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Varsha B

MVSc Graduate, Department
 of Veterinary Pharmacology
 and Toxicology, College of
 Veterinary and Animal
 Sciences, Kerala Veterinary
 and Animal Sciences
 University, Kerala, India

Nisha AR

Professor and Head,
 Department of Veterinary
 Pharmacology and Toxicology,
 College of Veterinary and
 Animal Sciences, Kerala
 Veterinary and Animal
 Sciences University, Kerala,
 India

Corresponding Author:**Nisha AR**

Professor and Head,
 Department of Veterinary
 Pharmacology and Toxicology,
 College of Veterinary and
 Animal Sciences, Kerala
 Veterinary and Animal
 Sciences University, Kerala,
 India

Squalene nanoparticles mitigate PFOA hepatotoxicity: Histopathological insights

Varsha B and Nisha AR

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Abstract

Perfluorooctanoic acid (PFOA), a persistent environmental pollutant, triggers hepatotoxicity via oxidative stress and antioxidant enzyme dysregulation. This study assessed the protective effects of squalene nanoparticles (SQ-NPs) against PFOA-induced liver damage in Wistar albino rats, benchmarked against N-acetyl cysteine (NAC). Thirty Wistar albino rats were allocated to six groups: control, PFOA (5 mg/kg, oral, 28 days), PFOA + NAC (50 mg/kg), PFOA + SQ (25 mg/kg), PFOA + low-dose SQ-NPs (25 mg/kg), and PFOA + high-dose SQ-NPs (25 mg/kg). PFOA significantly raised hepatic superoxide dismutase (SOD) and catalase levels, indicative of oxidative stress. SQ-NPs co-administration normalized SOD, and catalase activities to near-control levels, similar to NAC, as corroborated by histopathological evaluations revealing reduced necrosis, inflammation, and fibrosis. These results position SQ-NPs as a promising nanotherapeutic for mitigating PFOA hepatotoxicity, meriting advanced preclinical and clinical investigations.

Keywords: PFOA (Perfluorooctanoic acid), NAC (N-acetyl cysteine), SQ (squalene), SQNP (squalene nanoparticles), SOD (superoxide dismutase), CAT (Catalase)

1. Introduction

Per- and polyfluoroalkyl substances (PFAS) comprise a vast group of thousands of compounds [1, 2] manufactured since the 1940s for diverse consumer products and industrial uses [3]. The terminal transition products of PFAS are extremely persistent in the environment because of the C-F linkages in PFAS, which also produce very stable compounds. Moreover, many PFAS are efficient surfactants or surface protectors because their perfluorocarbon moieties are both hydrophobic and oleophobic [4]. The surface tension of water can be reduced by PFAS-based fluorosurfactants from around 72 mN m⁻¹ to less than 16 mN m⁻¹, which is half of what hydrocarbon surfactants can achieve [4, 5]. The thermal and chemical stability of PFAS compounds, as well as the various ways in which humans and biota may be exposed throughout their lives, are the reasons behind the growing worry for both human health and wildlife ecology [6, 7, 8, 9]. The chemical stability of PFAS, which results in environmental persistence [10], their high mobility, which gives them a long-range transport potential [11], causing their pervasive spreading into remote regions (e.g., the Arctic's or Antarctic's) and their propensity to bioaccumulate and biomagnify in biota through the contamination of food chains [15, 16, 17, 18, 19] are common characteristics of PFAS. A number of detrimental health effects have been linked to the presence of certain PFAS in the blood [20, 21], milk [22, 23], urine [24], tissues [25, 26, 27], and organs [28, 29, 30, 31, 32] of various human populations residing in developed nations. Prototypes of PFAS include perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA), which have strong surfactant activity, chemical inertness, and remarkable stability against environmental and metabolic degradation [3, 33]. The primary organ impacted when animals are exposed to large oral dosages of PFOS or PFOA is the liver. Several investigations have demonstrated that short-term or sub-chronic exposure to relatively high concentrations of PFOS or PFOA may decrease several components of adaptive immunity in addition to these liver effects [34, 35]. Environmental contaminants can cause liver damage primarily through oxidative stress [36] and mitochondrial dysfunction [37]. Actually, xenobiotics cause mitochondrial malfunction, which results in an overabundance of reactive oxygen species (ROS), a reduction in the potential of the mitochondrial membrane, and ultimately apoptosis [38].

According to recent research, PFOA damages cells and causes mitochondrial defects^[39].

Squalene (SQ), an unsaturated triterpene, is a crucial part of hormones, cholesterol, and fat-soluble vitamins. Japanese researchers separated SQ from shark liver in 1906. Research has demonstrated that SQ can alleviate dryness and eczema-related skin damage; it also helps with anti-oxidation (40), anti-inflammatory reactions (41), and cardiovascular health (42, 43). Furthermore, SQ reduced the production of iNOS and COX-2, which assisted in lowering the inflammatory response and repairing the damaged intestinal mucosal epithelial barrier (41, 44).

SQ also demonstrated strong antioxidant qualities in a study of alcohol-induced oxidative damage, mostly due to their defense against cell membrane lipid peroxidation^[45]. Alcohol-induced lipid and retinal structural damage may be lessened by SQ^[46]. This is explained by SQ's capacity to preserve normal cell membrane fluidity, lessen the loss of polyunsaturated fatty acids, and shield the layer of visual nerve fibers. Squalene's high degree of unsaturation makes it vulnerable to oxidation by a variety of substances, including light, oxygen, temperature, moisture, and metals. Squalene can be kept without compromising the end product's chemical and sensory qualities if it is successfully encapsulated^[47].

This study investigates the protective potential of squalene-loaded PLGA nanoparticles against PFOA induced hepatotoxicity in a rat model, focusing on histopathological changes in liver tissue.

2. Materials and Methods

Thirty Wistar albino rats (8-12 weeks of age, either sex, weighing 150-200 g) were sourced from the Small Animal Breeding Station at College of Veterinary and Animal Sciences (CVAS), Mannuthy, Kerala. Animal experiments were carried out ethically throughout, per the Institutional Animal Ethics Committee (IAEC)-approved protocol (certificate no. CVAS/MTY/IAEC/24/70) and in compliance with Committee for Control and Supervision of Experiments on Animals (CCSEA) guidelines, Government of India. Rats were housed three per plastic cage in the Department of Veterinary Pharmacology and Toxicology animal facility, CVAS, Mannuthy, and acclimatized for 14 days under standard conditions: 25°C ± 2°C, 70% ± 5% relative humidity, and 12-h light-dark cycle. They were fed standard rat pellets from the School of Animal Nutrition and Feed Technology, CVAS, Mannuthy, with tap water provided ad libitum. Data were analyzed statistically using GraphPad Prism version 5.0.

2.1 Test Substances

Perfluorooctanoic acid (95%, 171468-5G) was obtained from Sigma-Aldrich, India. Squalene (98%, 2.05 M, S3626-10ML) was also purchased from Sigma-Aldrich, India. N-acetylcysteine (technical grade) was procured from Cure and Care Agencies, Palakkad, Kerala. Squalene nanoparticles were synthesized and characterized in a prior study, with acute oral toxicity testing showing an LD50 > 2000 mg/kg.

Experimental groups were assigned as follows: Group I received PFOA at a dose of 5 mg/kg dissolved in 0.4% DMSO and served as the PFOA-exposed control group. Group II was treated with PFOA at 5 mg/kg in combination with N-acetylcysteine (NAC) at 50 mg/kg to evaluate the

protective effect of this antioxidant against PFOA-induced toxicity. Group III received PFOA at 5 mg/kg along with squalene at 25 mg/kg to assess the hepatoprotective potential of plain squalene. Group IV was administered PFOA at 5 mg/kg together with a low initial dose (50 µL) of squalene-loaded PLGA nanoparticles containing 25 mg/kg of squalene, while Group V received PFOA at 5 mg/kg plus a high initial dose (100 µL) of the same squalene-loaded PLGA nanoparticles, also at 25 mg/kg squalene equivalent, to compare the impact of nanoparticle dosing regimen on protection.

Animals were sacrificed *via* CO₂ chamber on day 29 after overnight fasting. Livers were rapidly harvested and washed in ice-cold 0.9% saline. Homogenates were prepared in chilled 1.15% (w/v) KCl using a glass homogenizer for antioxidant enzyme analyses, including catalase and SOD. Select liver portions were preserved in 10% neutral buffered formalin for histopathology.

2.2 Estimation of catalase

The level of catalase in tissue homogenate of the liver was assessed by the method of Hadwan and Ali (2018)^[48]. The chemicals used for the analysis of catalase were sodium hexametaphosphate (extrapure AR, 65-70%, 68915-31-1), cobalt (II) nitrate hexahydrate (extrapure AR, ACS, 99%, 10026-22-9), sodium bicarbonate (ACS, 99.7%, 144-55-8), potassium dihydrogen orthophosphate (extrapure AR, 99.5%, 7778-77-0), disodium hydrogen phosphate dihydrate (extrapure AR, 99.5%, 10028-24-7) procured from Sisco Research Laboratories Pvt. Ltd. Mumbai and Hydrogen peroxide (30%, 7722-84-1) was purchased from M/s Merck, India

2.3 Estimation of Superoxide Dismutase

SOD was determined by modified method of Misra and Fridovich, (1972)^[49]. The reagents used for the analysis of SOD were hydroxylamine hydrochloride (98%, 5470-11-1), nitro blue tetrazolium (extrapure 99%, 11207) ethylenediamine tetra acetic acid (extrapure AR, 99.5%, 6381-92-6), triton X-100 (9002-93-1) were procured from Sisco Research Laboratories Pvt. Ltd. Mumbai.

3. Results

3.1 Alterations in catalase activity

The level of catalase enzyme in the liver of all the experimental animals are shown in table 1. Group I (6.11±0.46) animals showed a reduction in catalase activity whereas there was no significant variation between group II (28.05±1.27) and IV (24.94±4.03) which resulted in restoration of catalase activity, as depicted in fig 1.

3.2 Superoxide dismutase levels in liver

The level of SOD enzyme in liver of all experimental animals is shown in table 2. Group I (4.75±0.32) animals showed significant reduction in SOD levels. Group II (11.54±0.94) and IV (10.02±0.52) showed no significant variation and increased levels of SOD compared to group I, as illustrated in fig 2.

3.3 Histopathology

The histological examination of the liver of all the experimental animals in the different groups was performed. In the histological examination of group I animals, the liver showed varying degrees of degeneration ranging from

microvesicular steatosis and single cell necrosis and in group II the normal architecture of hepatocyte was restored. The group III animals showed ballooning of hepatocytes and

obliteration hepatic cord space. The group IV animals showed normal histology of the liver comparable to that of the group II animals, as illustrated in fig 2.

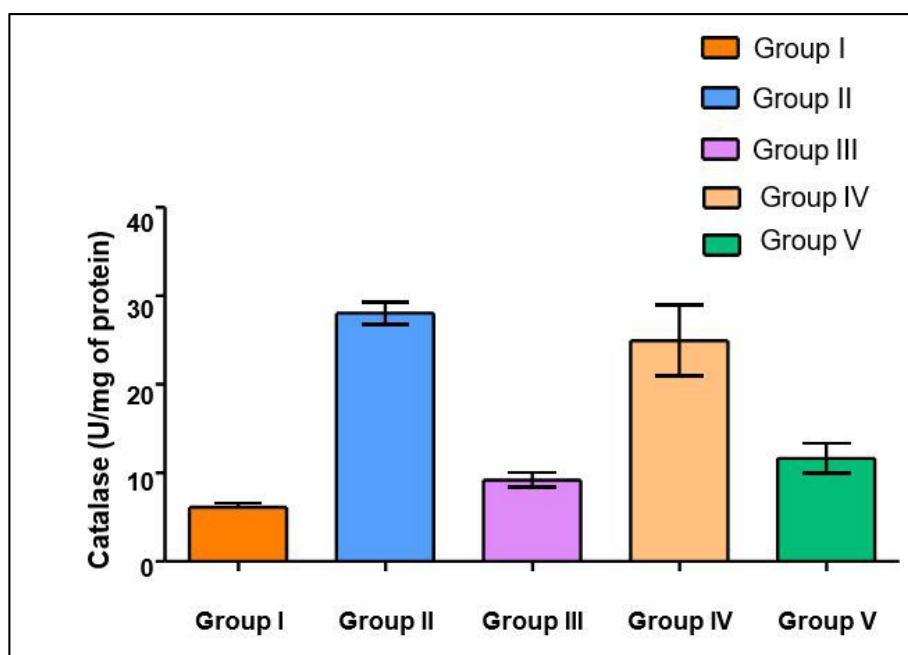
Table 1: Level of catalase in liver of different groups

Groups	Catalase (U/mg of protein)
I (PFOA)	6.11 ^a ±0.46
II (PFOA + NAC)	28.05 ^b ±1.27
III (PFOA + SQ)	9.18 ^a ±0.82
IV (PFOA + low dose SQ-NP)	24.94 ^b ±4.03
V (PFOA + high dose SQ-NP)	11.64 ^a ±1.7

Values are expressed as Mean ± SEM, n = 6

Values bearing different superscripts vary significantly at $p < 0.05$ in the column

PFOA-perfluorooctanoic acid; NAC-N-acetylcysteine; SQ-squalene; SQ-NP-Squalene loaded PLGA nanoparticles



Group I-PFOA; Group II-PFOA + NAC; Group III-PFOA + SQ; Group IV-PFOA + low dose SQ-NP; Group V-PFOA + high dose SQ-NP

PFOA-perfluorooctanoic acid; NAC-N-acetylcysteine; SQ-squalene; SQ-NP-squalene loaded PLGA nanoparticles

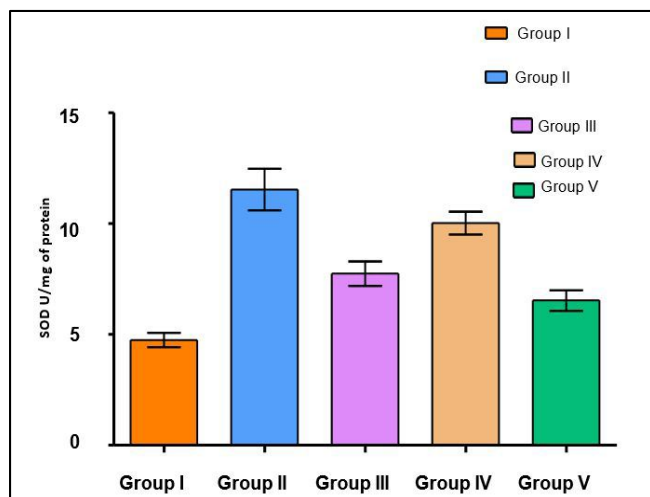
Fig 1: Level of catalase in liver of different groups

Table 2: Level of SOD in liver of different groups

Groups	SOD (U/mg of protein)
I (PFOA)	4.75 ^a ±0.32
II (PFOA + NAC)	11.54 ^c ±0.94
III (PFOA + SQ)	7.75 ^b ±0.55
IV (PFOA + low dose SQ-NP)	10.02 ^{bc} ±0.52
V (PFOA + high dose SQ-NP)	6.53 ^a ±0.46

Values are expressed as Mean ± SEM, n = 6

Values bearing different superscripts vary significantly at $p < 0.05$ in the column PFOA-perfluorooctanoic acid; NAC-N-acetylcysteine; SQ-squalene; SQ-NP-Squalene loaded PLGA nanoparticles



Group I-PFOA; Group II-PFOA + NAC; Group III-PFOA + SQ; Group IV-PFOA + low dose SQ-NP; Group V-PFOA + high dose SQ-NP

PFOA-perfluorooctanoic acid; NAC-N-acetylcysteine; SQ-squalene; SQ-NP-squalene loaded PLGA nanoparticles

Fig 2: Level of SOD in liver of different groups

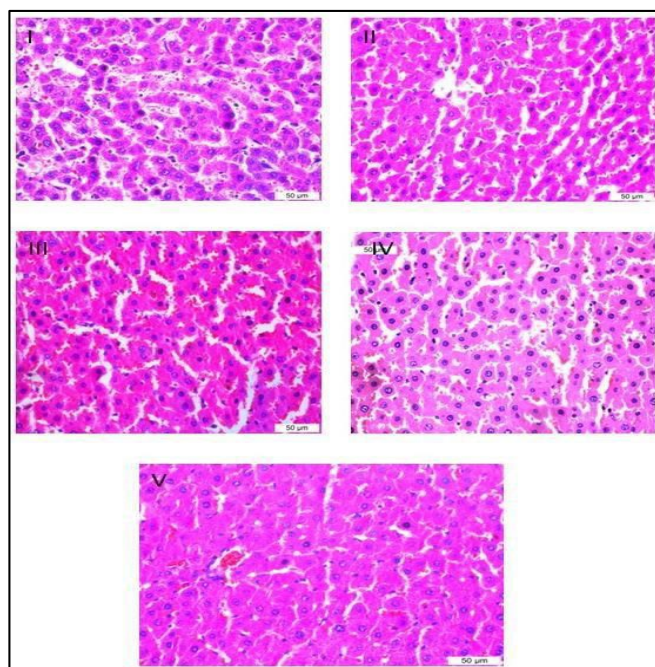


Fig 3: Histopathology of liver of different groups I) Section of liver (40X)-black arrows indicating microvesicular steatosis and hepatocellular necrosis II) Section of liver (40X) restoration of normal hepatocyte architecture III) Section of liver (40X)-black arrows indicating ballooning of hepatocyte with haemorrhagic sinusoids IV) Section of liver (40X)-normal hepatocyte integrity restored V) Section of liver (40X)-ballooning of hepatocytes with obliteration of sinusoidal space

4. Discussion

Human disorders such as cancer, ulcerative colitis, hyperuricemia, cardiovascular disease, thyroid disease, and liver damage are linked to PFOA exposure, which is a public health concern [50-54]. In addition to lowering hepatic triglycerides in mice, squalene treatment has been shown to cause notable changes in hepatic proteins implicated in oxidative stress, lipoprotein secretion, and lipid metabolism [55]. Squalene's unique and stable structure, which allows it to efficiently neutralize dangerous free radicals, is intimately linked to its antioxidant properties [56].

Additionally, due to its biocompatibility, inertness, and other beneficial qualities, it is widely employed as an excipient in pharmaceutical formulations for the treatment and management of diseases. Additionally, it has been shown to have cardioprotective and tumor-protective qualities, as well as a preventative effect on breast cancer [57]. Additionally, it lowers serum cholesterol levels [57, 58].

In the present study, we investigated the ameliorative effect of SQ-NP on PFOA-induced liver damage in rats. Hepatic histological analysis showed remarkable morphological changes in the liver of PFOA-treated mice. However, the histological changes induced by PFOA were obviously ameliorated by simultaneous treatment with SQ-NP similar to that of NAC.

The pathophysiology of several liver illnesses is thought to be significantly influenced by oxidative stress [59]. When the synthesis of ROS exceeds the cell's antioxidant capacity and there is an imbalance between the production of ROS and the antioxidant defense mechanisms, oxidative stress results. Research has shown that PFOA exposure causes oxidative stress in the kidney and liver [60]. Additionally, it has been shown that PFOA exposure produces ROS and oxidative stress [61, 62].

In this study, PFOA exposure caused a marked elevation in SOD and catalase levels in rat livers. As an effective antioxidant, NAC successfully normalized these elevated enzyme levels, with findings closely aligning with histopathological observations. Likewise, squalene nanoparticles (SQNP) restored SOD and catalase to baseline levels in the livers of PFOA-treated rats. This restoration underscores SQ-NP's potential as a potent antioxidant agent.

5. Conflict of interest statement

The authors declare that there are no conflicts of interest.

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