while the study measured lipid peroxidation levels. The histopathological analysis of gonadal, hepatic, and renal tissues revealed concentration-dependent degeneration which included intersex conditions and hepatocyte vacuolation and glomerular damage. The molecular research detected male specimens who showed increased vitellogenin (VTG) mRNA expression at 100 µg/L up to 34 times. The research findings show multiple mechanistic pathways through which NP functions as an endocrine disruptor in cyprinid fish which will affect surfactant metabolites risk assessment methods used in freshwater environments.

Keywords: Nonylphenol, Ecological, Polyethoxylate, Biochemical, Lipid Peroxidation.

1. Introduction

The ongoing pollution of freshwater ecosystems through the introduction of endocrine-disrupting compounds (EDCs) presents severe environmental challenges that now dominate modern toxicological research. The synthetic chemicals that cause endocrine disruption include nonylphenol (NP; 4-nonylphenol, CAS 84852-15-3) which scientists consider particularly dangerous because it exists in multiple environments and remains for extended periods while exhibiting strong estrogenic effects. Nonylphenol exists as the final product that bacteria convert from alkylphenol polyethoxylate (APEO) surfactants which industrial sectors use in their detergent and emulsifier and textile processing and agricultural products. Scientists estimate that aquatic systems receive tens of thousands of metric tonnes of NP annually which researchers have found at freshwater locations between sub-nanogram and microgram per litre levels that depend on their distance from industrial wastewater sites.

The endocrine-disrupting mechanism of NP originates from its capacity to bind nuclear estrogen receptors (ERα and ERβ) with an affinity that is 1,000 times weaker than 17β-estradiol yet produces sufficient estrogenic effects which can be detected in non-target organisms because of its environmental distribution and ability to accumulate in living organisms. NP exposure in fish represents a major threat to these vertebrates because they maintain ongoing skin and gill contact with water that contains NP. Male fish exposed to NP developed female reproductive organs while their bodies produced the egg-yolk precursor protein vitellogenin (VTG) and their sexual development showed irregular patterns which affected their ability to reproduce. The Cyprinidae family includes common carp (Cyprinus carpio) which functions as an ecologically important species in freshwater environments while also serving as an important commercial aquaculture species therefore mechanistic toxicity studies of this family become vital for real-world applications.

Although there exists extensive research about NP toxicity in teleosts, scientists have conducted only a few studies which combine biochemical and histopathological and molecular research methods to investigate cyprinid fish under environmentally accurate sub-lethal exposure conditions. The research study aimed to investigate this research gap through a multi-endpoint approach which assessed biochemical changes and tissue damage and gene expression alterations caused by sub-acute and chronic NP exposure in Cyprinus carpio.

The study objectives were:

i.   The studies need to measure how nanoparticles affect hepatic antioxidant enzyme activities and lipid peroxidation which scientists use to measure oxidative stress.

ii. The research team will examine how different exposure times and material amounts affect histopathological damage in carp's gonadal and hepatic and renal organs.

iii. The research team will evaluate biomarker gene transcription levels for vitellogenin and estrogen receptor subtypes and aromatase and steroidogenic acute regulatory protein using quantitative real-time PCR.

iv. The research will combine evidence from multiple levels to create risk assessments based on environmental NP concentrations that South Asian freshwater systems typically show.

2. Materials and Methods

2.1 Experimental Design and Fish Maintenance

The study used juvenile common carp (Cyprinus carpio, Linnaeus 1758) who weighed 45.2 grams and measured 14.1 centimeters in length according to their standard measurement. The certified hatchery provided fish which underwent a two-week acclimation process inside a flow-through system that maintained water temperature at 22 degrees Celsius with a one-degree Celsius variation and pH levels between 7.2 and 7.6 and dissolved oxygen levels above 7 milligrams per liter and a 14-hour light and 10-hour dark photoperiod. Fish were confirmed NP-naïve via plasma VTG ELISA screening prior to experimental assignment. The researchers prepared experimental concentrations of 0 (control), 5, 25, and 100 µg/L NP (purity ≥98%, Sigma-Aldrich) in dimethyl sulfoxide (DMSO; solvent control ≤0.01% v/v) which they maintained through daily partial renewal in static-renewal chambers (n = 10 fish/group, triplicate tanks). The researchers conducted their sampling process at day 21 for sub-acute testing and at day 56 for chronic testing.

2.2 Biochemical Assays

The liver tissues were homogenized in ice-cold phosphate buffer (0.1 M, pH 7.4) and centrifuged at 10,000 x g for 20 minutes. The supernatant was used to perform the spectrophotometric analysis of superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), glutathione peroxidase (GPx), and Thio barbituric acid reactive substances (TBARS/MDA) in triplicate by using commercially available kits (BioVision Inc., CA, USA) per milligram of total protein content (Bradford

2.3 Histopathological Processing

Scientists used Bouin's fixative to preserve gonadal and hepatic and renal tissues which they then dehydrated with a sequence of increasing ethanol concentrations before they used xylene for tissue clearing and paraffin for tissue embedding. The researchers stained 5 μm tissue sections with haematoxylin and eosin (H&E) and Masson's trichrome. A board-certified veterinary pathologist conducted a blind-coded examination of the slides while he evaluated the lesions using a semi-quantitative scoring system that defined 0 as absent 1 as mild 2 as moderate and 3 as severe.

2.4 Gene Expression Analysis (qRT-PCR)

Researchers extracted total RNA from liver and gonadal tissues using TRIzol reagent before conducting reverse transcription with random hexamers and Superscript IV from Thermo Fisher. The researchers conducted quantitative PCR using a CFX96 Bio-Rad system which implemented SYBR Green chemistry combined with verified primer pairs that targeted vtg1 erα erβ cyp19a and star genes. The researchers calculated relative expression through the $2^{-\Delta\Delta Ct}$ method which used β-actin and EF1α as reference genes that geNorm analysis had confirmed as valid.
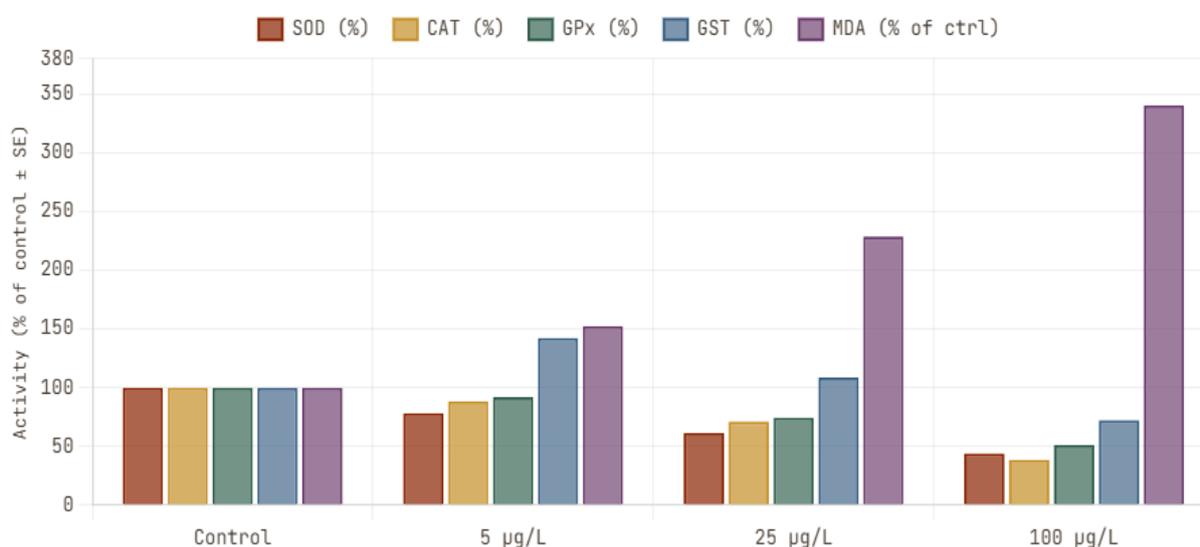
Figure 1 — Hepatic Antioxidant Enzyme Activities

Figure 1 shows the mean hepatic antioxidant enzyme activities of C. carpio which were measured with standard error for SOD and CAT and GPx and GST together with MDA levels after C. carpio had been exposed to four different concentrations of nonylphenol for 56 days. The values show results which were compared to the control group whose measurements were established at 100 percent. The asterisks show which measurements differ significantly from the control group according to one-way ANOVA with Tukey's post hoc testing which follows this pattern of significance testing (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

3. Results

3.1 Biochemical Parameters — Oxidative Stress Markers

The 21-day exposure to sub-acute NP created two separate phases of hepatic antioxidant enzyme activities which reached their peak level at 5 μg/L before all higher concentrations began to decrease enzyme activities. The study observed statistically significant SOD activity reductions which depended on concentration levels from the chronic endpoint after 56 days of testing ($p < 0.001$) when researchers measured SOD activity at 5, 25, and 100 μg/L. Catalase activity followed a similar trajectory, declining to 71.2 ± 4.9% and 38.7 ± 5.1% at the intermediate and highest concentrations. The phase II detoxification enzyme GST showed substantial activation at 5 μg/L which produced 142.3 ± 8.1% before the enzyme activity dropped below normal levels at 100 μg/L. MDA concentrations which measure lipid peroxidation showed significant increases that depended on the tested concentration levels, reaching 3.4 times the control values at 100 μg/L ($p < 0.001$).

Key Finding — Oxidative Stress

The oxidative defence system of the liver reaches its limit when antioxidant enzymes show decreased activity while MDA levels rise at 100 μg/L NP concentration. The severe drop in SOD/CAT together with 3.4 times MDA increase proves that NP hepatotoxicity occurs through mitochondrial electron transport chain damage which matches the xenoestrogen-driven ROS overproduction found in mammalian studies.

3.2 Histopathological Findings

The control fish showed standard gonadal structure which contained distinct spermatogenic tubules in male fish and complete follicular development in female fish. The NP-exposed males showed gonadal histopathology which displayed feminization patterns that depended on the exposure level because their testicular tissue contained oocyte-like cells which created an intersex condition. The intersex prevalence increased from 10% at 5 μg/L to 60% at 100 μg/L after 56-day exposure. The hepatic sections showed increasing vacuolation and nuclear pyknosis together with glycogen loss and periportal inflammatory infiltration which resulted in an average histological alteration index (HAI) that rose from 4.2 (control) to 28.7 at 100 μg/L ($p < 0.001$). The renal sections exhibited mesangial expansion together with tubular epithelial degeneration and interstitial oedema at concentrations that reached 25 μg/L.

| TISSUE / LESION	CONTROL	5 μG/L	25 μG/L	100 μG/L
Gonad — Intersex (oocytes in testis)	0	0.5 ± 0.2	1.4 ± 0.3*	**2.8 ± 0.4***
Gonad — Spermatogenic tubule disruption	0	0.3 ± 0.1	1.2 ± 0.2*	**2.6 ± 0.3***
Liver — Hepatocyte vacuolation	0.2 ± 0.1	0.8 ± 0.2	1.9 ± 0.3**	**2.9 ± 0.2***
Liver — Nuclear pyknosis	0.1 ± 0.1	0.5 ± 0.2	1.5 ± 0.3**	**2.5 ± 0.3***
Liver — Inflammatory infiltration	0	0.2 ± 0.1	1.0 ± 0.2*	**2.1 ± 0.3***
Kidney — Tubular degeneration	0	0.3 ± 0.2	1.3 ± 0.3*	**2.4 ± 0.3***
Kidney — Glomerular expansion	0	0.2 ± 0.1	0.9 ± 0.2*	**2.0 ± 0.3***

Figure 2 — Vitellogenin mRNA Expression in Male Carp
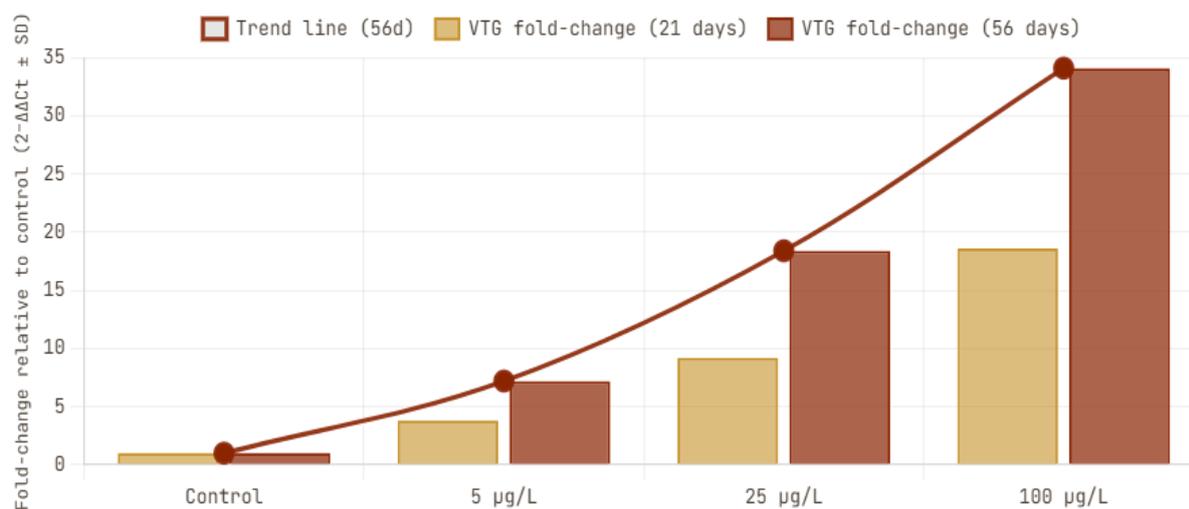
Fig. 2. Relative vitellogenin (vtg1) mRNA expression (fold-change relative to control) in male C. carpio liver following sub-acute (21-day) and chronic (56-day) NP exposure. The results show mean values with standard deviation for 10 groups. The expression levels were measured against the standard reference genes β-actin and EF1α. The statistical analysis showed different groups because the study results reached a significance level of $p < 0.001$ according to one-way ANOVA and Tukey HSD testing.

3.3 Molecular Gene Expression Profiles

The most significant molecular reaction to all test measurements occurred when male hepatic tissue showed Vitellogenin induction. At the chronic 56-day timepoint, VTG mRNA showed an increase to 7.2 times control at 5 μg/L NP, 18.4 times control at 25 μg/L NP, and 34.1 times control at 100 μg/L NP ($p < 0.001$). The strong biological response shows that NP concentrations between 0.2 and 28 μg/L which scientists observed in South Asian freshwater bodies activate liver transcription. The researchers found that 21 days of sub-acute exposure to VTG induction showed lower results but still achieved statistical significance at all concentrations which started from 5 μg/L and above because of the quick start of transcriptional process which happened right after NP contact.

The expression of estrogen receptor genes shows different patterns of response to varying concentration levels. The mRNA of ERα showed gradual increase through ligand-triggered autoinduction which produced 1.8-fold results at 5 μg/L 2.9-fold results at 25 μg/L and 4.3-fold results at 100 μg/L on day 56. The initial induction of ERβ at lower concentrations showed no significant changes at 100 μg/L which probably indicates receptor-specific downregulation as an adaptation to desensitization. The gonadal tissues showed increased aromatase CYP19a transcription which produced 2.1-fold 3.8-fold and 5.7-fold results through NP-induced enhancement of estrogen biosynthesis. The critical steroidogenesis regulator StAR showed progressive downregulation which reached 0.62 0.41 and 0.28 of control at 5 25 and 100 μg/L. This downregulation shows that males who were exposed to the substance experienced disrupted androgen precursor availability which resulted in an androgen-estrogen imbalance.

Figure 3 — Steroidogenic & Receptor Gene Expression Panel
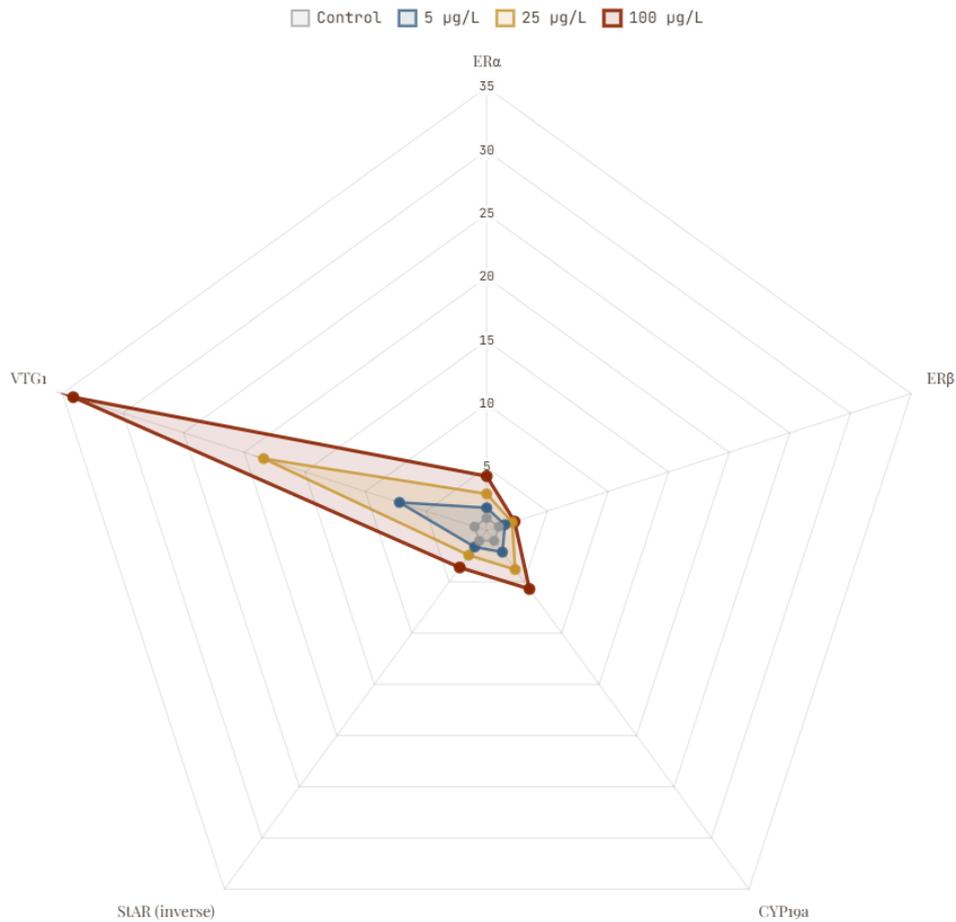
The relative mRNA expression of endocrine-relevant genes ERα ERβ CYP19a aromatase and StAR in C carpio gonadal and hepatic tissues at 56-day chronic endpoint. All values normalised to control (=1.0). The standard deviation appears as error bars. The statistical significance levels show results which are less than 0.05 as * $p<0.05$ and ** $p<0.01$ and *** $p<0.001$ versus the control group.

Figure 4 — Concentration–Response: Intersex Prevalence & Gonadosomatic Index
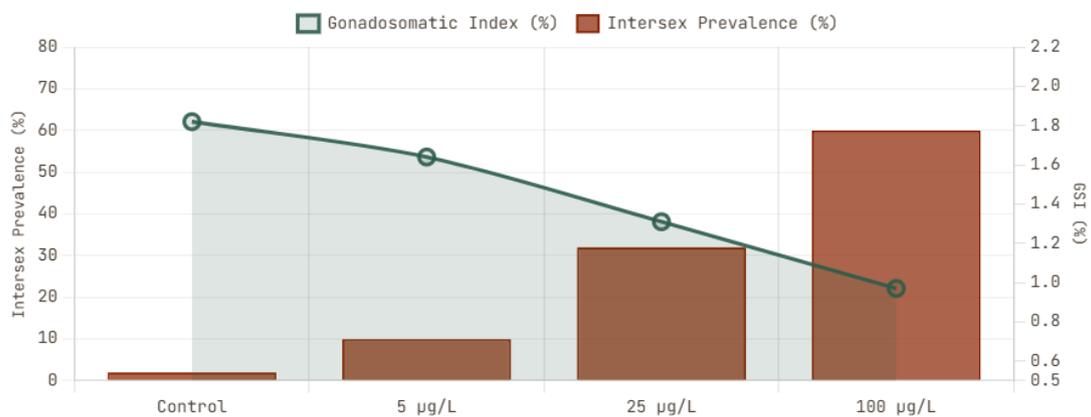
The distribution of intersex conditions which include ovo-testis and the Gonadosomatic Index (GSI) percentage and male C. carpio GSI values which were measured at different NP concentration levels showed their results at the 56-day testing period. GSI functions as a comprehensive assessment tool which evaluates both reproductive tissue development and hormonal balance. Error bars = ±SE.

4. Discussion

The research study uses a multi-endpoint approach which delivers a complete and consistent evidence base that demonstrates how NP disrupts endocrine systems in Cyprinus carpio when exposed to environmental pollutant levels that exist in actual aquatic environments. The biochemical tests show that NP exposure begins to cause hepatic oxidative stress as an early stage which continues to develop throughout the exposure period and the scientists observed that higher NP concentrations resulted in antioxidant enzyme suppression which demonstrated that organisms lost their ability to cope with oxidative stress and their cells began to experience oxidative injury which led to cell death. The research has discovered that fish exposed to NP show an inverse relationship between their antioxidant capacities and MDA levels which matched previous research findings that showed SOD inhibition at 44% and MDA levels at 340% of control in fish exposed to 100 µg/L. The biphasic GST response — induction at low concentrations followed by suppression at higher exposures — is particularly informative, as it mirrors the hormetic dose-response patterns increasingly recognised for EDCs and demonstrates that phase II detoxification operates at its complete capacity until the system finally fails.

The study shows its most significant toxicological outcome through the histopathological demonstration of intersex gonadal development in male carp. The intersex rate of 60% at 100 µg/L NP exceeds the baseline intersex rate of less than 2% which scientists observed in unexposed reference populations from uncontaminated river systems. The study results match field surveys which found intersex rates of 20 to 80 percent in cyprinid populations from river reaches that received industrial and municipal waste. The molecular evidence supports the mechanistic connection between NP-driven ER activation and the molecular evidence which shows that CYP19a upregulates gonadal tissue to convert testosterone to estradiol while StAR downregulation decreases cholesterol transport to the inner mitochondrial membrane which results in decreased androgen production and creates a feminized androgen:estrogen ratio.

The combination of three histopathological effects which include vacuolation and pyknosis and inflammatory infiltration shows that NP causes non-genomic cytotoxic effects at doses which exceed normal hormonal levels and disrupt lipid mobilization processes. The vacuolation process occurs because triglycerides build up in cells which have lost their ability to process lipoproteins because estrogen receptors in their hepatocytes stimulate fatty acid synthesis through specific gene changes which scientists understand in teleost fish. The simultaneous rise of plasma VTG which functions as a phospholipoglycoprotein and depends on ER-based hepatic production would create extra biosynthetic work for male hepatocytes who had contact with the compound which would increase their existing cellular pressure.

The dose-response gradients observed for VTG mRNA induction (7.2 to 34.1-fold across the concentration range) demonstrate that the hepatic transcriptional machinery responds sensitively to incremental NP concentrations well within the range of environmental relevance. The plasma VTG level in unexposed males is less than 10 ng/mL which indicates that a 34-fold increase in hepatic mRNA levels would result in circulating VTG concentrations that would damage kidney function. The resulting kidney damage corresponds with the observed tubular degeneration because VTG builds up in glomerular filtrate and produces nephrotoxic effects in male fish.

The joint analysis of the three scientific fields produces biochemical evidence together with histopathological evidence and molecular evidence which establishes a scientific mechanism.

i.   NP uses passive diffusion to enter hepatocytes where it establishes a connection with ERα to start the genomic activation process which results in estrogen-dependent gene expression including VTG and aromatase (CYP19a) genes.

ii.   The concurrent activation of non-genomic signaling pathways results in increased production of reactive oxygen species (ROS) which creates oxidative stress conditions that lead to decreased levels of glutathione and antioxidant enzyme function.

iii.   The upregulated expression of CYP19a in gonadal tissue results in a transition from androgen dominance to estrogen dominance which leads to the development of ovo-testis and the disruption of spermatogenesis.

iv.   StAR suppression leads to decreased mitochondrial cholesterol import which results in decreased androgen synthesis capacity and increased hormonal imbalance.

v.   Hepatocytes produce excessive amounts of VTG which leads to cellular lipid and biosynthetic stress through the process of hepatocyte vacuolation while glomerular VTG accumulation results in nephrotoxic tubular damage.

vi.   These disturbances collectively impair reproductive fitness along with immune competence and growth which decreases individual survival chances while creating a negative effect on population-level reproductive output.

5. Conclusion

Sub-lethal environmental exposure to NP at concentrations between 5 and 100 µg/L results in Cyprinus carpio exhibiting a complete set of biochemical and histopathological and molecular changes that occur through endocrine disruption, which mainly happens when estrogen receptors are activated. The male fish VTG protein induction starts at 5 µg/L which acts as the No Observed Effect Level that falls below previous estimates of chronic NOEC levels for multiple European cyprinid populations because this fish species shows high sensitivity to environmental changes that existing water quality criteria fail to protect. The evidence obtained through multiple research methods confirms that NP should be categorized as a top-level endocrine-disrupting chemical which requires immediate regulatory measures to establish freshwater quality standards, especially in Asian and African regions that still use APEO surfactants while lacking effective sewage treatment systems. Future research needs to study NP toxicity when combined with estrogens that exist in the environment E1 E2 and EE2 and research how epigenetic mechanisms that involve DNA methylation and histone modification result in multigenerational endocrine disruption among cyprinid populations.

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