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Research Article

Preclinical Evaluation of the Innovative MK-7 NE: Toxicity Assessment and Safety Optimization in *In-vivo* Models

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ABSTRACT

Vitamin K is crucial in blood clotting and bone health, yet its bioavailability is limited. NEs (NE) have emerged as promising delivery systems for fat-soluble vitamins like vitamin K because they enhance solubility, control drug release, prevent enzymatic degradation, and improve therapeutic efficacy. This study investigates a novel, cold-processed, acoustically stable menaquinon-7 nanoemulsion (MK-7 NE) derived from both fermented and standard MK-7. Preclinical optimization is essential for understanding potential toxicities and improving safety. A single oral dose of a placebo and MK-7 NE at varying concentrations, based on body weight, was administered to 35 Wistar albino rats, along with a 2 mg/kg dose of a standard MK-7 solution. Plasma MK-7 concentrations were monitored for 72 hours using HPLC analysis, while histopathological examination assessed tissue degradation in the rats' organs. To evaluate acute toxicity, an adult *Danio rerio* (Zebrafish) model was exposed to a 20 mg/kg concentration of the NE. The results indicated that MK-7 NE supplementation did not cause tissue or organ damage in either model, demonstrating its safety. These findings support the potential of MK-7 NE as an effective and safe formulation for enhancing vitamin K2 bioavailability and therapeutic benefits while minimizing potential risks.

INTRODUCTION

The advantages of utilizing NE as a medication administration method encompass various aspects. Initially, the innovative MK-7 NE features minute droplets that enable uniform deposition on surfaces. As a result, the system's overall low surface tension and the reduced interfacial tension between oil-in-water droplets allow for improved processes, such as wetting, spreading, and penetration. The diminutive size of the droplets significantly reduces the effect of gravity, where Brownian motion becomes adequate to counteract gravitational effects. NE exhibits thermodynamic stability, a property that supports the spontaneous self-emulsification of the system.^[1, 2] MK-7, or MK-7, is a kind of vitamin K2 that is fat-soluble and is mainly recognized for its function in bone

health, cardiovascular health, and calcium metabolism. MK-7's bioavailability is increased in an NE form, which enhances absorption, efficacy and minimizes side effects.^[3]

NE can enhance the stability of chemically sensitive compounds by protecting them from oxidative damage and light-induced degradation. They accelerate absorption, improve bioavailability, and reduce variations in absorption. Additionally, they aid in the dissolution of hydrophobic drugs such as fat-soluble vitamins and offer the potential for controlled release and targeted drug delivery.^[1-4] Identifying unexpected adverse events during clinical trials of a new drug can pose serious risks to both health and finances. Robust preclinical studies are essential to justify further clinical testing. Through

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meticulous pharmacokinetic analysis and preclinical safety assessments of the novel form NE MK-7, the pharmaceutical industry strives to reduce these risks. However, false-negative toxicity results in preclinical testing remain a persistent challenge.^[5] This pilot study aimed to conduct a preclinical evaluation of the novel MK-7 NE (single dose) by assessing its pharmacokinetics and toxicity *in-vivo* using wistar albino rats and adult Zebrafish. The research focused on specific organs, including the kidneys, lungs, uterus, and liver, examining potentially irreversible degeneration due to the fat-soluble nature and storage characteristics of MK-7. While also highlighting the usefulness of acute toxicity models that use adult zebrafish for seven days. The zebrafish model connects *in-vitro* tests with *in-vivo* research on mammals. When evaluating toxicological effects in adult zebrafish, the activity of biochemical enzymes such as catalase (CAT), lipid hydroperoxides (LOOH), lactate dehydrogenase (LDH), and superoxide dismutase (SOD) is essential. Zebrafish are a crucial tool in environmental and pharmaceutical toxicology investigations because monitoring these biomarkers provides important insights into oxidative stress and cellular damage induced by various toxicants.^[6] Creating cutting-edge drug delivery methods is essential in today's pharmaceutical environment to get around the drawbacks of traditional formulations. By enhancing the solubility, absorption, and bioavailability of vitamin K2, the innovative MK-7 NE facilitates its more efficient use in medicinal applications, resulting in a substantial improvement. The NE is a viable option for enhancing patient outcomes in terms of bone and cardiovascular health because of its capacity to prevent MK-7 from degrading while enabling regulated release. Furthermore, by offering crucial preclinical information on the pharmacokinetics and safety profile of MK-7 NE, this study advances the expanding field of pharmaceuticals driven by nanotechnology. Treatment plans can be significantly enhanced by integrating cutting-edge drug delivery techniques, including NE, into pharmaceutical formulations, particularly for fat-soluble substances with low bioavailability. In line with ongoing efforts to develop safer, more effective, and patient-friendly treatment options, our findings support further clinical research and suggest the potential of MK-7 NE as a superior alternative to traditional vitamin K2 administration.

MATERIALS AND METHODS

Materials

Standard MK-7 and Kolliphor RH-40 were acquired from Sigma Aldrich in Bengaluru, India. Vitamin E (dl- α -Tocopherol acetate) and Tween 20 were obtained from SRL Pvt. Ltd, India. Merck Chemicals Pvt. Ltd., Mumbai, India, supplied the HPLC-grade methanol, and West Coast Pharmaceutical Ltd., India, supplied the medium-chain

triglyceride (MCT) oil. The laboratory of Jamia Hamdard CIF developed deionized water. The analytical quality chemicals and reagents used were obtained from SD Fine Chem, located in Mumbai, India. Dialysis membrane was procured from Sigma Aldrich, Germany.

Methods

NE emulsification

MK-7 oil in water NE was prepared using the cold ultrasonication method (Supplementary Information).

In-vitro kinetics release study

The innovative MK-7 NE subjected to *in-vitro* study used a dialysis membrane having a molecular weight of 12 k Dalton with various pH buffers (1.2, 6.8, 7.2). At specific time intervals (0, 0.5, 1, 2, 3, and 4 hours) was withdrawn from the dialysis membrane, then HPLC analysis had been employed.^[7]

Dose Preparation

Sample (group 1) control

The control group was fed 1% carboxymethyl cellulose (CMC) solution.

Sample for (group 2) Standard (MK7) solution

To prepare the sample for group 2 (1g (2000 ppm) of MK-7 (2 mg/g) and 5 mg of CMC were mixed well with 5 mL of distilled water using a vortex for 3 minutes. The resulting emulsion contained approximately 0.4 mg/mL of MK-7. The sample was administered to each of the five animals in (group 2) according to their individual body weight.

Sample for (groups 3-7)

The resume groups use the same sample of MK-7 NE, but it is administered at different concentrations based on the animal's body weight, as shown in (Table 1). Estimating the right dosage for experimental rats based on an *in-vitro* kinetics release study and the human daily recommended dose of at least 100 μ g may be one of the criteria.^[8]

To obtain specimens of blood from the Albino Wistar rats at predetermined intervals (0, 2, 4, 6, 24, 48, and 72 hours).^[9] The specimens were immediately centrifuged for 12 minutes at 2000 rpm for plasma separation until high-performance liquid chromatography (HPLC) had been employed for additional examination (Supplementary Figs 1-4).

In-vivo high-dose acute toxicity evaluation in Wistar rat

The toxicity of the prepared MK-7 NE was evaluated in *Wistar albino* rats (n = 35) after ethical approval from Jamia Hamdard, New Delhi. Healthy male and female Wistar rats, weighing approximately 150 g, were obtained from the Central Animal House facility at Jamia Hamdard, New Delhi (ethical approval no. 1891, year 2022, from the Ethics Committee of India. The animals were divided

Table 1: Animal group for metabolomics and pharmacokinetic analysis

Group No.	Group	Treatment protocol	No. of Animals
1	Control (placebo)	1mg of carboxymethyl cellulose (CMC) to 1mL of distilled water single dose (Oral)	5
2	Standard (MK7)	VitK2-MK7 eq. single dose of 2 mg/kg (Oral)	5
3	NE. Formulation.1	VitK2-MK7 eq. single dose of 0.5 mg/kg (Oral)	5
4	NE. Formulation.2	VitK2-MK7 eq. single dose of 1 mg/kg (Oral)	5
5	NE. Formulation.3	VitK2-MK7 eq. single dose of 2 mg/kg (Oral)	5
6	NE. Formulation.4	VitK2-MK7 eq. single dose of 10 mg/kg (Oral)	5
7	NE. Formulation.5	VitK2-MK7 eq. single dose of 20 mg/kg (Oral)	5

into different groups, provided free access to water and a normal diet, and allowed a 7-day acclimatization period. All the animals in (group 1) received vehicle control, (group 2) received 2 mg/kg, 0.5 mL of standard MK-7 solution, while (group 7) received the innovated MK-7 NE 20 mg/kg single dose, 14 days of observation were maintained.

Histopathology study

After the experiment was completed, the animals were sacrificed under CO₂ anesthesia. The kidneys, liver, lungs, and uterus were chosen from different animal groups (placebo, standard, and toxic dose). These organs undergo histopathological examination, during which they are sectioned to prepare tissue samples for analysis.^[10]

Zebrafish as a pattern for inspection toxicity and evaluating the effectiveness of drugs

The toxicological evaluation of the prepared MK-7 NE, with a high dose, was performed in adult zebrafish. The adult zebra fish were divided into six groups and subjected to the experiment for seven days as follows.^[6]

Experimental Setups

Group I

Zebra fishes in this group served as controls and were fed with normal fish pellet feed (n=10). Group II: Zebra Fishes were fed with 20 mg/Kg. Body wt. of an emulsion of vitamin K2 was mixed with normal fish feed powder (n = 10) and fed for 7 days. After the experimental period, the fish were sacrificed by the hypothermal method, and the whole-mount fish were fixed with formalin to study the histological changes and also to assess biochemical parameters commonly studied to evaluate toxicity. The pharmacological substances are lactate dehydrogenase (LDH), superoxide dismutase (SOD), catalase (CAT), and lipid peroxidation (LOOH).^[6]

Histopathology of zebrafish

The control and vitamin K2-treated fish were fixed as whole mounts. Sections were deparaffined in xylene at 65°C for 20 minutes, followed by two clearings in xylene (10 minutes each). Slides were transferred through a series

of alcohol (100, 90, 70, 50, and 30%) and finally hydrated to distilled water (10 minutes each). After 15 minutes of incubation in Mayer's haematoxylin stain, the slides were rinsed for 10 minutes under running tap water. Slides were then counterstained in eosin for 2 minutes. After removing excess eosin, the slides were dehydrated in a series of alcohol (30–100%) followed by two changes in xylene. The sections were fixed using DPX mount ant and examined under the light microscope.

Biochemical Markers in the Zebrafish Model for Assessing Toxicity and Drug Effectiveness

Lactate dehydrogenase (LDH)

To perform the LDH assay, 0.1 mL of muscle tissue and 1-mL of buffered substrate were added to a set of tubes, which were then incubated for 15 minutes at 37°C. The incubation was carried out for a further 15 minutes following the addition of 0.2 mL of NAD solution. About 1-mL of DNPH reagent was added to halt the reaction, and the tubes were then incubated at 37°C for an additional 15 minutes. Following the DNPH halt of the reaction, 0.1 mL of serum was added to the blank tubes. A Shimadzu UV spectrometer was then used to measure the generated color at λ 420 nm after adding 7 mL of sodium hydroxide solution. The same process was applied to suitable standard aliquots for the analysis. The enzyme activity was expressed IU/L.^[6, 11]

Lipid hydroperoxides analysis (LOOH)

The ferrous oxidation in xylenol orange (FOX) reagent 0.9 mL was mixed with 0.1 mL of methanol-extracted tissue lipid sample and cultivated for 30 minutes at ambient temperature. The absorbance was measured at 560 nm. The amount of hydroperoxides produced was calculated by using the molar extinction coefficient of $4.6 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$. The unit of measurement for LOOH levels is nmol/g tissue.^[12,13]

Superoxide dismutase (SOD)

In the reaction, 0.1 mL of tissue homogenate was combined with 0.5 mL of EDTA solution and 0.5 mL of carbonate buffer. Additionally, the required volume of the



enzyme was added, and the final volume was adjusted to 2.5 mL. A UV spectrophotometer was applied to quantify the increase in absorbance at 480 nm after 0.4 mL of epinephrine was added to start the reaction. In a control tube without the enzyme, 50% of the autoxidation of epinephrine to adrenochrome was performed. The enzyme activity was expressed in IU/L.^[14]

Catalase enzyme (CAT)

The tissue was homogenized with isotonic buffer and centrifuged at 3000 rpm for 10 minutes at 4°C. The pellet was disposed of, and the supernatant was gathered for additional examination. To the supernatant, 0.01 mL of ethanol was added per mL of supernatant liquid. Then, the samples were incubated in a bath of ice-cold water for 30 minutes. After incubation, 10% Triton X-100 was gathered to the 0.1 mL of supernatant and used for catalase estimation. 2 mL of sample diluted in phosphate buffer and 1-mL of H₂O₂ were blended to the reaction mixture, and extinction was read at 240 nm at 15-seconds intervals for 3 minutes. The catalase action is expressed as μ moles of H₂O₂ decomposed per min per mg protein.^[15]

RESULTS AND DISCUSSION

Histopathological Examination of Rat Tissue

To assess how a novel MK-7 formulation affects the organs and tissues of wistar albino rats (specifically renal, lung, uterus, and liver), a histopathological examination was conducted. Animals were treated with different concentrations of MK-7 and a placebo (Control), and their tissues were examined using a microscope to identify any structural or cellular changes. This histopathological analysis plays a vital role in assessing the potential toxicity, efficacy, and safety profile of the MK-7 formulation (Figs 1-4).

Histopathology of renal

H & E kidney slides of groups (1, 2, and 7) (Table 1) are respectively illustrated in (Fig. 1a, b, and c). The analysis of renal tissue from group 1, which received a placebo,

at 40x magnification revealed a normal structural architecture. There were no signs of pyknosis, apoptosis, cellular disintegration, or hypertrophic changes. The glomerulus, Bowman's capsule, mesangial cells, and podocytes appeared normal. Furthermore, there was no damage observed in the proximal convoluted tubules or distal convoluted tubules (Fig. 1a).

The examination of renal tissue from group 2 at 40x magnification, which received a single oral dose of 2 mg/kg of vitamin K2-MK7 solution, revealed significant damage to the structural architecture. There were notable signs of pyknosis and cellular disintegration. The glomerulus, Bowman's capsule, mesangial cells, and podocytes appeared disintegrated and fibrotic. Both the proximal convoluted tubules and distal convoluted tubules were also damaged (Fig. 1b). The results obtained in our study were consistent with the results obtained from the study conducted by Pucaj *et al.*, 2011, the authors found that low toxic changes were observed in the Sprague-Dawley rats for 90 days after 10 mg/kg daily and in the animals treated with 2000 mg/kg single-dose administration.^[16] The third section examined group 7 at 40x magnification, which received a single oral dose of 20 mg/kg MK-7 NE from kidney tissues (Cortex and medulla). Several normal glomeruli with regular capillary loops were observed. There was no increase in mesangial cells, and the deposition of mesangial matrix was within normal limits. The cortex and medulla exhibited scattered, dilated and congested blood vessels, while the proximal convoluted tubules, medulla, and interstitium appeared normal. There were no signs of ischemia or necrosis (Fig. 1c). MK-7 obtained from fermented natto is considered one of the safest products, as it does not produce any toxicity in animals or during clinical trials in healthy subjects.^[16,17]

Histopathology of lung

The sections examined from the lungs of groups 1, 2, and 7 are illustrated in Fig. 2a-c, respectively. The section was examined under 100X magnification from group 1 (placebo) and showed lung tissue with normal histological attributes. There were no signs of congestion, hemorrhage,

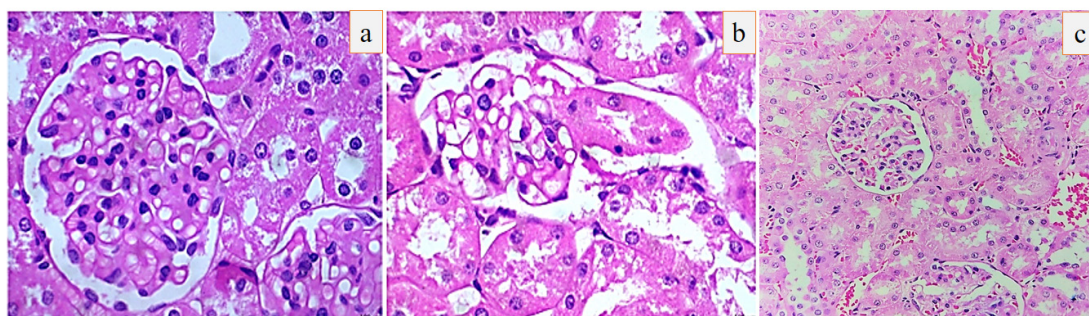


Fig. 1: a) Section examined from the group (1) (placebo) showing Renal tissue (Glomerulus, Bowman's capsule, mesangial cells, and podocytes). b) Section examined from the group (2) at a dose of 2 mg/kg (Standard MK-7 solution) showing renal tissue. (Glomerulus, Bowman's capsule, mesangial cells, and podocytes). c) Section examined the group (7) of a single mouthful of 20 mg/kg MK-7 NE from kidney tissues (Cortex, medulla)

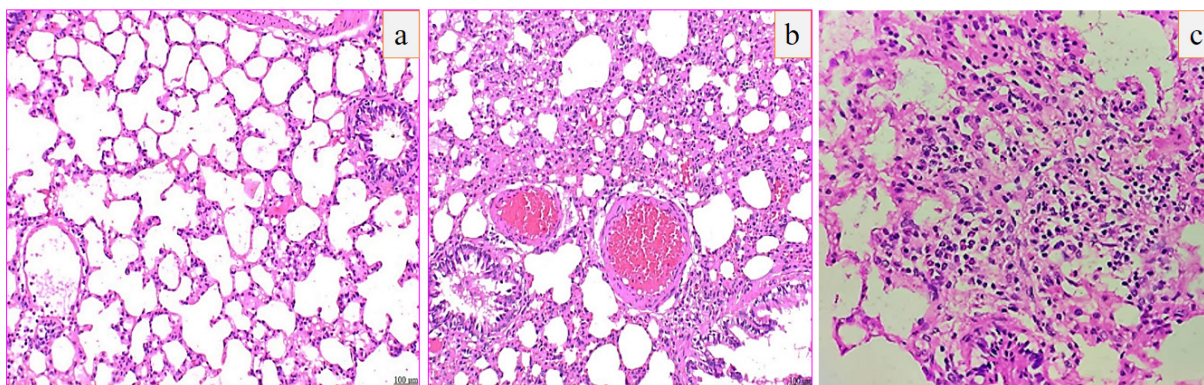


Fig. 2: a) Section examined from the group (1) [placebo] shows lung tissue (alveolar). b) A section examined from a group (2) at a single mouthful of 2 mg/kg (standard MK-7 solution) shows lung tissue (alveolar). c) The section examined from group (7), a single dose of 20 mg/kg vitamin K2-MK7 NE), shows lung tissue (alveolar)

neutrophilic or macrophagic infiltration, or bronchial damage observed (Fig. 2a). The second section examined group 2, which received a single oral dose of 2 mg/kg of (standard MK-7 solution), demonstrates lung tissue with significant damage, and congestion in the histological attributes of the lung tissue. There was severe congestion, hemorrhage, neutrophilic infiltration, bronchial damage, and mild fatty changes observed (Fig. 2b). The third section examined group 7, which received a single oral dose of 20 mg/kg MK-7 NE at 100 x magnification, showing lung tissue (alveolar), reveals increased alveolar spatial thickness due to inflammatory cell infiltration and hyperplasia of pneumocytes. Neutrophils were observed in both the alveolar space and the alveolar wall. The interspersed bronchi and bronchioles appeared normal (Fig. 2c). In our findings, both fermented MK-7 NE formulations showed small infiltration and hyperplasia of pneumocytes, while in the case of the standard MK-7 solution, the changes were more remarkable.^[19,20]

Histopathology of uterus

Sections examined from the uterus of groups (1, 2, and 7) are illustrated in (Fig. 3a-c) respectively. The section examined from group 1 at 100x magnification shows uterine tissue, which received a [placebo] (Table 1), and revealed no estrogenic changes. Both the endometrium and myometrium appeared normal. There was no edema observed in the endometrial stroma or the endometrial epithelium. Additionally, there were no signs of glandular hyperplasia, congestion, or myometrial hypertrophy (Fig. 3a). The second section examined from group 2 at a single dose of 2 mg/kg (standard MK-7 solution), shows uterine tissue, shows mild edema in the endometrial stroma, with the presence of endometrial epithelium and mild glandular hyperplasia and congestion. However, there was no hypertrophy of the myometrium (Fig. 3b). The third section examined from group 7 at a single dose of 20 mg/kg MK-7 NE shows uterine tissues with variable-

sized muscle bundles arranged in fascicles and syncytial formations. These cells had indistinct cell borders, abundant eosinophilic cytoplasm, and elongated nuclei with bland chromatin. Intervening areas showed fibro-connective tissue, edema, and scattered blood vessels (Fig. 3c). The histological changes were observed within group 7.^[21,22]

Histopathology of liver

The section examined from the liver of groups 1, 2, and 7 are illustrated in (Fig. 4). The section examined from group 1 at 40x magnification shows parenchymal liver tissue and displays a typical structural architecture of the liver parenchyma. No pyknosis, apoptosis, mild cellular disintegration, or hypertrophic changes were observed. The central vein and hepatocytes appeared normal without any signs of congestion or hemorrhage (Fig. 4a). The section examined from group 2 at a single dose of 2 mg/kg (standard MK-7 solution) at 40x magnification, shows liver parenchymal tissue, and revealed significant damage to the liver tissue. There were evident signs of pyknosis and cellular disintegration. The central vein and hepatocytes appeared disintegrated without any congestion or hemorrhage (Fig. 4b). Section examined from group 7 at a single dose of 20 mg/kg MK-7 NE showed from liver parenchymal tissues at 40x magnification, shows preserved lobular architecture. Hepatocytes were arranged in a trabecular pattern and maintained their polarity. The hepatocytes were polygonal in shape, with cytoplasm ranging from clear to eosinophilic. The nucleus was round to oval, with coarse chromatin and noticeable nucleoli. Some central veins were dilated, and focal areas exhibited periportal inflammation and mild fibrosis. No necrosis, biliary cell damage, or fatty changes were observed (Fig. 4c). Vitamin K₂ has a beneficial role in the non-alcoholic fatty liver and cholestasis and has also been found to prevent the development of cancer at a therapeutic dose.^[22,24]



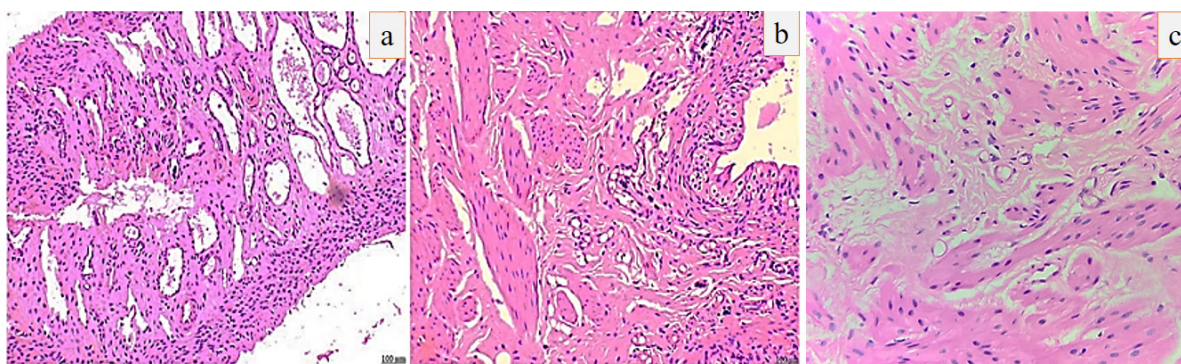


Fig. 3: a) Section examined from the group (1) [placebo] shows uterine tissue. b) The section examined from a group (2) at a single mouthful of 2 mg/kg (Standard (MK-7) solution), shows uterine tissue. c) Section examined from the group (7) as a single mouthful of 20 mg/kg MK-7 NE shows uterine tissues were seen at (400x)

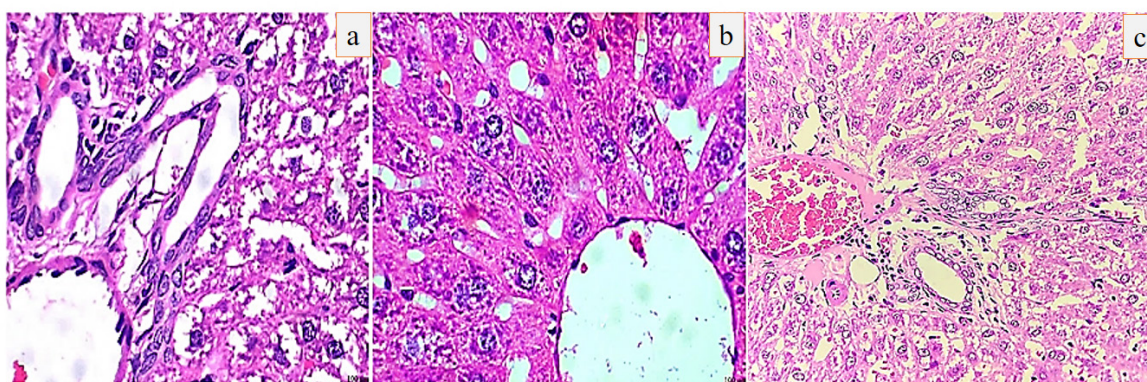


Fig. 4: a) The section examined from the group (1) Placebo] shows parenchymal liver tissue. b) The section examined from group (2) as a single mouthful of 2 mg/kg (Standard (MK-7) solution), shows liver parenchymal tissue. c) The section examined from a group (7) of a single mouthful of 20 mg/kg MK-7 NE shows liver parenchymal tissues

Histopathological Examination of Zebrafishes Tissue

The formalin-fixed sections of control and treated zebrafish indicated that there were no histopathological changes in the liver and muscle architecture of either the control or the experimental groups. The acute administration of vitamin K₂ NE did not show any inflammatory cell infiltration, edema, or nuclear condensation in the liver and other organs of the fish. Therefore, vitamin K₂ emulsion supplementation caused no tissue or organ damage of control and treated fish. Examination of formalin-fixed sections from both control and treated zebrafish indicated that there were no histopathological alterations in the liver and muscle structures of either group. The acute administration of MK-7 NE did not reveal any signs of inflammatory cell infiltration, edema, or nuclear condensation in the liver or other organs of the fish. Consequently, the supplementation of vitamin K₂ NE showed no adverse effects on the tissues and organs of both the control and treated fish as shown in (Fig. 5a, b), zebrafish gills tissue, (Fig. 5c, d), zebrafish liver tissue, and (Fig. 5e, f) zebrafish muscle tissue.

Biochemical Markers in the Zebrafish Model for Assessing Toxicity and Drug Effectiveness

Lactate dehydrogenase test (LDH)

The graph depicts that there were no notable variations between the levels of lactate dehydrogenase in the control group, with a range of 85.5 to 86.5 IU/L, and the treated group exposed to a toxic dose of MK-7 NE 20 mg/kg, with a range of 87.5 to 88 IU/L (Fig. 6a). In our study, the levels of lactate dehydrogenase in the control group and the treatment group were graphed, and the results revealed no significant increase in their production. This suggests that the toxic dose of MK-7 NE did not exhibit any signs of toxicity in the tested subjects.^[10]

Lipid hydroperoxides (LOOH)

The treated group, exposed to a toxic dose of MK-7 NE 20 mg/kg, produced approximately 14.5 nmoles of lipid hydroperoxides (LOOH), slightly higher than the control group, which recorded 13.75 nmole (Fig. 6b). Malondialdehyde (MDA) is the end-product of peroxidized

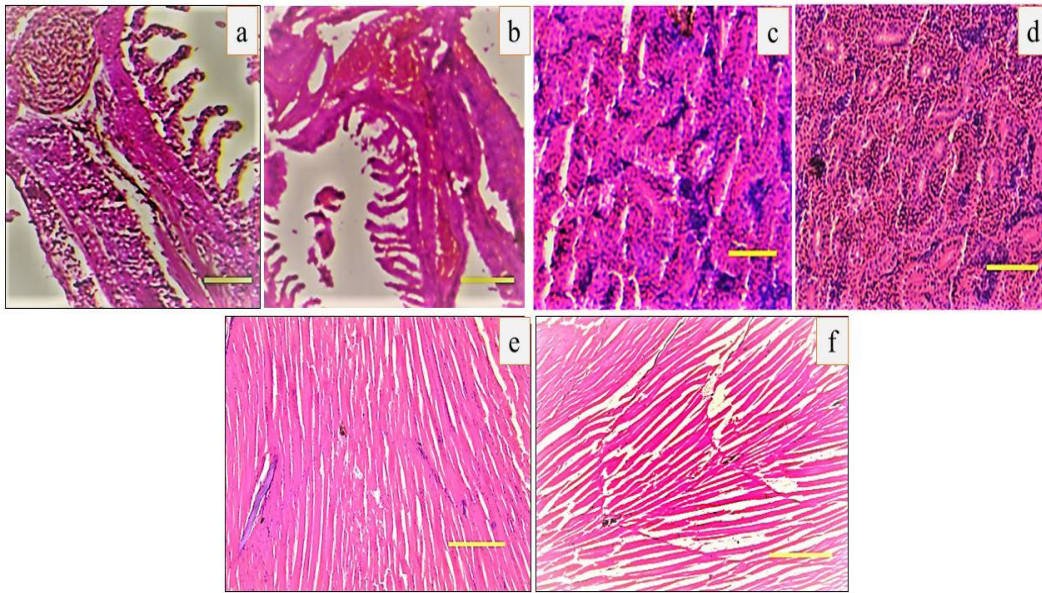


Fig. 5: a) Zebrafish in the control group exhibited normal pathological morphology of Gills. b) Gills of zebrafish treated with a dose of 20 mg/kg MK-7 NE. c) Zebrafish in the control group exhibited normal pathological morphology of the liver parenchymal. d) liver parenchymal of zebrafish treated with a dose of 20 mg/kg MK-7 NE. e) Zebrafish in the control group exhibited normal pathological morphology of muscle fibre, f) Muscle fibre of zebrafish treated with a toxic dose of 20 mg/kg MK-7 NE

polyunsaturated fatty acids and acts as a trustworthy marker for lipid peroxidation.^[25]

Superoxide dismutase (SOD)

Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST), and reduced glutathione (GSH) play a pivotal role in mitigating oxidative stress. These enzymes act as crucial biomarkers in toxicity assessments, particularly in response to potential toxicants.^[26,27] The treated group, exposed to a toxic dose of MK-7 NE 20 mg/kg, produced (SOD) approximately '0.4 to 0.45' mole/min/mg of protein, slightly lower than the control group, which recorded '0.45 to 0.54' mole/min/mg of protein (Fig. 6c).

Catalase enzyme (CAT)

Catalase (CAT) is a crucial antioxidant enzyme that plays a significant role in protecting cells from oxidative stress by catalyzing the breakdown of hydrogen peroxide (H_2O_2) into water and oxygen. This process prevents the accumulation of reactive oxygen species (ROS), which can cause cellular damage.^[6,10,15]

The treated group, exposed to a toxic dose of MK-7 NE 20 mg/kg, produced catalase (CAT) enzyme at approximately 2500 mole/min/mg of protein, slightly higher than the control group, which recorded 240 moles/min/mg of protein (Fig. 6d). Catalase (CAT) is another essential antioxidant enzyme found in nearly all living organisms. Its primary function is to break down hydrogen peroxide (H_2O_2), a harmful reactive oxygen species (ROS), into water and oxygen, thereby protecting cells from oxidative damage.

DISCUSSION

The histopathological examination of H&E stained kidney slides from groups 1, 2, and 7 (Table 1) is illustrated in Fig. 1a, b, and c, respectively. The analysis of renal tissue from group 1 (placebo) at 40x magnification revealed normal structural architecture. There were no signs of pyknosis, apoptosis, cellular disintegration, or hypertrophic changes. The glomerulus, Bowman's capsule, mesangial cells, and podocytes appeared intact, with no damage observed in the proximal or distal convoluted tubules (Fig. 1a). In contrast, renal tissue from group 2, which received a single oral dose of 2 mg/kg vitamin K2-MK7 solution, exhibited significant structural damage at 40x magnification. Marked pyknosis and cellular disintegration were observed, along with fibrosis in the glomerulus, Bowman's capsule, mesangial cells, and podocytes. Damage was also evident in both the proximal and distal convoluted tubules (Fig. 1b). These findings align with the study conducted by Pucaj *et al.* (2011), which reported low toxic changes in Sprague-Dawley rats over 90 days at 10 mg/kg daily and following a single dose of 2000 mg/kg. Renal tissue from group 7, which received a single oral dose of 20 mg/kg MK-7 NE, exhibited mostly normal glomeruli with intact capillary loops at 40x magnification. There was no mesangial cell proliferation, and mesangial matrix deposition remained within normal limits. The cortex and medulla displayed scattered dilated and congested blood vessels, but the proximal convoluted tubules, medulla, and interstitium appeared normal, with no signs of ischemia or necrosis (Fig. 1c). These results suggest that MK-7 NE, particularly in its fermented form



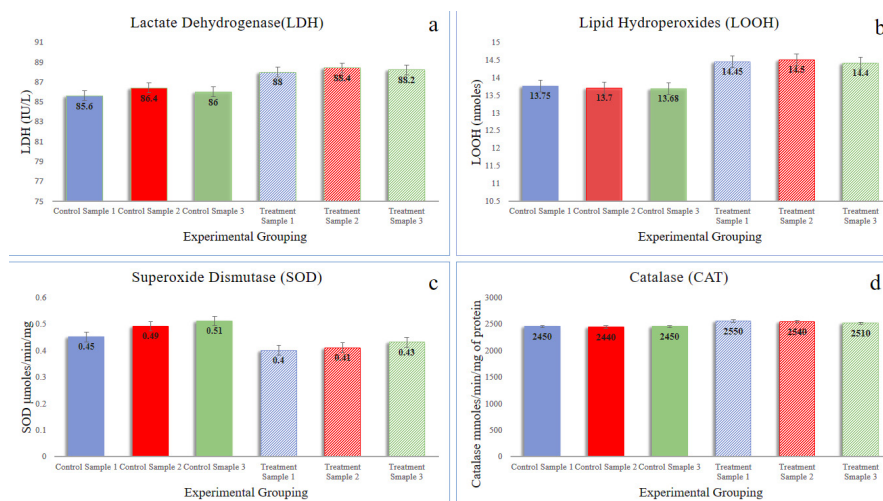


Fig. 6: a) Bar graphs illustrated lactate dehydrogenase IU/L levels for (Control and treated Zebrafish groups). b) Lipid hydroperoxides (LOOH) nmol/L level for (Control and treated Zebrafish groups). c) Superoxide dismutase (SOD) mmol/min/mg of protein level for (Control and treated Zebrafish groups). d) Catalase (CAT) mmol/min/mg of protein level for (Control and treated Zebrafish groups)

from natto, is a safe formulation with no observed renal toxicity in experimental animals. [18]

The histopathological analysis of lung sections from groups 1, 2, and 7 is illustrated in Fig. 2a, b, and c, respectively. The lung tissue from group 1 (placebo) examined at 100x magnification exhibited normal histological features with no signs of congestion, hemorrhage, neutrophilic or macrophagic infiltration, or bronchial damage (Fig. 2a). In contrast, the lung tissue from group 2, which received a single oral dose of 2 mg/kg of standard MK-7 solution, showed significant histopathological damage. Severe congestion, hemorrhage, neutrophilic infiltration, bronchial damage, and mild fatty changes were observed, indicating substantial pulmonary stress and inflammation (Fig. 2b). The lung tissue from group 7, which received a single oral dose of 20 mg/kg MK-7 NE, exhibited increased alveolar spatial thickness due to inflammatory cell infiltration and pneumocyte hyperplasia. Neutrophils were present in both the alveolar space and alveolar wall, while the bronchi and bronchioles appeared normal (Fig. 2c). These findings suggest that fermented MK-7 NE formulations induced only minor pneumocyte infiltration and hyperplasia, whereas the standard MK-7 solution caused more pronounced lung tissue alterations. [18] The histopathological analysis of uterine sections from groups 1, 2, and 7 is illustrated in Fig. 3a, b, and c, respectively. The uterine tissue from group 1 (placebo), examined at 100X magnification, exhibited normal histological architecture with no estrogenic changes. Both the endometrium and myometrium appeared intact, with no signs of edema in the endometrial stroma or epithelium. Furthermore, no glandular hyperplasia, congestion, or myometrial hypertrophy was observed (Fig. 3a). In contrast, the uterine tissue from group 2, which received a single oral dose of 2 mg/kg of standard MK-7 solution, displayed

mild edema in the endometrial stroma. The presence of endometrial epithelium, mild glandular hyperplasia, and congestion was noted, though no hypertrophy of the myometrium was detected (Fig. 3b). The uterine tissue from group 7, which received a single oral dose of 20 mg/kg MK-7 NE, exhibited variable-sized muscle bundles with fascicles and syncytially arranged cells. These cells had indistinct cell borders, abundant eosinophilic cytoplasm, and elongated nuclei with bland chromatin. Intervening areas displayed fibro-connective tissue, edema, and scattered blood vessels (Fig. 3c). [18]

The histopathological examination of liver sections from groups 1, 2, and 7 is illustrated in Fig. 4a, b, and c, respectively. The liver tissue from group 1 (placebo), examined at 40x magnification, exhibited a typical parenchymal structure with no signs of pyknosis, apoptosis, cellular disintegration, or hypertrophic changes. The central vein and hepatocytes appeared normal, with no indications of congestion or hemorrhage (Fig. 4a). In contrast, liver tissue from group 2, which received a single oral dose of 2 mg/kg of standard MK-7 solution, showed significant structural damage. Pronounced pyknosis and cellular disintegration were observed, although congestion and hemorrhage were absent. The central vein and hepatocytes appeared disorganized and structurally compromised (Fig. 4b). Liver tissue from group 7, which received a single oral dose of 20 mg/kg MK-7 NE, exhibited preserved lobular architecture. Hepatocytes were arranged in a trabecular pattern, maintaining their polarity and polygonal shape, with cytoplasm ranging from clear to eosinophilic. The nuclei were round to oval, with coarse chromatin and prominent nucleoli. While some central veins appeared dilated, and focal areas showed periportal inflammation and mild fibrosis, no necrosis, biliary cell damage, or fatty

changes were detected (Fig. 4c). These findings align with previous studies highlighting the hepatoprotective role of vitamin K2. It has been reported to offer therapeutic benefits in conditions such as non-alcoholic fatty liver disease (NAFLD) and cholestasis and may also play a role in inhibiting cancer progression at therapeutic doses.^[18] The assessment of the acute toxicity of the innovative MK-7 NE in zebrafish was used to examine histopathological changes by comparing the control group with those exposed to a toxic dose.^[28] Histopathological analysis of formalin-fixed sections from both control and treated zebrafish demonstrated no significant alterations in liver, muscle, or gill architecture. These findings suggest that acute administration of a high dose of MK-7 NE (20 mg/kg) did not induce observable toxicity in zebrafish tissues.

The liver, a critical organ for metabolism and detoxification, maintained its normal structural integrity across all groups. No signs of inflammatory cell infiltration, edema, or nuclear condensation were observed in the liver parenchyma of treated zebrafish (Fig. 5c, d). Similarly, muscle tissues from both control and treated groups retained normal morphology, with no evidence of degenerative changes or structural disruptions (Fig. 5e, f). Furthermore, Gill tissues showed no histopathological abnormalities, confirming that MK-7 NE did not affect respiratory structures (Fig. 5a, b). These results indicate that even at high doses, MK-7 NE does not cause tissue damage or toxicity in zebrafish, supporting its safety profile. The absence of inflammatory responses or structural alterations aligns with previous findings on the biocompatibility of vitamin K2 formulations. However, further long-term studies may be necessary to confirm these findings under chronic exposure conditions.^[29]

The graph (Fig. 6a) illustrates the levels of lactate dehydrogenase (LDH) in the control group and the group exposed to a high dose of MK-7 NE (20 mg/kg). The control group exhibited LDH levels within a range of 85.5 to 86.5 IU/L, while the treated group showed a slightly elevated but statistically insignificant range of 87.5 to 88 IU/L. These findings indicate that exposure to a high dose of MK-7 NE did not induce a significant increase in LDH production, suggesting an absence of notable cytotoxic effects. LDH is a key biomarker of cellular damage and metabolic stress, and its stable levels in both groups further support the safety profile of MK-7 NE at this dosage.^[30]

The levels of lipid hydroperoxides (LOOH) were slightly elevated in the treated group exposed to a high dose of MK-7 NE (20 mg/kg), measuring approximately 14.5 nmoles, compared to 13.75 nmoles in the control group (Fig. 6b). This minimal increase suggests that MK-7 NE did not induce significant oxidative stress at this dosage. Lipid hydroperoxides are primary products of lipid peroxidation, which can lead to cellular damage if excessively accumulated. However, the observed LOOH levels remained within a normal physiological range,

indicating the absence of substantial oxidative damage. Additionally, malondialdehyde (MDA), a reliable marker of lipid peroxidation and oxidative stress, was not elevated to a concerning extent.^[31]

In this study, the treated group exposed to a high dose of MK-7 NE (20 mg/kg) exhibited SOD activity ranging from 0.4 to 0.45 moles/min/mg of protein, slightly lower than the control group, which recorded 0.45 to 0.54 moles/min/mg of protein (Fig. 6c). Despite this minor reduction, the difference was not substantial enough to indicate significant oxidative stress or toxicity. Superoxide dismutase (SOD) is a critical antioxidant enzyme that neutralizes superoxide radicals, thereby preventing cellular damage. A slight decrease in SOD activity may suggest a mild oxidative response to MK-7 NE, but given that the reduction was minimal, the findings suggest that MK-7 NE does not exert substantial oxidative stress at the tested dose.^[32]

In toxicology studies, measuring CAT activity provides valuable information about the organism's capacity to mitigate oxidative stress and detoxify harmful substances. Lipid peroxidation is the term used to describe the oxidative deterioration of fats, or lipids, in cell membranes, resulting in the formation of lipid peroxides and other reactive aldehyde products. In this study, the treated group exposed to a high dose of MK-7 NE (20 mg/kg) exhibited a CAT enzyme activity of approximately 2500 moles/min/mg of protein, which was slightly higher than the control group, which recorded 240 moles/min/mg of protein (Fig. 6d). The increase in CAT activity suggests a potential adaptive response of the antioxidant defense system to counteract mild oxidative stress. Measuring CAT activity in toxicology studies is crucial for assessing the organism's capacity to detoxify harmful substances and mitigate oxidative stress. Since lipid peroxidation results in the oxidative degradation of lipids, leading to the formation of lipid peroxides and reactive aldehydes, the observed increase in CAT activity may indicate an enhanced defense mechanism in response to MK-7 NE exposure. However, the findings also suggest that the toxic dose did not induce severe oxidative damage, as the increase in CAT activity remained within a physiological range.^[26,27]

CONCLUSION

Regarding (a single dose of 20, 2 mg/kg of the standard solution of MK-7 and a placebo), the histopathology of the Wistar Albino rat's kidney, lung, uterus, and liver was discussed. It was evident from the histology slides that MK7 NE had little or no harmful side effects. The examination of formalin-fixed tissue sections from both control and treated zebrafish indicated that there were no histopathological alterations in the liver and muscle structures of either group. The acute administration of vitamin K2 emulsion did not reveal any signs of inflammatory cell infiltration, edema, or nuclear condensation in the liver or other organs of the



fish. Consequently, the supplementation of vitamin K2 emulsion showed no adverse effects on the tissues and organs of both the control and treated zebrafish and rats. This work showed promise as a less hazardous and more bioavailable substitute for conventional MK-7 solutions, revealing important new information on the safety and effectiveness of a novel MK-7 NE formulation. The results are strengthened by the use of robust statistical techniques in conjunction with histopathological and biochemical studies. A narrow dose range, brief exposure, and a lack of mechanistic insights are some of the study's drawbacks. By investigating longer-term effects, wider dose ranges, and molecular mechanisms, as well as by validating the results in clinical trials, future research should fill in these gaps.

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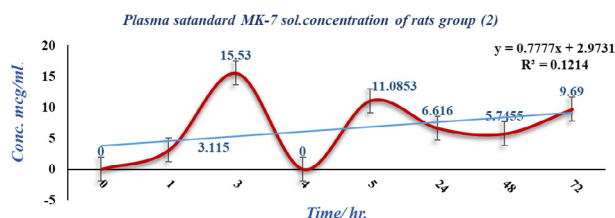


Fig. S1: Pharmacokinetic profile, time-dependent concentration accumulation and depilation of standard VitK2 MK-7 at concentration solution of 2mg/kg (Oral) in rats plasma for group (2)

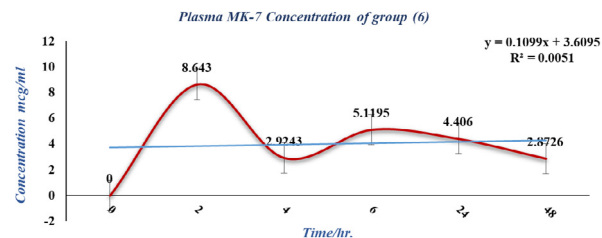


Fig. S3: Pharmacokinetic profile, time-dependent concentration accumulation and depilation of VitK2 MK-7 NE at concentration of 10 mg/kg (Oral) in rats plasma for group (6)

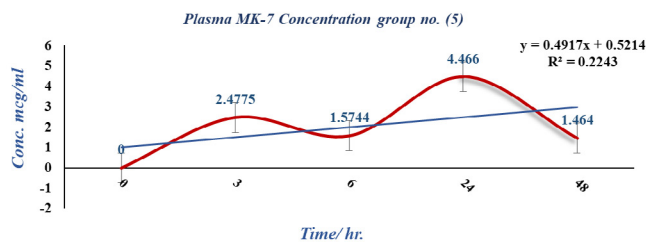


Fig. S2: Pharmacokinetic profile, time-dependent concentration accumulation and depilation of standard VitK2 MK-7 NE at concentration of 2 mg/kg (Oral) in rats plasma for group (5)

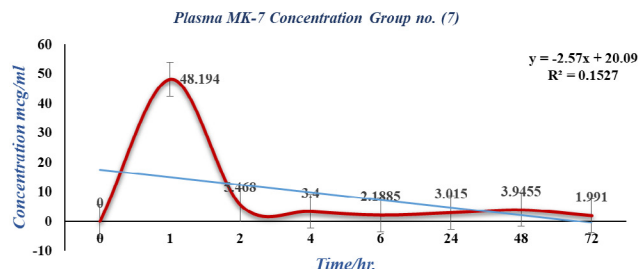


Fig. S4: Pharmacokinetic profile, time-dependent concentration accumulation and depilation of VitK2 MK-7 NE at concentration of 20 mg/kg (Oral) in rats plasma for group (7)

