Article

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Amelioration of Organophosphate Poisoning Using Red Blood Cell Membrane-Cloaked Oil Nano-Sponge

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Background: Organophosphate poisoning poses a significant health burden due to the absence of specific pharmacological antidotes. To address this issue, we explored the potential of nanoparticles for organophosphate detoxification, focusing on biomimetic nanoparticles with natural cell membrane coatings.

Methods: We developed membrane-cloaked oil nano-sponges by combining red blood cell (RBC) membranes and oil to create a dual-model detoxification system for organophosphates. The oil component non-specifically absorbed organophosphate poisons, while the membrane-bound acetylcholinesterase enzyme is specifically bound to the toxic molecules, preserving intrinsic acetylcholinesterase function. We evaluated the detoxification capacity of this system using chlorpyrifos as a model insecticide in a rabbit model.

Results: The prepared oil nano-sponge exhibited potent antidote capabilities against organophosphate poisoning and demonstrated efficacy in poisoning prevention. The safety evaluation revealed no adverse effects on the gross appearance or histopathological sections of the liver, kidneys, spleen, lungs, and heart.

Conclusions: In summary, our novel membrane-cloaked oil nano-sponge proved to be a safe and effective antidote for organophosphate poisoning in rabbits. These findings have the potential for extrapolation to other species, offering a promising solution to mitigate inadvertent organophosphate poisoning.

Keywords: organophosphate; RBCs; poisoning; nano-sponge; improvement

Introduction

Organophosphates are esters of phosphoric acid. These chemicals were originally produced by the reaction of alcohol and phosphoric acid. The most potent organophosphates are G and V series agents which were developed for military use as chemical warfare agents (nerve agents) [1]. Civilians are exposed to less toxic organophosphates. Synthetic organophosphates are extensively used in crop production (as pesticides) and in veterinary medicine (as anthelmintics or ectoparasiticides) [2,3]. These chemicals exert significant toxic effects in non-target species by phosphorylation and subsequent inactivation of acetylcholinesterase enzyme. The result is an accumulation of neurotransmitter acetylcholine and continuous stimulation of acetylcholine receptors at neuroeffector junctions, at autonomic ganglia and neuronal synapses in the central nervous system [4] leading to a spectrum of clinical signs collectively termed as a cholinergic syndrome. Organophosphates are by far the most common cause of poisoning. According to estimates in 2014, three million people around the globe were annually intoxicated out of which one million died [5]. These chemicals account for 30% of all suicides worldwide [6]. The extent of chronic and low-level poisoning is far above the estimates of acute toxicity [7].

The signs and symptoms of acute toxicity (within minutes to hours) can be broadly classified as (i) muscarinic effects, (ii) nicotinic effects, and (iii) central nervous system effects. Muscarinic effects include hypersalivation, lacrimation urinary incontinence, fecal incon-

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tinence, diarrhea, emesis, diaphoresis, meiosis, bradycardia, and bronchospasm. Nicotinic effects include cramping, muscle fasciculation, weakness, and diaphragmatic failure. Autonomic nicotinic effects include tachycardia, hypertension, mydriasis, and pallor. Central nervous system effects include emotional lability, restlessness, anxiety, confusion, tremors, ataxia, apnea, seizures, and coma [8,9]. Organophosphate toxicity is not limited to the acute phase, but delayed effects (24 hours to 2 weeks) can also become life-threatening due to neuronal excitotoxicity [10]. Chronic toxicity can result in persistent oxidative stress, selective organ failure, reproductive incapacitation, delayed neuropathy, mutagenicity, carcinogenicity, and teratogenicity [11,12].

Available treatment options for organophosphate poisoning include muscarinic antagonists (atropine), cholinesterase reactivators (oximes), reversible blockers of cholinesterase (carbamates), exogenous enzymes (human butyryl-cholinesterase, bacterial phosphotriesterase, etc.) and supportive therapy. These treatment options are effective in saving a life, yet each of these has some limitations and drawbacks [13,14]. For instance, atropine cannot be considered a specific antidote because it is a muscarinic antagonist and ineffective in controlling nicotinic effects. Atropine resistance in some individuals and toxicity in others is a limitation in predicting the outcome of therapy. It may prevent cholinergic neuronal toxicity if administered within a very limited therapeutic time window (2–30 minutes) after poisoning. If this time lapses, delayed neuropathy is inevitable [15]. Oximes are purported to function as cholinesterase reactivators, but oxime therapy fails after the aging of the cholinesterase enzyme. The aging half-life of soman is about 2 min and that of the VR compound is 140 hours [16,17]. Hence, oximes have a limited spectrum of activity, and they must be administered within a narrow time window. Carbamate compounds reversibly block cholinesterase enzyme to prevent it from irreversible inhibition by organophosphates on subsequent exposure. Hence, they can be used as a pre-treatment option and their protection lasts for 24 hours or less. The dose cannot exceed a limit because they cause cholinergic crises by themselves. Exogenous enzymes are of value as a pre-treatment option but, their continuous administration is required to continue having any effect [18].

Here arises the need to develop a safe and potent antidote for organophosphate poisoning. The human body produces two types of cholinesterases, butyryl-cholinesterase, and acetylcholinesterase. The former has less catalytic efficiency and is found in plasma and liver while acetylcholinesterase is efficient hydrolytic enzyme and is found at nerve terminals and on red blood cell (RBC) membranes [19,20]. Researchers have availed the benefit of the membrane-bound enzyme to construct biomimetic nanoparticles that capture organophosphates, hence sparing intrinsic enzymes to function. Once stoichiometric binding of enzyme and substrate occurs, both are eliminated. RBC membrane-coated polymeric nanoparticles have been proven to be an effective antidote to organophosphate poisoning [21]. To add value to biomimetic nanoparticles, the present study incorporates two detoxifying modalities; membranes, and oils, to form membrane-cloaked oil nanodroplets which are hereafter termed oil nano-sponge. Membranes furnish acetylcholinesterase enzyme which irreversibly binds and eliminates organophosphate compounds. Organophosphates being lipophilic can cross the membrane barrier and get absorbed in oil which serves as a non-specific absorbent of lipophilic compounds. Hence, the dual model detoxifying platform serves as a potent antidote for organophosphate poisoning.

Materials and Methods

Materials

Chlorpyrifos (97% crystals in solid form), ethanol (99% anhydrous), soybean oil (edible soybean oil 1-litre pouch, Glaze®, FarmFields, Pakistan), fish oil (edible fish oil 150 mL bottle, Icelandic Cod Liver Oil®, Nutrifactor, Pakistan), Eppendorf tubes (1.5 mL microcentrifuge tubes), bath sonicator (Elma®), normal saline, deionized water (Victor Liners, Lahore, Pakistan), Fourier transform infrared spectroscopy (FTIR) analysis was performed with instrument Perkin-Elmer, Spectrum II in a dry atmosphere. Data were collected in the range of 600–4000 wavenumbers (cm^{-1}) with a resolution of 4 cm^{-1} and processed with Spectrum 10 software (version 10.4.3, PerkinElmer Inc., Bucks, UK), UV-Vis spectrophotometer (https://biobase.en.made-in-china.com/product-group/CePJudncXRpF/UV-VIS-Spectrophotometer-1.html) (Thermoscientific Multiskan GO). The experimental data will be analysed by GraphPad Prism software (version 6.0, GraphPad Software, Inc., San Diego, CA, USA).

Rabbit RBCs Membrane Collection

Red blood cell membranes were collected from rabbit blood to coat the membranes over the oil droplets for the formulation of biomimetic oil nano-sponge. Blood (5 mL) was collected with a heparinized syringe from the rabbit's jugular vein after the area was properly shaved off and sanitized. Briefly, 1 mL of collected blood was taken in each Eppendorf tube (1.5 mL) and centrifuged at 3000 rpm for 5 minutes. Plasma was removed and RBCs were collected and treated with deionized distilled water. The contents of the tube were vortex mixed so that all cells freely float in the hypotonic environment and then centrifugation was done at 13,000 rpm for 5 min. This process allows hypotonic lysis of RBCs, removing all the content of the cells leaving behind the hollow cells which sediment because of higher density. The fluid portion becomes red due to the leaked haemoglobin and the red-tinged fluid was removed.

Distilled water was added again for another round of centrifuge. This step was repeated until the fluid portion becomes colourless and membranes form a light pink pallet in the sediment. The distilled water of the membrane suspension was replaced with normal saline. The protein content of membrane suspension was quantified by Bradford's method and it was taken as an index of the quantity of membranes [22]. In this method, the membrane suspension was treated with Coomassie Brilliant Blue dye and absorbance was taken by spectrophotometer at 595 nm. The detected protein concentration of RBC membrane suspension from 1 mL of rabbit blood was 0.2 mg/mL. Thus, the suspensions with higher or lower protein content was standardized to 0.2 mg/mL and then frozen at –20 °C for further use.

Preparation of Oil Nano-Sponge

For getting a precise quantity of oil, 8 mg fish or soyabean oil was mixed with normal saline to make up 1 mL volume and the mixture was subjected to bath sonication for 10 min so that homogenous oil nano-emulsion was obtained. From the nano-emulsion, 0.1 mL was taken which was precisely equivalent to 0.8 mg oil. In 0.2 mg membrane suspension, 0.8 mg oil nano-emulsion was added so that a membrane-to-oil ratio (w/w) of 1:4 was ensured. The volume of the mixture was adjusted to 1 mL in the Eppendorf tube. The mixture was vigorously shaken, tubes were fixed in a stand, placed in a bath sonicator, and covered with ice packs. The mixture was then subjected to sonication for 20 min (3 min sonication and 1 min pause, 5 cycles) and then passed through a mini-extruder. This step allowed the cloaking of membranes over nano-droplets of oil to form oil nano-sponge. At standard protocol, the oil nano-sponge was obtained at 1 mg/mL concentration and stored at 4 °C until used.

Verification of Coating of RBC Membranes onto the Oil Droplets

One of the important aspects to confirm the successful synthesis of oil nano-sponge is by verifying the coating of the cell membrane over the oil droplets. For this purpose, the suspension of oil nano-sponge was centrifuged at 13,000 rpm for 5 minutes, and nano-sponges accumulated at the surface. This confirms oil to membrane association because membranes' density is higher than water and forms pallets in sediment. However, oil nano-sponge density is lower than water so it rises to the surface after centrifugation [22]. The same procedure was followed for the preparation of both fish oil nano-sponge (FONS) and soybean oil nano-sponge (SONS).

Characterization of Oil Nano-Sponge

The characterization of prepared oil-nanosponge was done, to confirm the successful synthesis of oil-nanosponge. Zeta size and zeta potential of prepared fish oil nano-emulsion (FONE), soybean oil nano-emulsion (SONE), FONS, and SONS were determined with dynamic light scattering [23]. Briefly, Fourier transform infrared spectroscopy (FTIR) spectroscopy was done in the same way for FONE, SONE, FONS and SONS. Surface adsorption of chlorpyrifos onto nano-sponge was confirmed by FTIR spectroscopy of the nano-sponge-chlorpyrifos complex. This was done by mixing 10 mg chlorpyrifos (in acetone solution) with 1 mg nano-sponge or nano-emulsion. The mixture immediately formed a precipitate which was subjected to FTIR analysis.

Measurement of Acetylcholinesterase Activity

Cholinesterase activity of membrane suspension and FONS or SONS (with the same membrane content as of suspension) was determined to check if there was any loss of activity during the preparation of nano-sponges. Each measurement was taken in triplicate. The assay was based on a modified Ellman's method [24]. Prepared reagents and procedures were adopted from Elabscience® Acetylcholinesterase Assay Kit (https://www.sigmaaldrich.com/US/en/product/sigma/cs0003, catalogue No. E-BC-K174). The statistical analysis was done through paired *t*-test using GraphPad Prism software (version 6.0, GraphPad Software, Inc., San Diego, CA, USA).

Measurement of Chlorpyrifos Adsorption into Oil Nano-Sponge

The *in-vitro* testing was done to determine the organophosphate adsorption capability of oil nano-sponge. Chlorpyrifos, being sparingly soluble in water, was dissolved in ethyl alcohol. To get a standard absorbance curve, ethanol solutions of chlorpyrifos in different dilutions in the range of 2–10 mg/mL were prepared and the absorbance curve was plotted using a 290 nm wavelength by UV-Vis spectrophotometer [25]. The volume of the mixture in each tube was made up to 1 mL with ethanol. The mixture was bath sonicated at 40 °C for 15 min. This allows maximum adsorption of chlorpyrifos with oil nano-sponge in a concentration-dependent manner resulting in precipitation of nano-sponge after sonication. The precipitate of the laden nano-sponge was removed by centrifugation at 13,000 rpm for 5 min whereupon the oil nano-sponge gets separated along the eccentric side of the tubes. The solution was withdrawn with a micropipette and labeled as a 'cleared solution'. Chlorpyrifos quantity in each cleared solution was determined by their absorbance at 290 nm wavelength. The quantity of adsorbed chlorpyrifos was calculated by subtracting the remaining quantity from the initial quantity (*i.e.,* 10 mg). The adsorbed quantity was related to the quantity of nano-sponge applied for clearing chlorpyrifos.

Oil Nano-Sponge-Mediated Detoxification Potential

The *in-vivo* analysis was performed to evaluate the therapeutic efficacy of oil nano-sponge by detoxifying

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chlorpyrifos. Rabbits of 11–15 months of age were used as experimental animals. All rabbits were purchased from a local market in Faisalabad and weighed in the range of 1.25–1.75 kg. A total of 42 rabbits were recruited for the study. No pregnant female was used for any purpose in the study. All animals were kept in cages at room temperature and were fed a normal diet and *ad-libitum* water. Furthermore, the animals were acclimatized for two weeks whereupon the treatment period began. Allotment to groups and allocation of treatments were completely randomized. Every possible care was taken to minimize suffering. All the experiments involving animals were performed according to guidelines for the care and use of experimental animals. The guidelines were approved by the Biosafety and Bioethics Committee (IBC) of the University of Agriculture, Faisalabad, Pakistan (Certificate No: 1760/ORIC). Detoxification potential was validated by firstly oral LD_{100} dose of chlorpyrifos (1200 mg/kg) followed by intravenous dose of treatment (2 mg/kg). Rabbits (4 groups, n = 6 in each) death/survival rate was taken as the index of failure/success of treatment. Group-I animals were served with a normal diet only and considered as negative control, Group-II animals received a normal diet along with organophosphate (chlorpyrifos) and were denoted as positive control. Group-III and Group-IV served as treatment groups which received fish oil nano-sponge (FONS) and soybean oil nano-sponge (SONS) respectively. Treatment was administered 30 min after oral dosing of chlorpyrifos. Animals were kept under observation until 14 days post-treatment. A survival curve was plotted through Kaplan Meier survival analysis using GraphPad Prism, to represent the results. Animals in Group-I and II were euthanized painlessly by holding the rabbit's body in one hand and its head in the other. By swiftly snapping the head upward and backward, you can effectively break the spinal column, resulting in a quick and painless death for the rabbit. Their major organs (kidneys, liver, lungs, spleen and heart) were photographed *in situ*, collected, and fixed in 10% neutral buffered formalin for further analysis.

Safety Evaluation of Oil Nano-Sponge

A successful treatment has to be safe to become applicable. Safety evaluation of oil nano-sponge was performed by intravenous administration of a mixture of chlorpyrifos and oil nano-sponge and then histopathological comparison of treatment groups with negative and positive control groups. Four groups (n = 6 in each) were subject to trial for comparison. Organs of negative and positive control (Group-I and II) were harvested as described in section 2.5 above. Group-III and IV comprised animals which were slaughtered on 3rd and 7th day after treatment. It was figured out that 50 mg of chlorpyrifos is completely adsorbed by 1 mg nano-sponge therefore, a mixture of same proportions was given to rabbits by intravenous route. After slaughtering, five major organs (liver, kidney, heart, lung, spleen) were immediately fixed in 10% neutral buffered formalin. Thereafter, the samples were dehydrated, cleared, impregnated, embedded in wax, sectioned by microtome, mounted on glass slides, and stained with haematoxylin and eosin stains according to standard protocols of tissue sectioning and staining [26]. The histopathological comparison was made by photographs of slides under 10× magnification of the objective lens of the light microscope. The result is given as descriptive analysis.

Results

Preparation of Oil Nano-Sponges

RBC membranes were collected according to the protocol and the membranes sediment at the bottom of the Eppendorf tube in the form of a pinkish-white pellet. The protein content of RBC membrane suspension was found to be 201.3538 µg/mL and 199.4307 µg/mL in two replicates (Table 1). Fish oil nano-sponge (FONS) and soybean oil nano-sponge (SONS) were prepared. When the suspension of the oil nano-sponge was centrifuged at 13,000 rpm for 5 min, nano-sponges accumulated at the surface as shown in Fig. 1. This confirms oil to membrane association because membranes' density is higher than water and they form pellets in sediment but when oil nano-sponge is formed their density is lower than water due to which they rise to the surface after centrifugation.

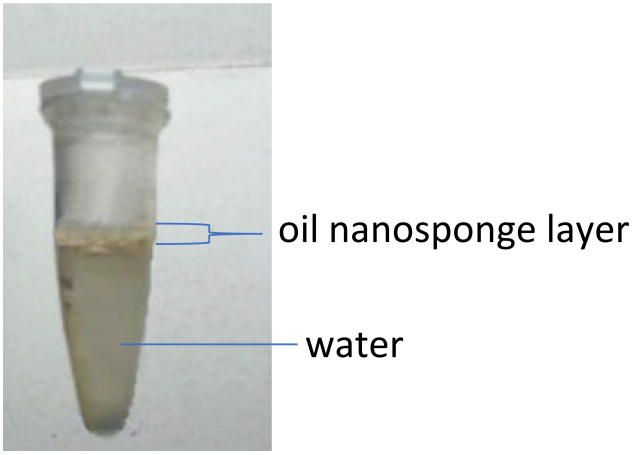

Fig. 1. Suspension of oil nano-sponge.

Table 1. Protein quantification by Bradford method.

Sr.	Absorbance 1	Absorbance 2	Protein (µg/mL)
1	1.309	1.3088	201.3538
2	1.308	1.3079	199.4307

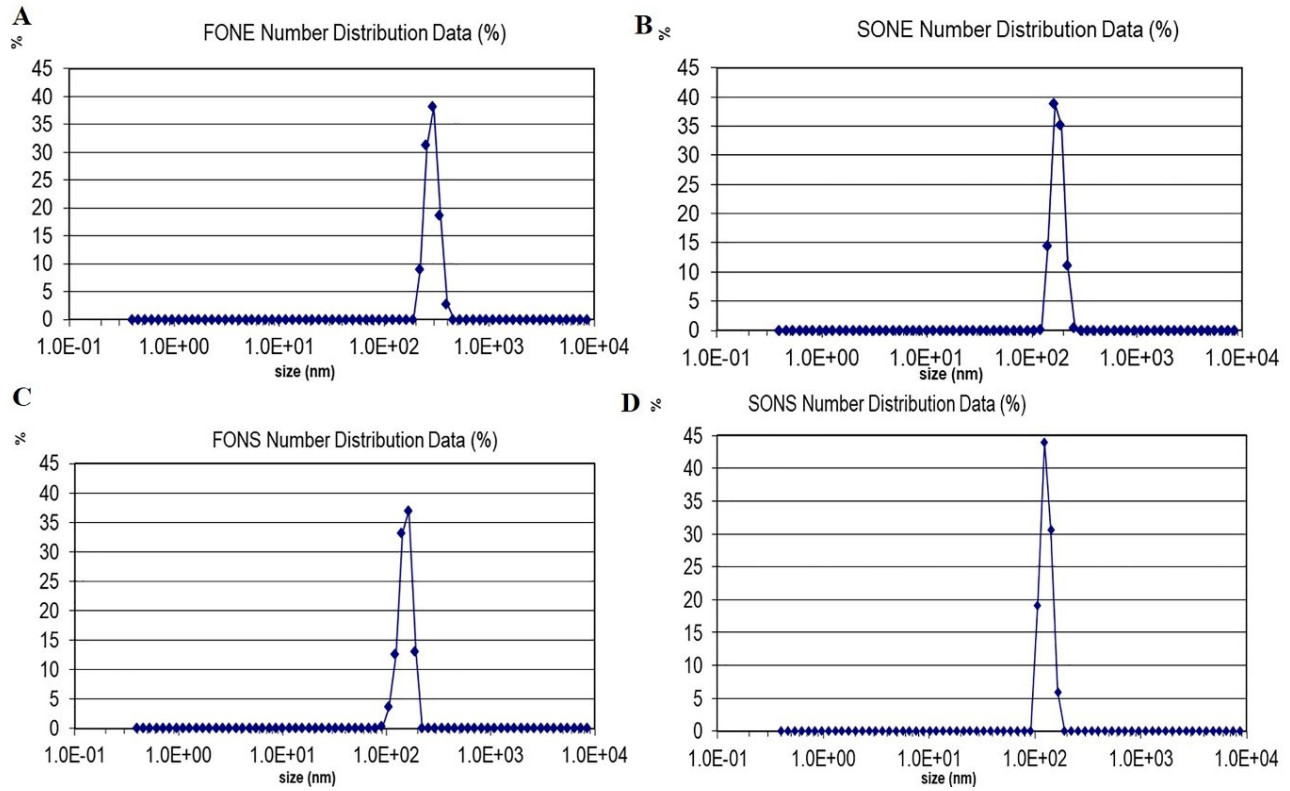

Fig. 2. Zeta size of nanoformulations. (A) Zeta size of Fish oil nano-emulsion. (B) Zeta size of soya bean oil nano-emulsion. (C) Zeta size of fish oil nano-suspension. (D) Zeta size of soya bean oil nano-suspension. FONE, fish oil nano-emulsion; SONE, soybean oil nano-emulsion; FONS, fish oil nano-sponge; SONS, soybean oil nano-sponge.

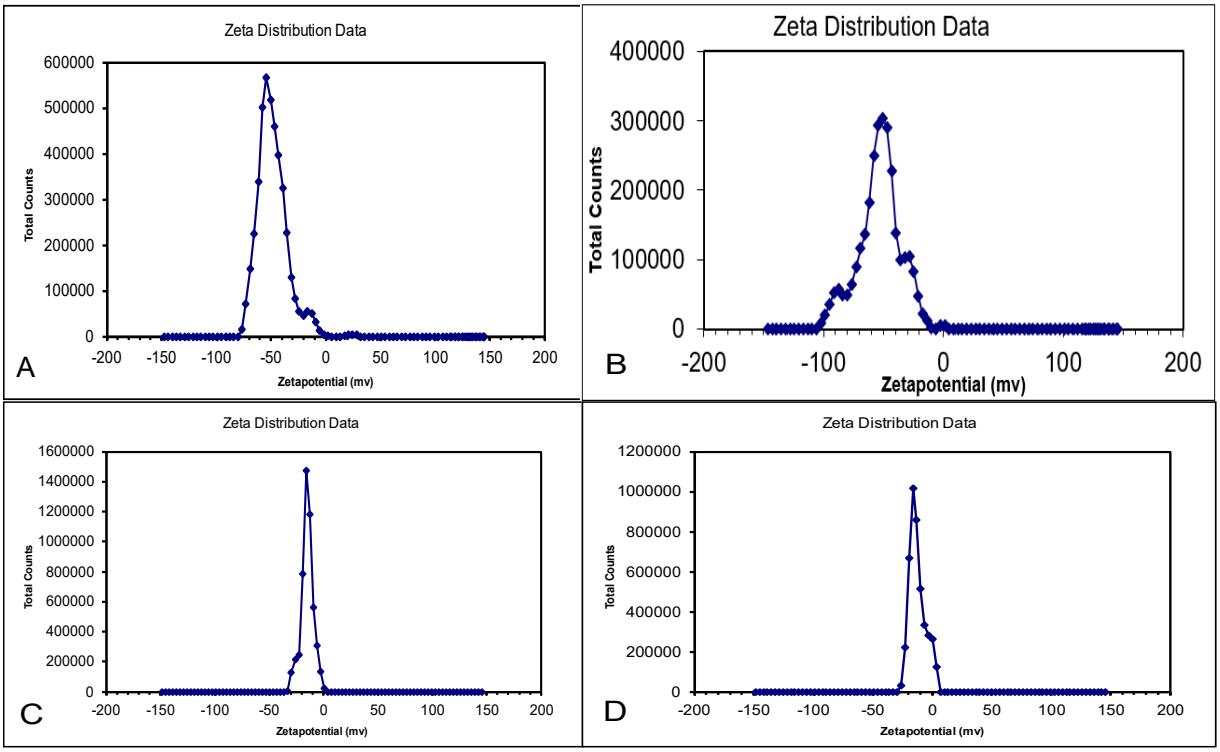

Fig. 3. Zeta potential of nanoformulations. (A) Zeta Distribution Data of fish oil nano-emulsion (FONE). (B) Zeta potential of soybean oil nano-emulsion (SONE). (C) Zeta potential of fish oil nano-sponge (FONS). (D) Zeta potential of soybean oil nano-sponge (SONS).

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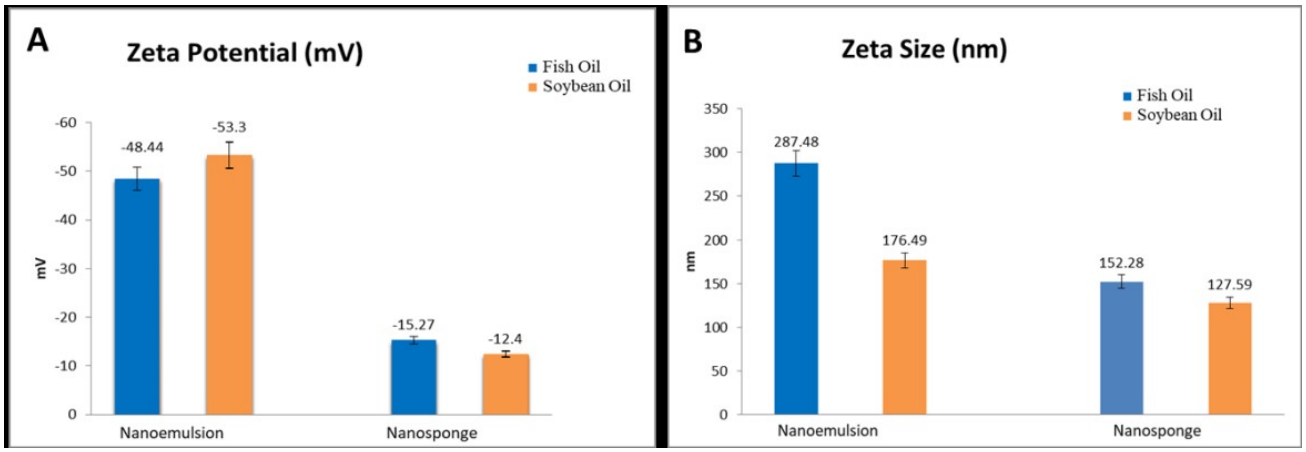

Fig. 4. Comparative analysis between nano-emulsion and nano-sponge. (A) Average zeta potential. (B) Average zeta size.

Characterization of Oil Nano-Sponges

Zeta Size

The zeta size of oil nano-emulsions (NE) and oil nano-sponges (NS) were determined separately. Oil nano-droplets of fish oil nano-emulsion and soybean oil nano-emulsion had an average size of 287.48 nm and 176.49 nm, respectively (Fig. 2A,B) while that of fish oil nano-sponge and soybean oil nano-sponge was 152.28 nm and 127.59 nm, respectively (Fig. 2C,D).

Zeta Potential

The Zeta potential of fish oil nano-emulsion (FONE) and soybean oil nano-emulsion (SONE) was –48.44 mV and –53.3 mV respectively (Fig. 3A,B), while that of fish oil nano-sponge (FONS) and soybean oil nano-sponge (SONS) was –15.27 mV and –12.4 mV (Fig. 3C,D).

Fig. 4A shows a comparison of size between fish oil and soybean oil nano-emulsions and nano-sponges while Fig. 4B makes a comparison of zeta potential between nano-emulsion and nano-sponges. Furthermore, the comparison between the average zeta size of nano-emulsions and oil nano-sponges and the comparison between the average zeta potential of nano-emulsions and oil nano-sponges were also shown in Fig. 4.

FTIR Spectroscopy (Surface Analysis)

Fourier-transform infrared spectroscopy was done for chlorpyrifos (CPS), both oils, both nano-sponges (FONS and SONS), a mixture of fish oil nano-sponge with chlorpyrifos (FONS-CPS complex), and a mixture of soybean oil nano-sponge with chlorpyrifos (SONS-CPS complex) (Figs. 5,6).

The transmittance peaks of oils differ from their respective oil nano-sponges, hence, confirming the complete cloaking of oils by membranes (Fig. 5A,B and Fig. 6A,B). Moreover, the same transmittance pattern of chlorpyrifos is shown in Figs. 5C,6C. A mixture of chlorpyrifos and oil nano-sponge (FONS and SONS separately) immediately forms a precipitate whose transmittance pattern contains peaks of nano-sponge as well as of chlorpyrifos (Fig. 5D,E and Fig. 6D,E). These results confirm that (i) membranes surround both types of nano-sponges while the oil core is completely 'masked' by membranes, and (ii) chlorpyrifos gets adsorbed onto the surface of both oil nano-sponges.

Transmittance of fish oil nano-sponge and soybean oil nano-sponge has a similar pattern. Likewise, the combined graph of FTIR spectra shows overlapping curves which speak of identical surface chemistry of both oil nano-sponges (Fig. 7).

Measurement of Acetylcholinesterase Activity

Ellman's method was used for the determination of cholinesterase activity of hollow RBC membranes suspension, fish oil nano-sponge, and soybean oil nano-sponge. Each assay was repeated in triplicate. The average cholinesterase activity of hollow RBC membranes suspension was 293.51 U/mL at a protein concentration of 0.2 mg/mL, while that of fish oil nano-sponge and soybean oil nano-sponge was 291.79 and 292.13 U/mL respectively at equal protein concentrations. Fig. 8A gives the graphical representation of the results. Fig. 8B,C graphically compares the cholinesterase activity of membranes with both oil nano-sponges with a 95% confidence interval.

Measurement of Chlorpyrifos Adsorption onto Oil Nano-Sponge

As demonstrated by FTIR spectroscopy that chlorpyrifos gets adsorbed onto the surface of both oil nano-sponges, the present study quantifies *in-vitro* clearance of chlorpyrifos by both oil nano-sponges. Fig. 9A,B gives a graphical representation of the quantity of adsorbed chlorpyrifos with different quantities of FONS and SONS respectively.

Prevention of Chlorpyrifos Intoxication

The preventive potential of oil nano-sponges against organophosphate poisoning was evaluated *in-vivo*, by ad-

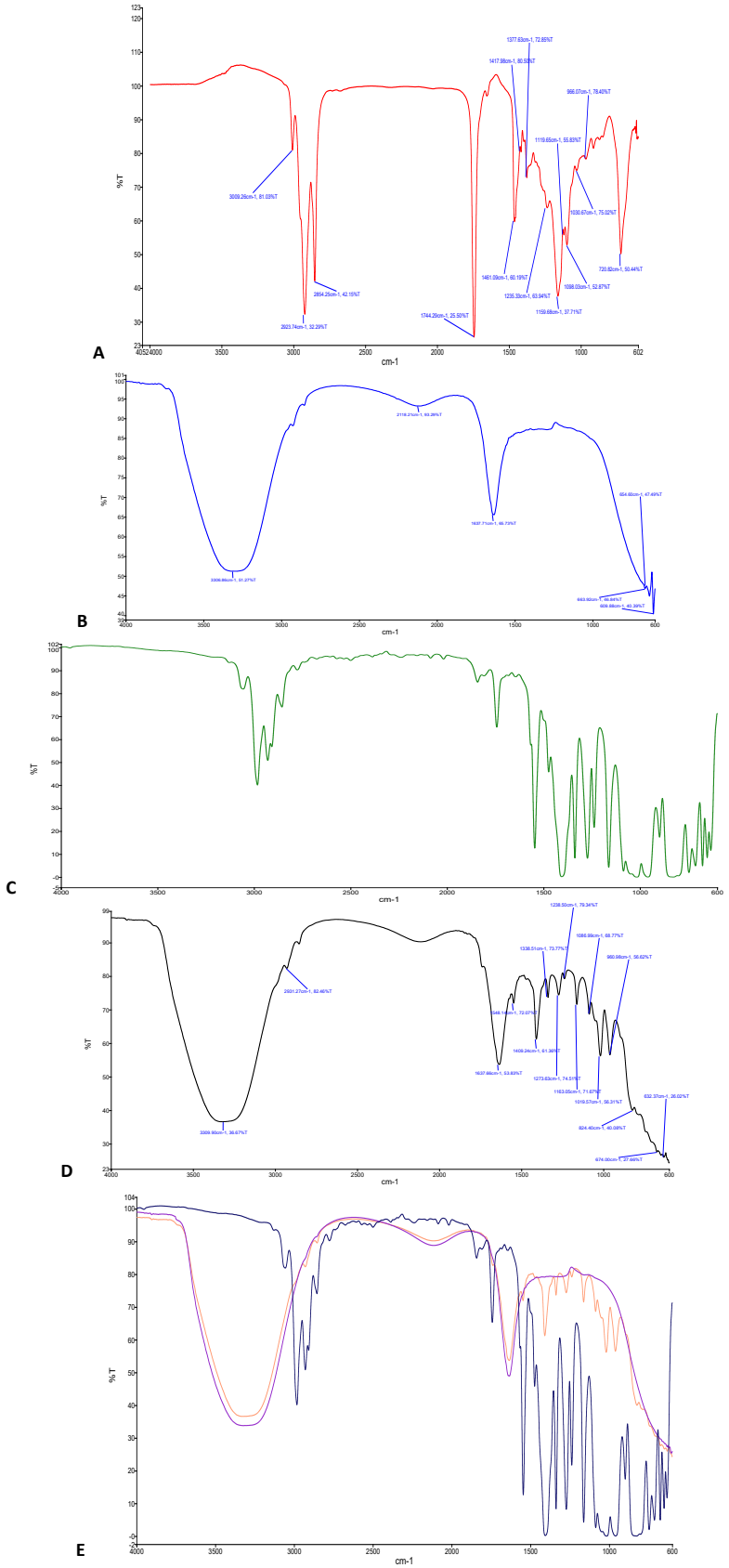

Fig. 5. FTIR spectrum analysis of (A) fish oil, (B) fish oil nano-sponge, (C) chlorpyrifos, (D) mixture of chlorpyrifos and fish oil nano-sponge, (E) combined FTIR spectra analysis of chlorpyrifos, fish oil NS and a mixture of both. FTIR, Fourier transform infrared spectroscopy.

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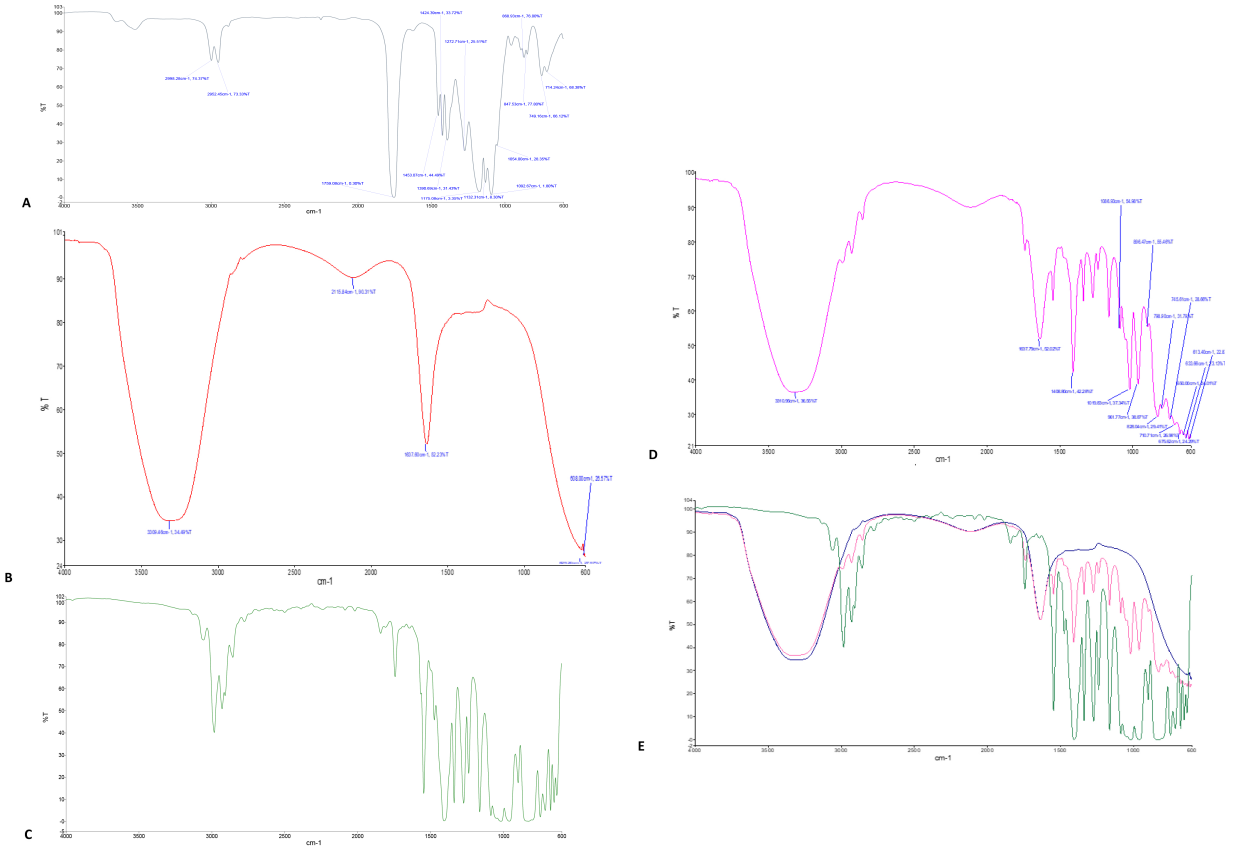

Fig. 6. FTIR spectrum of (A) soybean oil, (B) soybean oil nano-sponge, (C) chlorpyrifos, (D) a mixture of chlorpyrifos and soybean oil nano-sponge, (E) combined chlorpyrifos, soybean oil nano-sponge and a mixture of both.

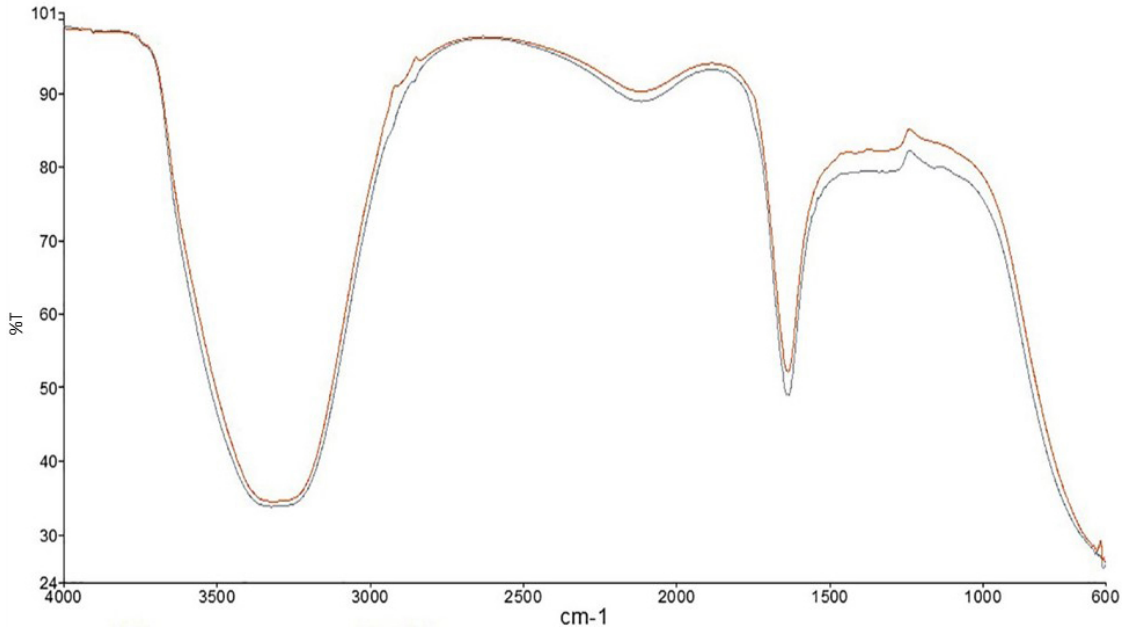

Fig. 7. Combined spectra of fish oil and soybean oil nano-sponges.

ministering the variable intravenous dose of oil nano-sponge before the administration of a fixed subcutaneous dose of chlorpyrifos. Animals were kept under observation for 14 days. Fig. 10A,B represent the probability of survival with FONS and SONS respectively as preventive treatment at different dose rates. Positive and negative controls are included for comparison.

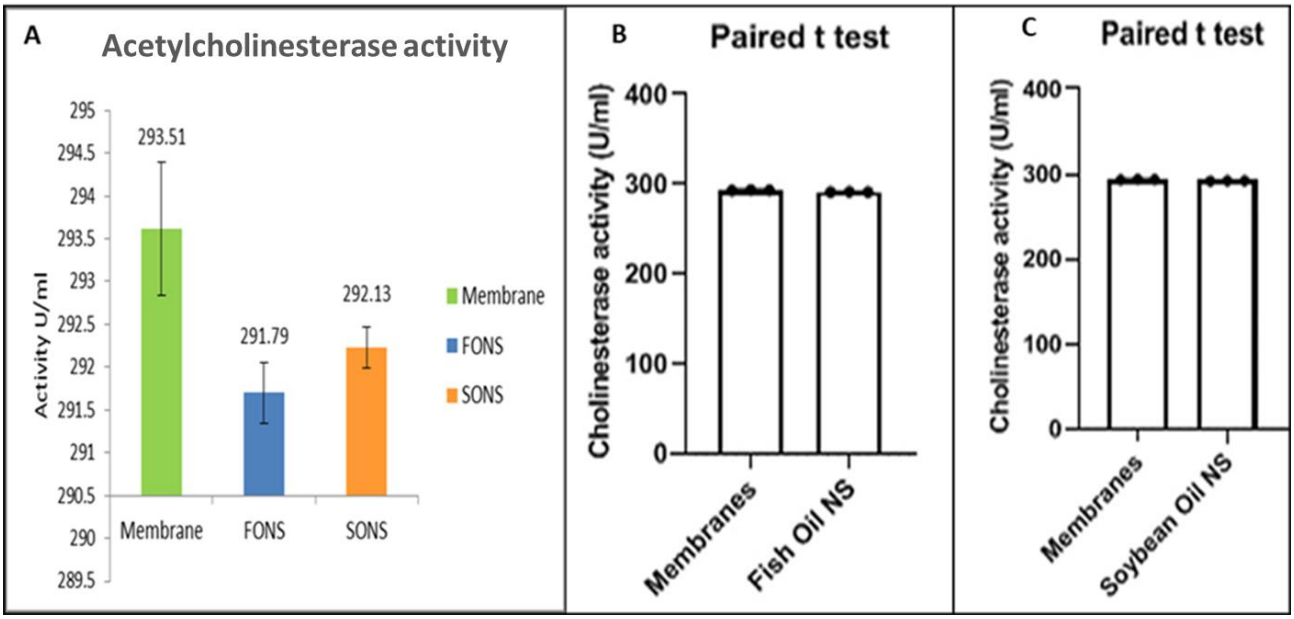

Fig. 8. Acetylcholinesterase activity. (A) Acetylcholinesterase activity of membrane suspension and oil nano-sponges. (B) Comparison of cholinesterase activity between membranes and fish oil nano-sponge with 95% confidence interval. (C) Comparison of cholinesterase activity between membranes and soybean oil nano-sponge with 95% confidence interval.

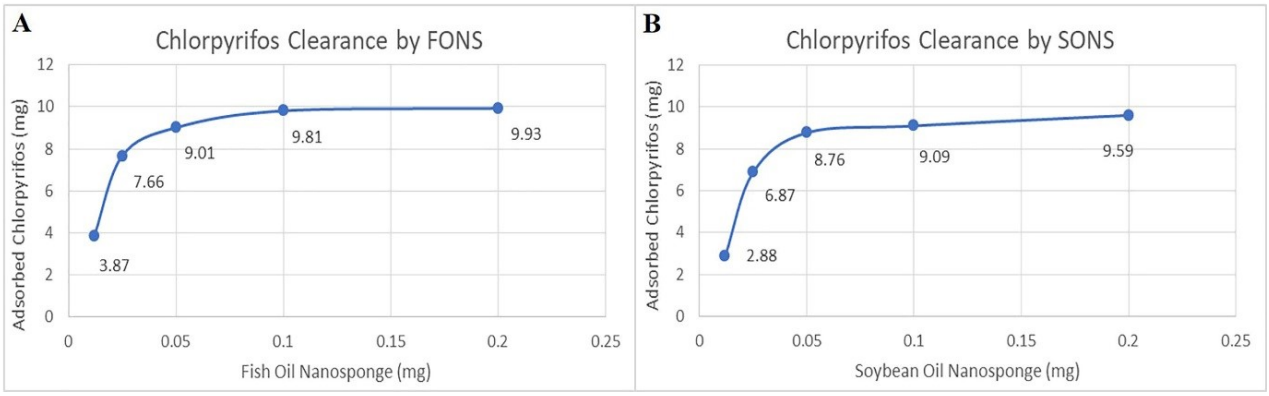

Fig. 9. Chlorpyrifos clearance by nanoformulations. (A) Chlorpyrifos clearance by fish oil nano-sponge. (B) Chlorpyrifos clearance by soybean oil nano-sponge.

Safety Evaluation of Oil Nano-Sponge

As demonstrated by the measurement of chlorpyrifos adsorption onto oil nano-sponge oil nano-sponges successfully detoxified 50 times the weight of chlorpyrifos, the same ratio was used for safety evaluation. Animals in two groups were given a mixture of 50 mg/kg chlorpyrifos and 1 mg/kg oil nano-sponge by intravenous route. Animals in Group-I were slaughtered on day-3 while those in Group II were slaughtered on day-7 after treatment. Images of major organs liver, kidney, heart, lungs, and spleen with negative and positive control groups are given after histopathology for comparison. These comparisons were shown in Fig. 11.

Histopathology

i. Liver

(Negative control) normal. (3rd day post-treatment) Normal. (7th day post-treatment) cellular infiltration in a few fields. (Positive control) the hepatic parenchyma indicated a moderate to severe degree of fibrosis which was indicated by fibroblast proliferation. Moderate congestion was present. Mild to moderate degree of vacuolar degeneration was present. Vacuoles had a hazy appearance. A moderate degree of haemorrhage was present. Sinusoidal spaces were normal. Cellular infiltration was present with necrotic zones. Individual cell necrosis was evident in the pyknotic nuclei of hepatocytes (Fig. 11).

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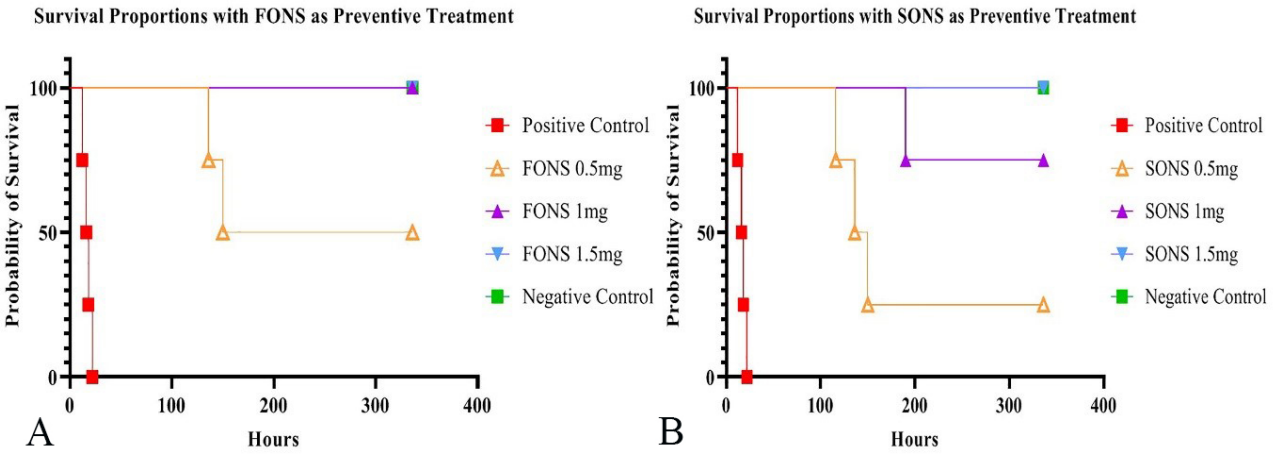

Fig. 10. ***In-vivo*** **prevention from poisoning.** (A) Survival proportions with fish oil nano-sponge as preventive treatment at different dose rates. (B) Survival proportions with soybean oil nano-sponge as preventive treatment at different dose rates.

ii. Kidney

(Negative control) normal appearance of glomeruli, Bowman's capsule, and collecting tubules. (3rd day post-treatment) normal. (7th day post-treatment) the normal arrangement of cells with pyknotic changes at a few places. (Positive Control) the renal parenchyma indicates a moderate to severe degree of necrosis indicated by acute tubular necrosis (dotted circle represented necrotic glomerulus). Nuclei of tubular epithelial cells show pyknotic and necrotic changes throughout the renal parenchyma. Haemorrhages and congestion were present. Mostly, tubules were detached from basal membranes (Fig. 11).

iii. Heart

(Negative control) normal appearance of myocardial fibres in longitudinal and cross sections. (3rd day post-treatment) normal. (7th day post-treatment) normal. (Positive control) cardiac parenchyma indicated necrotic changes in myofibres, both in longitudinal and cross sections. The nuclei of myofibres were condensed and pyknotic. In a few places, congestion was also present (Fig. 11).

iv. Lungs

(Negative control) normal air spaces, thin alveolar walls and no cellular infiltration. (3rd day post-treatment) cellular infiltration at a few places. (7th day post-treatment) minor oedematous changes in a few places. Air spaces were reduced by pneumonic changes. (Positive control) pulmonary parenchyma indicated moderate to severe pneumonic changes. In a few places, lung fields looked like liver (hepatization). Mild to moderate congestion was present in a few places. Proteinaceous material (exudate) indicated that these were oedematous changes (Fig. 11).

v. Spleen

(Negative control) normal appearance of red and white pulp. (3rd day post-treatment) normal. (7th day post-treatment) normal. (Positive control) a severe degree of necrosis was present with the sloughing of tissue from some places during processing. Tissue debris was also present. Red and white pulp was not differentiable in some places (Fig. 11).

The histopathology of all the organs appeared normal in negative control group. However the positive group showed necrotic necrotic glomerulus in kidney, pyknotic nuclei of hepatocytes, necrotic changes in myofibres, pneumonic changes in lungs, and tissue debris in spleen as shown by black marks. The post treatment (Day 3) group showed normal appearance of all organs except cellular infiltration in lung tissues. Whereas, the post-treatment group (Day 7) represent normal appearance of all organs while a little inflammation in the lung tissues (Fig. 11).

Discussion

The mechanism of the toxic action of organophosphate involves the inhibition of acetylcholinesterase (AChE) enzyme at nerve terminals. Organophosphates are highly lipophilic compounds and once in the bloodstream, they quickly migrate to nervous tissue where intoxication is initiated through binding and subsequent inactivation of the AChE enzyme. As the active hydrolytic AChE enzyme level falls, the neurotransmitter acetylcholine pools at muscarinic and nicotinic receptors in the nervous system, autonomic ganglia, and myoneural junctions. Unregulated stimulation of receptors results in a state described as a cholinergic syndrome. It is clinically presented with hyper-salivation, lacrimation, urination, defecation, gastrointestinal distress, emesis, and meiosis. Acute toxicity results in extended periods of neuromuscular de-

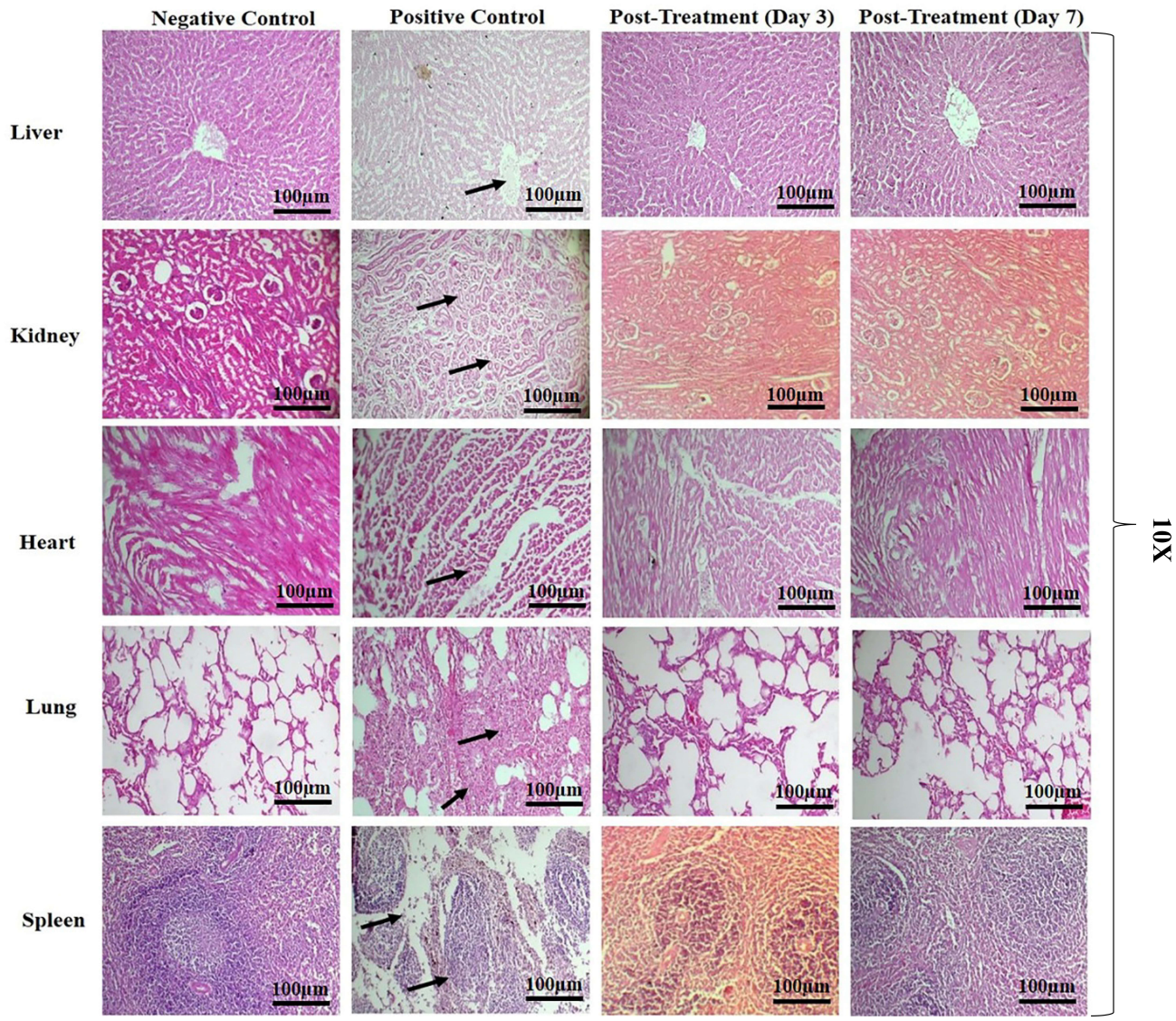

Fig. 11. Hematoxylin and Eosin staining of the liver, kidneys, heart, lungs, and spleen; comparison of histopathology for treatment groups with negative and positive control groups. Arrows indicate the changes in organs for treatment.

polarization characterized by uncontrolled muscle spasms or twitches which may lead to tachycardia, muscle weakness, or in extreme cases, respiratory muscle paralysis and acute death of the victim [27]. The clinical presentation of poisoned cases depends on the nature of the organophosphate compound, magnitude, frequency, route of exposure, age and health status of victim and solvent in formulation, etc. Available treatment options for organophosphate include the following [28,29]. (I) Competitive muscarinic antagonist (atropine), (II) Acetylcholinesterase reactivators (oximes, etc.), (III) Reversible blockers of acetylcholinesterase (pyridostigmine, physostigmine, and other carbamates), (IV) Exogenous enzymes (human butyrylcholinesterase, huBChE, bacterial phosphor-tri-esterase, etc.) and (V) Supportive treatment (anticonvulsants, emetics, fluid therapy, gastric lavage, etc.).

Atropine is the most widely used drug in organophosphate poisoning. Although effective in preventing lethality, atropine cannot be considered a specific antidote because of some limitations. Firstly, atropine is a muscarinic-blocking agent and is successful in preventing muscarinic effects while nicotinic effects continue. Therefore, nicotinic effects on muscles and central nervous system cannot be blocked by atropine alone and delayed neuropathy is inevitable [15]. Further, atropine resistance in some individuals and toxicity in others is a limiting factor in its boundless use and the situation becomes exacerbated because in acute toxicity cases, clinicians are unaware of the magnitude of exposure and hence the quantity of atropine required. Oximes, on the other hand, are purported to function as cholinesterase reactivators but they are of insufficient effectiveness in some poisonings and offer a variable spectrum of efficacy in different nerve agents and pesti-

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cide poisoning. It has been reported that oximes, although reactivate AChE to various extents but fail to reactivate 'aged' AChE therefore, have an uncertain therapeutic effect in various organophosphate poisoning [16]. Some fast-aging organophosphates (for example, soman) have an aging half-life as short as several minutes. Hence, oxime therapy in such poisoned patients could be either immediate or useless [30]. Carbamates are spontaneous reactivators of the acetylcholinesterase enzyme. These compounds block the enzyme temporarily to deny access of irreversible organophosphates to the active site of the enzyme on subsequent exposure. Hence, carbamates can only be used as a pretreatment option. The duration of protection by carbamates is less than 24 hours and also the dose cannot exceed a defined limit because carbamate poisoning itself leads to cholinergic syndrome [31]. Prophylactic administration of human butyryl-cholinesterase can prevent the cholinergic crisis from ever occurring. This plasma circulation enzyme has the ability of 1:1 stoichiometric binding with a wide range of organophosphates resulting in the deactivation of both the enzyme and substrate therefore, larger doses are required if acute poisoning is anticipated. Another drawback with this strategy is that constant replenishment of enzymes will be required to continue having any benefit. Also, the mass production of the enzyme with an extended lifetime remains a challenge. Another prophylactic strategy employs catalytic bio-scavenger in the form of phosphotriesterase from the bacterial origin, but its use is not clinically successful because of rapid clearance from tissues [18]. From all the above discussion, it can be derived that none of the available treatment options is a specific antidote for organophosphate poisoning. Although effective in preventing lethality, each one has some limitations and drawbacks which raise the need to develop a safe, potent, and specific antidote for organophosphate poisoning.

One of the applications of nanomedicine is that it makes use of nanoparticles for the treatment of different ailments [32]. The lack of a pharmaceutical antidote for organophosphate poisoning necessitates the use of nanoparticles to absorb or somehow neutralize the toxins. Researchers have explored the benefit of biomimetic nanoparticles which behave like a cell in the living system, hence they evade the immune response, have extended retention time *in-vivo* have deep penetration into tissues depending upon their size, and are biodegradable. Further, their augmented detoxification capacity makes them a fitting choice for organophosphate poisoning. Pang *et al*., (2015) [33] reported that a polymeric core surrounded by RBC membranes can successfully rescue the mice after a lethal dose of dichlorvos insecticide. Chen *et al*., (2019) [22] formulated a dual model RBC membrane-cloaked oil nano-sponge by wrapping oil nano-droplets with red blood cell membranes. Oil nano-sponge was shown to be a safe and effective antidote in therapeutic as well as prophylactic regimens in mouse models of organophosphate (viz., dichlorvos, paraoxon, and di-isopropyl fluorophosphate) poisoning [22]. Altaf *et al*., (2021) [21] demonstrated the detoxification capacity of RBC-coated polymeric nano-sponge for chlorpyrifos insecticide poisoning in rabbits. Though cell membrane-covered polymeric nanoparticles are effective detoxifying platforms, a multimodal platform is more of a value, especially in the case of severe and acute poisoning.

The present study combines the nonspecific absorbing capacity of oils with an organophosphate-capturing capacity of RBC membranes to formulate a dual model oil nano-sponge. Fish oil and soybean oil were used to formulate two types of oil nano-sponge. Chlorpyrifos, the most widely used pesticide in Pakistan [12,34], was selected as the model organophosphate, and detoxification capacity was demonstrated in rabbits. For reliable results on detoxification capacity, an oral LD_{100} dose was to be administered but there is much disagreement in the literature about the dose rate in rabbits. The toxicity depends, at large, on the solvent since chlorpyrifos is insoluble in water. One study reports LD_{100} as 500 mg/kg using ethyl alcohol as a solvent [35] while another study reports 2000 mg/kg using corn oil as a solvent [36]. Some other studies report LD_{100} values in the range that is mentioned here. Therefore, oral LD_{100} dose rates are crude estimates and different studies report different dose rates with their context. In the present study, ethyl alcohol was used as a solvent for oral dosing of chlorpyrifos, and the LD_{100} value was estimated to be 1200 mg/kg. No animal in the positive control group survived at this dose rate which increases the reliability of results in treated groups. The design of the study and results are condensed in the following text.

Rabbit's RBCs membranes were collected by hypotonic lysis using deionized distilled water. The cellular contents leaked and membranes were collected in sediment after centrifugation. Protein contents of the membrane suspension were taken as a measure of the weight of membranes and the Bradford method was used to quantify protein contents of membrane suspension. 1 mL blood of rabbit yielded RBC membrane suspension with a protein concentration of 0.2 mg/mL. Any suspension having higher or lower protein content was standardized to 0.2 mg/mL. As demonstrated by Chen *et al*., (2019) [22] that the size of the oil nano-sponge depends upon membrane to oil ratio, and the least size was obtained at a 1:4 ratio, the same ratio was used in the present study. Oil nano-sponge was formulated by mixing 0.2 mg membranes and 0.8 mg oil and subjecting the mixture to bath sonication for 20 minutes. Sonication allows membranes to completely wrap over oil nano-droplets. The same procedure was used for fish oil nano-sponge (FONS) and soybean oil nano-sponge (SONS). Dynamic light scattering measured the zeta size of FONS and SONS as 152.28 nm and 127.59 nm. The zeta size of fish oil nano-droplets and soybean oil nano-droplets was 287.48 and 176.49 nm respectively. Oil nano-droplets adjusted to

the reduced size of nano-sponges. This observation is in agreement with earlier studies which reported that the size of oil nano-sponge is variable and depends upon membrane-to-oil ratio and the addition of membranes results in reduced size of nanoparticles [22]. Likewise, the zeta potential of fish oil nano-emulsion and soybean oil nano-emulsion was –48.44 mV and –53.3 mV respectively, while the zeta potential of FONS and SONS was –15.27 mV and –12.4 mV. Here, more negatively charged oil nano-droplets became less negative after the nano-sponge was formed. This is due to the shielding effect of membranes over oil nano-droplets as was observed and reported by Dehaini *et al*., (2017) [37] that when the highly negative core of nanoparticles is shielded with less negative cover, the zeta potential of resultant nanoparticles rises. Fourier transform infrared spectroscopy (FTIR) analysis reveals that absorbance curves of FONS and SONS are nearly overlapping, thereby confirming the same surface chemistry and complete membrane cloaking on nano-droplets of two different oils. The absorbance of oils differs from their respective nano-sponge which is again evidence of membrane cover over oil nano-droplets. FTIR spectrum of chlorpyrifos and oil nano-sponges differed in absorbance peaks, but when a mixture of chlorpyrifos and nano-sponge was subjected to analysis, it generated peaks of both the nano-sponge as well as chlorpyrifos. This result confirms the adsorption of chlorpyrifos onto oil nano-sponge. Next, the cholinesterase activity of the membrane suspension and that of both oil nano-sponges was determined. The result showed that there was a little loss of activity in oil nano-sponges. Then, *in-vitro* clearance of chlorpyrifos by both types of nano-sponges was evaluated by mixing 10 mg chlorpyrifos with twofold serial dilutions of nano-sponges ranging from 0.2 mg to 0.012 mg. The mixture was bath sonicated at 40 °C for 15 minutes. This allows maximum adsorption of chlorpyrifos with oil nano-sponges resulting in precipitates of oil nano-sponges. The precipitates were removed by centrifugation and the remaining quantity of chlorpyrifos in the solution was determined by taking absorbance at 290 nm wavelength in a UV-Vis spectrophotometer. The quantity of adsorbed chlorpyrifos was calculated by subtracting the remaining quantity from the initial quantity. The results showed that increasing the quantity of oil nano-sponge results in increased clearance of chlorpyrifos from the solution. Fish oil nano-sponge was more efficient in clearance than soybean oil nano-sponge. These results confirm *in-vitro* capacity of oil nano-sponges to clear chlorpyrifos from a solution.

Oil nano-sponge capacity for *in-vivo* detoxification of chlorpyrifos was validated in rabbits. Treatment groups of FONS and SONS were given an LD_{100} dose (1200 mg/kg) of chlorpyrifos (CPS) followed for 30 min by a 2 mg/kg intravenous dose of treatment. All treated animals survived till the 14th day of observation with minimal weight loss. While animals in the positive control group died in the range of 2–5 days after oral gavage of CPS. This result was conclusive evidence of the detoxification efficacy of oil nano-sponge in CPS poisoning. To confirm the preventive potential of oil nano-sponge from poisoning, the treatment was administered before the poisoning. FONS and SONS treatment groups along with positive and negative controls were studied in parallel. Treatment groups were given IV doses of nano-sponges at three dose rates (1.5 mg/kg, 1 mg/kg, and 0.5 mg/kg) followed 30 min by a fixed (300 mg/kg) subcutaneous dose of CPS. Each treatment was repeated in quadruplicate. Results indicate that a 1.5 mg/kg dose of oil nano-sponge saved animals from poisoning while mortality occurred in the 1 mg/kg group and 0.5 mg/kg group. It can be inferred that increasing the dose of oil nano-sponge increases the chances of survival.

As already mentioned 0.2 mg oil nano-sponge can detoxify 10 mg of chlorpyrifos, the same quantities in the mixture were evaluated for safety in rabbits. Thus, the safety profile of the nano-sponge was evaluated and histopathological analysis revealed that oil nano-sponges saved vital organs from the damage of chlorpyrifos.

To sum up the results, it can be said that oil nano-sponges offered a safe and potent platform for the detoxification of organophosphate poisons. Since edible oils and cell membranes are used, the nano-sponge is a perfectly biocompatible and biodegradable platform. The present study is a step forward toward protecting the health of poisoned patients. This study, however, has not explored dose-response relations in poisoned patients. The keeping life of nano-sponge is also not determined. Nano-sponge formulated for one species was not used in other species. Although nano-sponge was proven to be effective in chlorpyrifos poisoning, its efficacy for other organophosphates is yet to be determined. These are some lacunae for further research which will pave the way towards the clinical use of oil nano-sponges for the amelioration of organophosphate poisoning.

Conclusions

In conclusion, our study successfully developed and characterized oil nano-sponges, namely fish oil nano-sponge (FONS) and soybean oil nano-sponge (SONS), for potential applications in the detoxification of organophosphate compounds. We demonstrated that these nano-sponges effectively cloaked the oil core with RBC membranes and adsorbed chlorpyrifos, a common organophosphate pesticide, onto their surfaces. Furthermore, in an *in-vivo* experiment, the oil nano-sponges showed promising potential in preventing chlorpyrifos intoxication when administered intravenously. Importantly, safety evaluations revealed that these nano-sponges could detoxify a substantial amount of chlorpyrifos without causing significant harm to major organs. These findings highlight the potential of oil nano-sponges as a novel approach for the prevention and treatment of organophosphate poisoning, with

further research and development offering the prospect of enhancing their effectiveness and safety for clinical applications.

Availability of Data and Materials

All data included in this study are available upon request by contacting with the corresponding author.

Author Contributions

Conceptualization: SA. Data formation: MUI, SA, TI, MAN. Data interpretation: SA, AM, MA, MAN, AAA, MAAM, AMH. Writing original draft: MUI, MA, MAN, AAA, MAAM, AM, AMH. Writing assistance, article submission: MAN, MA, MAAM, AMH. Review & editing: TI, MAN, AMH, SA, AAA, AM. Supervision: SA. All authors read and approved the final manuscript. All authors contributed to the important editorial changes in the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The guidelines were approved by the Biosafety and Bioethics Committee (IBC) of the University of Agriculture, Faisalabad, Pakistan (Certificate No: 1760/ORIC).

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Conflict of Interest

The authors declare no conflict of interest.

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