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Original Article

Safety assessment of menaquinone-7 for use in human nutrition

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ABSTRACT

Vitamin K occurs widely in foods and has been shown to have a beneficial effect on the cardiovascular system, as well as anticancer, anti-inflammatory, and antiosteoporosis properties. A previous study indicates that long-chain menaquinone-7 may be more bioavailable than vitamin K and short-chain menaquinones. In the present study, acute, subacute toxicity and genotoxicity assays were carried out to evaluate the safety of oral menaquinone-7 in albino Wistar rats. Oral administration of menaquinone-7, at a concentration of 2000 mg/kg, did not cause toxic symptoms in either male or female rats. A subacute toxicity study also proved the safety and tolerance of prolonged treatment (for 90 days) with menaquinone-7 in rats, as evidenced by biochemical, hematological, and urine parameters as well as by histopathological analysis. Genotoxicity and mutagenicity studies were performed by comet, micronucleus, and Ames tests on *Salmonella typhimurium* strains, which showed cellular safety and nonmutagenicity of menaquinone-7. The results indicate the safety of menaquinone-7 for human consumption.

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1. Introduction

It was discovered over 80 years ago that food-derived components are essential factors in blood coagulation. In 1929, experimental studies at the University of Copenhagen using chicks fed a diet extremely low in fat revealed that low-fat-diet birds developed a hemorrhagic condition and their blood coagulation time was slower than that in the control group [1]. Further investigations showed that the deficiency of a fat-soluble vitamin, vitamin K, was responsible for the hemorrhagic tendency. Vitamin K occurs in two biologically active forms: phylloquinone (vitamin K1) and menaquinones (vitamin K2). Vitamin K1 is produced by green and leafy vegetables, and algae, whereas vitamin K2 is predominantly of microbial origin [2,3]. The major form of dietary vitamin K is considered to be K1, accounting for ~90% of total vitamin K intake [3]. Vitamin K2 is found in animal products, meat, dairy, eggs, and fermented foods such as cheese, yoghurt, and the traditional Japanese food natto (*Bacillus natto* fermented soya beans); vitamin K2 is also synthesized by intestinal microflora [4].

In addition to its role in the synthesis of hepatic blood-coagulation proteins, vitamin K has been found to play a role in bone health, cardiovascular health, prevention of cancer, suppression of inflammation, prevention of brain oxidative damage, sphingolipid synthesis, and osteoporosis [5–9]. Epidemiological studies from Japan and Europe suggest an association between poor vitamin K2 (but not vitamin K1) intake and increased postmenopausal bone loss, arterial calcification (notably in diabetes), end-stage renal disease, and cardiovascular disease during normal aging, as well as an increased risk of bone fracture [10,11]. Supplementation with vitamin K2 may prevent age-related bone loss and improve bone strength, arterial elasticity, and cardiovascular health [12–15]. Available information indicates that current intake of vitamin K, particularly K2, may not be sufficient for the maintenance of bone health and cardiovascular health [16,17].

Compared to phylloquinone, menaquinones, especially long-chain menaquinone-7, may be beneficial for bone and soft tissue, specifically for protein carboxylation and production. This is probably because menaquinone-7 is better absorbed and is more bioavailable than vitamin K1 or short-chain menaquinones such as menaquinone-4. Because there is a paucity of studies on the toxicity of menaquinones, especially long-chain menaquinones, we present in this paper a systematic toxicity and safety study of oral vitamin K2–7 in rodents.

2. Materials and methods

2.1. Chemicals

Vitamin K2–7 (MenaquinGold) was provided by Viridis Biopharma Pvt. Ltd. (Mumbai, India). All other chemicals used were commercially available in the purest form.

2.2. Animals

Male and female 7–8-week-old albino Wistar rats were obtained from the experimental animal facility at Gujarat Ayurved University, Jamnagar, India. Animals were housed in polypropylene cages under a controlled atmosphere (25 ± 1°C at 60–70% relative humidity) with a 12-hour light–12-hour dark cycle [18]. All animals were fed with Amrut brand rat pellet feed supplied by Pranav Agro Ltd. (Pune, India) and tap water *ad libitum*. Following at least 1 week of acclimatization, the rats were divided randomly into seven groups of eight animals each for experiments, as discussed below. Menaquinone-7 compound was suspended in a propylene glycol vehicle. Test formulations were administered to the animals through oral gavage. All procedures were in accordance with the guiding principles established by the Animal Care Committee of our University.

2.3. Oral acute toxicity

The toxicity study was conducted according to the modified the Organisation for Economic Co-operation and Development (OECD) protocol as practiced by the Institute for Post Graduate Teaching and Research in Ayurveda and was cleared by the Institutional Review Board (IRB) (IAEC-04/09-10/Proj-02) of Gujarat Ayurveda University, Udupi, India. Healthy, 10–12-week-old Albino Wistar rats ($n = 40$) of average weight (150–210 g) were randomly divided into five groups, with four male and four female rats in each group, and kept in separate cages. The animals were supplied *ad libitum* with water and pellet feed throughout the study, and subjected to overnight fasting prior to dosing with menaquinone-7 or placebo. The control group received propylene glycol vehicle, and each treated group received menaquinone-7, at doses of 0.5 mg/kg, 1.0 mg/kg, 10 mg/kg, or 20 mg/kg, once daily for 14 days. LD_{50} value was determined by administration of a single dose of 2000 mg/kg menaquinone-7 for 14 days. The animals were monitored for general behavior, toxic signs and symptoms, or mortality during the experimental period. At the end of the study, the animals were sacrificed by CO_2 asphyxiation and examined for gross changes in vital organs.

2.4. Ninety-day subacute oral toxicity in rats

2.4.1. Animal treatment

The protocol employed was a modified version of the OECD guideline 408. Ten animals per group, five male and five female Wistar Albino rats, were used instead of 10 male and 10 female rats as in the original OECD protocol. The animals were oral treated with 0.1 mg/kg, 0.5 mg/kg, and 1.0 mg/kg menaquinone-7 once daily for a period of 90 days. Animals of the control group received propylene glycol as the vehicle. Menaquinone-7 solution was prepared fresh every day and administered at a constant volume of 1 mL/100 g body weight between 8 AM and 9 AM. Behavior, mortality, and changes in body weight and food consumption were recorded. Upon completion of 90-day subchronic protocol sacrifice, autopsy of animals and histopathology of selected organs were performed. The number of experimental animals per group was approved by the Ethics Committee of the Institutional Review Board of Gujarat Ayurveda University (IAEC-04/09-10/Proj-02).

2.4.2. Biochemical and hematological analysis

Evaluation of blood biochemistry was carried out in all the animals on Day 15, Day 45, and Day 91 at the time of sacrifice.

On Day 90, all animals were fasted overnight. Blood was collected by supraorbital puncture, with the help of microcapillary tubes under mild ether anesthesia. Blood sugar, serum urea, creatinine, uric acid, total cholesterol, triglycerides, total protein, albumin-to-globulin ratio (A/G ratio), liver enzymes serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT), alkaline phosphatase activity, and serum calcium levels were estimated with a clinical analyzer (ERBA CHEM-5; GMI, Inc., Ramsey, MN, USA).

Hematological parameters were measured using an autocell counter (MS-9 veterinary hematology cell counter; Melet Schloesing, France), including total white blood cell (WBC) count, total lymphocyte count, total monocyte count, total granulocyte count, lymphocyte percentage, monocyte percentage, granulocyte percentage, red blood cell (RBC) count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, RBC distribution width, and clotting time.

2.4.3. *Urine analysis*

Qualitative urine analysis of the experimental animals was carried out using AMES multiple kits on Day 15 and Day 45, and at the time of sacrifice on Day 91. Urine was collected in dry-ice-cooled beakers. After collection, the urine samples were thawed, vigorously mixed, and analyzed for specific gravity and pH.

2.4.4. *Histopathology analysis*

Male and female rats were sacrificed on Day 91 and autopsied. The abdomen was opened through midline incision, followed by dissection of the selected organs that were then cleaned and weighed. The organs were placed in containers filled with 10% formalin for tissue preservation, and histopathology of the brain, pituitary, thymus, lymph node, heart, lungs, spleen, seminal vesicles, uterus, skin, trachea, liver, stomach, jejunum, kidney, testis, prostate, and ovary was determined.

2.4.5. *Statistical analysis*

The Student *t* test for paired data was employed for comparing differences within the group and one-way analysis of variance (one-way ANOVA) was employed with Dunnet's multiple *t* test as a *post hoc* test for comparison of observed changes among different groups. For this purpose, Sigmastat software (version 3.1) was used.

2.5. *Genotoxicity study*

Albino Wistar rats ($n = 60$; 30 males and 30 females), average weight 200–300 g, were randomly divided into six groups, each group containing five male and five female rats, and placed in separate cages. All animals had *ad libitum* tap water and pellet feed. The animals received menaquinone-7 at a dose of 0.1 mg/kg or 1.0 mg/kg once daily for 28 days. Two groups were maintained as controls for comparative assessment of genotoxicity through comet assay and micronucleus test. Animals in control groups received cyclophosphamide at a dose of 40 mg/kg body weight intraperitoneally on Day 29. Colchicine was administered on Day 30 to three groups and one control group, and 0.1 mg/kg or 1.0 mg/kg menaquinone-7 to the other groups. Two hours after colchicine administration, blood was collected from all animals for genotoxicity assessment using comet assay. The animals were sacrificed by cervical dislocation and evaluated for gross pathology of internal organs, and femur bones were collected for chromosomal aberration and micronucleus tests.

2.6. *Ames test*

Mutagenic effect was examined by *Salmonella typhimurium* reverse mutation assay at selected histidine loci in five tester strains, viz. TA1535, TA97a, TA98, T100, and TA102, in the presence and absence of a metabolic activation system (S9). The tester strains were exposed to menaquinone-7 in triplicate cultures at doses of 0.02 mg, 0.06 mg, 0.2 mg, 0.6 mg, or 2 mg. The plate incorporation method was followed for the test.

Suspensions of bacterial cells were exposed to the test article in the presence and absence of an exogenous metabolic activation system. These suspensions were mixed with an overlay agar and plated immediately onto minimal medium. After a 48–72-hours incubation period, revertant colonies on vehicle control plates were checked for sterility. The plates were observed for uniform lawn of auxotrophs and the colonies for histidine revertants as prototrophs. Histidine revertant colonies per plate were counted, and the mean number of colonies at each test point was calculated.

2.7. *Statistical analysis*

Results were expressed as the means ± standard deviation. Differences between groups were evaluated using one-way analysis of variance, followed by Dunett's *t* test. All statistical analyses were performed using the statistical software SPSS 11.0 (SPSS Ltd., Working, Surrey, UK). A p value <0.05 was considered statistically significant [19].

3. Results

3.1. *Acute toxicity study*

No physical or behavioral changes and no mortality were recorded for all experimental rats after 14 days' gavage administration of various concentrations of menaquinone-7. A gradual physiological gain in body weight occurred in both male and female animals of all experimental groups, including the control group. No statistically significant difference in body weight gain was observed among the test groups and between test groups and the control group. The test compound did not produce any mortality or clinical signs of toxicity at the highest treatment dose of menaquinone-7 (20 mg/kg). The test compound did not produce any observable toxic effects except for mild irritability in two animals out of eight (25%) in the dose group of 1 mg/kg of menaquinone-7. Water and food consumptions were not affected by administration of the formula in all dosing groups and the control group. The results of 14-day oral administration of menaquinone-7 in an acute toxicity study showed no adverse effects of the test compound on either sex of Wistar rats. For

LD_{50} analysis, animals survived the 14-day observation period with no significant change in body weight, and no symptoms of distress or toxic effects. The study showed that the LD_{50} value of the formula is more than 2000 mg/kg body weight.

3.2. *Ninety-day subacute oral toxicity*

3.2.1. *General observation*

All treatment and placebo receiving groups exhibited a normal body weight gain pattern. In male rats of the control group, the average body weight gain from the baseline to Day 90 was 66 g, with 82 g, 87 g, and 62 g gains using menaquinone-7 at concentrations of 0.1 mg/kg, 0.5 mg/kg, and 1 mg/kg, respectively. In female rats, the average body weight gain by Day 90 was 30 g in the control group, and 35 g, 33 g, and 48 g in the menaquinone-7 group at concentrations 0.1 mg/kg, 0.5 mg/kg, and 1 mg/kg, respectively. These weight gains are not statistically significant. Moreover, administration of 0.1 mg/kg, 0.5 mg/kg, and 1 mg/kg menaqinone-7 to male rats did not statistically affect the average weight of the liver, thymus, kidney, spleen, testis, seminal vesicles, prostate, and uterus to a significant extent in comparison to the sham-fed control group. Female rats receiving menaquinone-7 at a dose of 0.5 mg/kg showed a statistically significant ($p < 0.05$) decrease in heart weight in comparison to the control group. The average heart weight was 0.333 ± 0.009 g in the control group and 0.292 ± 0.010 g in the menaquinone-7 group (0.5 mg/kg).

3.2.2. *Effect of menaquinone-7 on biochemical parameters in rats*

Male and female rats administered menaquinone-7 at concentrations of 0.1 mg/kg, 0.5 mg/kg, and 1 mg/kg did not show a statistically significant effect on the blood levels of liver enzymes, including SGPT, SGOT, and alkaline phosphatase (ALP); serum glucose; total protein; creatinine; and blood urea levels in comparison to the control group. In the male group receiving menaquinone-7 at a dose of 0.1 mg/kg, a statistically significant decrease in uric acid level was observed on Day 45. A comparison of baseline values (3.24 ± 0.22 mg/dL) compared to baseline value of 3.82 ± 0.31 (mg/dL) ($p < 0.05$), but did not show a statistically significant difference from the corresponding values in the control group (3.30 ± 0.31 mg/dL) (Table 1). In the 0.5 mg/kg and 1 mg/kg menaquinone-7 groups, a statistically significant ($p < 0.05$) increase was observed in the uric acid level when compared to the baseline levels (from 2.70 ± 0.22 mg/dL to 3.82 ± 0.24 mg/dL on Day 90 in the 0.5 mg/kg group, and from 3.82 ± 00.31 to 4.64 ± 0.20 on Day 45 and from 3.82 ± 0.31 to 4.02 ± 0.14 on Day 90 in the 1 mg/kg group). The increase of uric acid level observed in the 1 mg/kg menaquinone-7 male group on Day 45 was also statistically significantly higher ($p < 0.05$) than that in control male rats. There was no change in the uric acid levels in the female rats as compared to the baseline and control values (Table 1).

The A/G ratio did not change statistically significantly in 0.5 mg/kg and 1 mg/kg male rat groups on Day 45 and Day 90 in comparison to the respective baseline values as well as control group values. However, in the 0.1 mg/kg menaquinone-7 group, a statistically significant decrease in the A/G ratio was observed on Day 45 as compared to the baseline (from 1.04 ± 0.06 to 0.59 ± 0.07; $p < 0.01$) and the control values (0.59 ± 0.07 in the 0.1 mg/kg menaquinone-7 group compared to 1.16 ± 0.08 in the control group). In female rats, the control group showed a statistically significant ($p < 0.05$) increase in the A/G ratio on Day 90 in comparison to the baseline values (1.29 ± 0.01 on Day 90 compared to the baseline value of 1.06 ± 0.07). In addition, on Day 90, a statistically significant increase in the A/G ratio was observed in the 0.5 mg/kg group in comparison to the baseline values (1.33 ± 0.05 compared to the baseline value of 1.06 ± 0.03) and in the 0.1 mg/kg group in comparison to the control group value (1.45 ± 0.04 compared to 1.29 ± 0.01 in the control group; $p < 0.01$; Table 2). In female rats treated with menaquinone-7 at a dose of 0.1 mg/kg, a statistically significant decrease in the A/G ratio was observed on Day 45 in comparison to the baseline values (from 1.30 ± 0.33 to 0.69 ± 0.02; $p < 0.01$).

The effects of menaquinone-7 administration on serum albumin and globulin levels are shown in Table 2. In female rats treated with 0.1 mg/kg menaquinone-7, serum albumin level on Day 45 was statistically significantly lower in comparison to the baseline values ($p < 0.01$), with no statistically significant differences between control group and menaquinone-7-receiving groups (0.5 mg/kg and 1 mg/kg). The serum globulin level in male rats of the control and 0.5 mg/kg

Table 1 – Effect of the 90-day subacute toxicity of menaquinone-7 on uric acid (mg/dL) level in rats.

Uric acid (mg/dL)	Menaquinone-7 concentrations			
	Control	0.1 mg/kg	0.5 mg/kg	1 mg/kg
Male rats				
Day 0	3.50 ± 0.02	3.96 ± 0.16	2.70 ± 0.22	3.82 ± 0.31
Day 45	3.30 ± 0.31	$3.24 \pm 0.22^{*}$	3.78 ± 0.29	$4.64 \pm 0.02^{*,\#\#}$
Day 90	3.40 ± 0.14	0.42 ± 0.21	$3.82 \pm 0.24^{**}$	$4.02 \pm 0.14^{\#}$
Female rats				
Day 0	3.54 ± 0.16	3.30 ± 0.17	2.96 ± 0.75	3.54 ± 0.29
Day 45	3.64 ± 0.12	3.16 ± 0.24	3.88 ± 0.57	3.66 ± 0.44
Day 90	3.76 ± 0.14	3.74 ± 0.20	4.14 ± 0.10	3.68 ± 0.17

$^{*}p < 0.05$ with reference to initial values in the concerned group (paired *t* test).
$^{**}p < 0.01$ with reference to initial values in the concerned group (paired *t* test).
$^{\#}p < 0.05$ with reference to values in the control group (paired *t* test).
$^{\#\#}p < 0.01$ with reference to values in the control group (paired *t* test).

Table 2 – Effect of 90-day subacute toxicity of menaquinone-7 on serum albumin, globulin levels, and A/G ratio in rats.

Parameters	Menaquinone-7 concentrations			
	Control	0.1 mg/kg	0.5 mg/kg	1 mg/kg
Serum albumin (mg/dL)				
Male rats				
Day 0	4.56 ± 0.19	4.84 ± 0.14	4.46 ± 0.09	4.36 ± 0.18
Day 45	4.28 ± 0.22	3.04 ± 0.30**	4.53 ± 0.12	4.50 ± 0.22
Day 90	4.66 ± 0.20	4.52 ± 0.37	4.32 ± 0.14	4.72 ± 0.15
Female rats				
Day 0	4.72 ± 0.09	4.58 ± 0.09	4.60 ± 0.12	4.58 ± 0.10
Day 45	4.34 ± 0.14**	3.10 ± 0.11***,##	4.28 ± 0.26	4.64 ± 0.17
Day 90	4.46 ± 0.16	4.95 ± 0.09	4.78 ± 0.13	4.66 ± 0.17
Serum globulin (mg/dL)				
Male rats				
Day 0	4.80 ± 0.11	4.68 ± 0.18	4.54 ± 0.17	4.40 ± 0.35
Day 45	3.62 ± 0.18**	5.18 ± 0.09*,##	3.60 ± 0.16*	4.00 ± 0.18
Day 90	3.84 ± 0.14*	3.54 ± 0.21*	3.34 ± 0.13**	3.70 ± 0.07
Female rats				
Day 0	4.48 ± 0.21	3.64 ± 0.34	4.32 ± 0.08	4.58 ± 0.15
Day 45	3.62 ± 0.24	4.42 ± 0.10	3.84 ± 0.16*	4.02 ± 0.15*
Day 90	3.50 ± 0.24	3.34 ± 0.12	3.58 ± 0.07**	3.58 ± 0.13**
A/G ratio				
Male rats				
Day 0	0.95 ± 0.04	1.04 ± 0.06	1.00 ± 0.06	1.01 ± 0.09
Day 45	1.16 ± 0.08	0.59 ± 0.07**	1.26 ± 0.07	1.13 ± 0.09
Day 90	1.22 ± 0.08	1.30 ± 0.17	1.30 ± 0.07	1.27 ± 0.03
Female rats				
Day 0	1.06 ± 0.07	1.30 ± 0.32	1.06 ± 0.03	1.00 ± 0.04
Day 45	1.21 ± 0.08	0.69 ± 0.02##	1.11 ± 0.55	1.16 ± 0.08
Day 90	1.29 ± 0.01*	1.45 ± 0.04##	1.33 ± 0.05*	1.31 ± 0.08

*$p < 0.05$ (paired t test) with reference to initial values in the concerned group.
**$p < 0.01$ (paired t test) with reference to initial values in the concerned group.
***$p < 0.001$ (paired t test) with reference to initial values in the concerned group.
##$p < 0.01$ with reference to values in the control group (paired t test).
A/G ratio = albumin to globulin ratio.

menaquinone-7 groups was found to be statistically significantly decreased on Day 45 and Day 90 in comparison to the respective baseline values ($p < 0.01$). Male rats who received menaquinone-7 at a dose of 0.1 mg/kg demonstrated a statistically significant increase of globulin levels on Day 45 ($p < 0.01$) but a statistically significant decrease in globulin values on Day 90 ($p < 0.01$), as compared to the baseline and control group. No statistically significant changes in globulin levels were found in the 1 mg/kg menaquinone-7 group, in either male or female rats, as compared to the respective baseline and control values. A statistically significant increase in globulin levels in comparison to the control group was observed on Day 45 in female rats at 0.1 mg/kg dose of menaquinone-7 ($p < 0.01$). In the 0.5 mg/kg and 1 mg/kg dose groups, female rats showed a statistically significant decrease in globulin levels on Day 45 and Day 90 in comparison to the respective baseline values ($p < 0.001$ and $p < 0.05$). However, no statistically significant difference was observed in globulin levels between the control group and menaquinone-7-administered group (Table 2).

The results of total cholesterol analysis of male rats in both the control group and menaquinone-7-administered group displayed a statistically significant decrease in the total serum cholesterol on Day 45 in comparison to the baseline levels ($p < 0.05$ in the control group and $p < 0.01$ in the menaquinone-7 group; Table 3). The blood cholesterol levels in menaquinone-7-receiving groups (all dose groups) did not differ significantly from those of the control group, in both male and female rats, on Day 90. Female rats treated with 0.5 mg/kg menaquinone-7 showed a statistically significant decrease ($p < 0.05$) in blood cholesterol on Day 45 in comparison to the baseline values. In addition, the level of blood cholesterol in the 1 mg/kg menaquinone-7 group on Day 45 was significantly lower than that in the control group ($p < 0.05$). High-density lipoprotein (HDL)-cholesterol levels in male rats at all time points were not statistically significantly changed from the baseline and in comparison to the control group (Table 3). In female rats, there was no statistically significant change in HDL-cholesterol levels from the baseline. However, in comparison to the control group, HDL-cholesterol levels in all dose groups were found to be statistically significantly lower on Day 90 ($p < 0.05$). On Day 45, serum triglyceride levels in male rats of the control and 0.1 mg/kg menaquinone-7 groups were found to be statistically significantly lower than the respective baseline levels ($p < 0.05$; Table 3). Triglyceride levels on Day 45 in male rats administered the 1 mg/kg dose were statistically significantly increased in comparison to the baseline ($p < 0.05$), but showed no statistically significant difference on Day 90 from the baseline and control levels.

Table 3 – Effect of 90-day subacute toxicity of menaquinone-7 on serum total cholesterol, HDL-cholesterol, and triglyceride levels in rats.

Parameters	Menaquinone-7 concentrations			
	Control	0.1 mg/kg	0.5 mg/kg	1 mg/kg
Total cholesterol (mg/dL)				
Male rats				
Day 0	180.82 ± 5.62	194.72 ± 0.30	180.20 ± 4.12	186.00 ± 5.10
Day 45	164.08 ± 4.12*	176.44 ± 5.22**	164.84 ± 9.49*	157.80 ± 7.20
Day 90	168.72 ± 18.99	185.72 ± 2.80	181.10 ± 3.45	183.24 ± 3.77
Female rats				
Day 0	195.78 ± 1.43	173.40 ± 2.29	186.20 ± 3.60	180.20 ± 3.51
Day 45	185.94 ± 5.05	171.40 ± 3.54	161.48 ± 5.99*	161.66 ± 5.11#
Day 90	188.52 ± 2.74	181.96 ± 4.64	183.20 ± 2.31	184.80 ± 2.03
HDL-cholesterol (mg/dL)				
Male rats				
Day 0	34.94 ± 1.86	36.02 ± 1.32	35.48 ± 1.63	33.00 ± 3.10
Day 45	30.94 ± 1.15	35.72 ± 1.85	34.36 ± 2.08	33.54 ± 1.41
Day 90	37.14 ± 2.01	33.24 ± 0.65	32.54 ± 1.01	31.10 ± 1.12
Female rats				
Day 0	35.66 ± 1.22	30.40 ± 1.03	35.40 ± 1.86	35.60 ± 0.98
Day 45	35.92 ± 1.60	32.82 ± 1.89	37.16 ± 0.53	37.04 ± 1.04
Day 90	39.10 ± 0.57	31.98 ± 1.54	32.88 ± 0.76	34.80 ± 0.50#
Triglyceride (mg/dL)				
Male rats				
Day 0	107.20 ± 4.85	105.12 ± 4.87	112.60 ± 13.65	94.20 ± 8.48
Day 45	71.64 ± 5.83*	92.18 ± 3.39*	107.20 ± 2.52#	100.90 ± 2.74#
Day 90	110.00 ± 5.00	108.70 ± 3.85	116.60 ± 7.01	122.00 ± 16.66
Female rats				
Day 0	100.46 ± 4.14	93.00 ± 3.39	104.80 ± 5.27	118.20 ± 15.04
Day 45	111.40 ± 4.93	94.04 ± 5.76	111.30 ± 3.53	97.80 ± 2.08
Day 90	129.20 ± 5.71*	110.50 ± 4.06	113.70 ± 4.10	118.54 ± 4.49

*$p < 0.05$ with reference to initial values in the concerned group (paired t test).
**$p < 0.01$ with reference to initial values in the concerned group (paired t test).
#$p < 0.05$ with reference to values in the control group (paired t test).
HDL = high-density lipoprotein.

3.2.3. *Effect of menaquinone-7 on hematological parameters in rats*

A statistically significant decrease in total WBC count was observed on Day 90 in both male and female rats of the control group in comparison to the baseline counts (Table 4). Rats receiving menaquinone-7 at a dose of 0.1 mg/kg showed a significantly decreased WBC count on Day 45 in comparison to the baseline count ($p < 0.01$). The percentage of lymphocytes in the control male and female rats was not statistically significantly different on Day 45 and Day 90 in comparison to the baseline and menaquinone-7-receiving groups. Menaquinone-7 treatment (0.1 mg/kg) caused a statistically significant decrease of lymphocytes in male rats ($p < 0.05$) on Day 90. A statistically significant increase in lymphocytes was seen in female rats receiving 1 mg/kg of menaquinone-7 in comparison to the baseline on Day 45 ($p < 0.05$). The percent of granulocytes did not change statistically significantly in male rats of the 0.5 mg/kg and 1 mg/kg dose groups on Day 45 and Day 90 in comparison to the baseline values. However, male rats treated with menaquinone-7 at a dose of 0.1 mg/kg showed a significant increase of granulocytes in comparison to the baseline on Day 45 ($p < 0.05$).

Table 5 shows the RBC count and hematocrit values after menaquinone-7 treatment. RBC count and hematocrit values in both male and female rats in the control group were significantly decreased on Day 90 in comparison to the baseline. Male rats treated with 0.1 mg/kg menaquinone-7 demonstrated a significant increase in RBC count and hematocrit values on Day 45 in comparison to the baseline. A significant increase of RBC count on Day 45 was also observed in both male and female rats treated with 1 mg/kg menaquinone-7 in comparison to the baseline ($p < 0.01$). No statistically significant change in RBC and hematocrit values was observed between the control and three dosing male and female groups at any testing time point.

Hemoglobin levels were statistically significantly decreased on Day 45 and Day 90 in male control rats in comparison to the baseline ($p < 0.01$). Female rats in the control group showed a significant decrease of hemoglobin levels on Day 90 ($p < 0.05$). A significant increase of hemoglobin levels was noted on Day 45 in male rats treated with 0.1 mg/kg menaquinone-7 ($p < 0.05$), whereas a decrease was seen at a dose of 1 mg/kg ($p < 0.05$), in comparison to the baseline. There was no statistically significant change in hemoglobin levels between the control and three dosing male and female groups at any testing time point. Additionally, there was no statistically significant difference in the mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, mean

Table 4 – Effect of 90-day subacute toxicity of menaquinone-7 on hematological parameters in rats.

Parameters	Menaquinone-7 concentrations			
	Control	0.1 mg/kg	0.5 mg/kg	1 mg/kg
Total WBC count ($10^3/\mu L$)				
Male rats				
Day 0	8.44 ± 1.30	6.32 ± 0.71	7.32 ± 0.10	8.72 ± 1.06
Day 45	6.50 ± 0.14	5.06 ± 0.57	5.14 ± 0.54	7.48 ± 0.90
Day 90	4.28 ± 0.99*	4.74 ± 0.99	7.76 ± 2.47	6.32 ± 2.23
Female rats				
Day 0	8.52 ± 1.16	9.08 ± 1.37	7.88 ± 1.58	7.80 ± 1.01
Day 45	5.76 ± 0.29	5.26 ± 0.22**	8.48 ± 1.80	8.24 ± 0.31
Day 90	3.14 ± 0.33***	4.30 ± 0.88	4.08 ± 0.58	4.50 ± 1.14
Lymphocytes (%)				
Male rats				
Day 0	67.22 ± 5.75	50.48 ± 3.73	59.88 ± 7.07	49.26 ± 6.08
Day 45	64.34 ± 3.89	52.92 ± 0.30	66.46 ± 2.06	55.58 ± 9.92
Day 90	59.40 ± 10.98	23.34 ± 7.89*	55.08 ± 2.43	47.52 ± 7.11
Female rats				
Day 0	50.52 ± 5.78	56.16 ± 9.64	61.46 ± 10.29	50.96 ± 4.31
Day 45	66.14 ± 3.29	56.58 ± 2.99	70.98 ± 4.08	71.88 ± 1.07*
Day 90	52.96 ± 10.63	36.93 ± 14.44	56.98 ± 3.53	46.16 ± 5.49
Granulocyte (%)				
Male rats				
Day 0	13.94 ± 6.54	13.90 ± 7.24	17.80 ± 8.97	29.92 ± 7.24
Day 45	24.66 ± 5.09	36.68 ± 0.54*	23.70 ± 2.02	33.04 ± 9.93
Day 90	24.58 ± 10.68	59.28 ± 11.97	26.28 ± 4.28	32.48 ± 8.93
Female rats				
Day 0	29.10 ± 9.74	29.14 ± 9.98	21.48 ± 9.75	27.88 ± 8.93
Day 45	21.60 ± 5.26	35.62 ± 4.26	25.98 ± 2.89	16.78 ± 1.39
Day 90	32.54 ± 11.55	41.75 ± 18.07	26.26 ± 5.02	35.94 ± 7.21

*$p < 0.05$ with reference to initial values in the concerned group (paired t test).
**$p < 0.01$ with reference to initial values in the concerned group (paired t test).
***$p < 0.001$ with reference to initial values in the concerned group (paired t test).
WBC = white blood cell.

corpuscular volume, and RBC distribution width values and in clotting time between the control and three dosing male and female groups at any testing time point.

3.2.4. *Effect of menaquinone-7 on urine parameters in rats*

The analysis of urine on Day 45 and Day 90 did not show statistically significant changes in specific gravity and pH in either sex of all the treatment groups in comparison to the baseline and control group. In the 0.5 mg/kg and 1 mg/kg test drug-administered groups (in females), a significant increase in specific gravity of urine was observed on Day 45 in comparison to initial values. However, no such increase could be observed on Day 90. Change in pH at the dose level of 1000 μg/kg was significant in comparison to the control group on Day 45.

3.2.5. *Histopathology evaluation*

No remarkable histopathological changes were observed in the organs of the control animals and animals treated with menaquinone-7 (either sex), including the forebrain, midbrain, hindbrain, cerebellum, pituitary gland, trachea, lung, spleen, thymus, lymph node, heart, liver, kidney, stomach, intestine (jejunum), testis, seminal vesicle, ventral prostate, skin, and bone marrow. However, an increase in the size of the organ and proliferation of epithelium in uterine were found in one to two rats in menaquinone-7-treated groups at all concentrations, and the remaining rats exhibited normal cytoarchitecture.

3.3. *Genotoxicity testing*

The administered menaquinone-7 and placebo did not cause any symptoms of toxicity, and response of all animals to handling was normal at testing intervals. There were no gross pathological findings in the body organs of any experimental animal. Potential genotoxic effects were examined using chromosomal aberration, and micronucleus and comet assay tests. The mean frequency of chromosomal aberrations and mean number of micronuclei did not differ statistically significantly between the groups receiving 10 times the recommended dose of menaquinone-7 (1 mg/kg for 15 consecutive days) as compared to the group that had not received menaquinone-7 supplementation. The olive tail movement/cell in the comet assay also revealed no qualitative or quantitative DNA damage in the cells of the animals supplemented with menaquinone-7 as compared to control animals.

3.4. *Mutagenicity*

Menaquinone-7 was found to be nonmutagenic for tested *S. typhimurium* strains TA 1535, TA97a, TA98, TA100, and TA102. In Ames test, the same frequencies of histamine

Table 5 – Effect of 90-day subacute toxicity of menaquinone-7 on RBC count, hemoglobin content, and hematocrit in rats.

Parameters	Menaquinone-7 concentrations			
	Control	0.1 mg/kg	0.5 mg/kg	1 mg/kg
RBC count (10^6/μL)				
Male rats				
Day 0	12.38 ± 0.46	9.80 ± 0.65	8.75 ± 0.63	9.53 ± 0.99
Day 45	13.16 ± 0.77	13.22 ± 0.22*	10.27 ± 1.55	10.07 ± 1.50
Day 90	9.46 ± 0.62*	13.25 ± 1.91	9.56 ± 0.73	13.57 ± 1.46
Female rats				
Day 0	9.78 ± 0.21	7.79 ± 0.56	9.51 ± 1.16	8.17 ± 1.40
Day 45	10.82 ± 0.08	9.88 ± 1.61	8.96 ± 0.04	11.92 ± 2.10**
Day 90	8.74 ± 0.37**	11.41 ± 1.90	9.94 ± 0.58	11.43 ± 1.42
Hemoglobin (g/dL)				
Male rats				
Day 0	23.04 ± 0.17	19.12 ± 1.20	17.76 ± 0.93	19.94 ± 1.54
Day 45	21.18 ± 0.37**	21.58 ± 0.74*	17.30 ± 1.91	15.98 ± 2.38*
Day 90	15.60 ± 0.33***	21.20 ± 2.10	18.04 ± 1.37	21.96 ± 1.12
Female rats				
Day 0	18.30 ± 0.74	16.20 ± 1.01	18.60 ± 1.96	16.30 ± 2.36
Day 45	17.86 ± 1.05	16.70 ± 2.08	16.06 ± 0.73	18.46 ± 1.95
Day 90	15.18 ± 0.23*	18.62 ± 1.92	18.22 ± 1.11	20.08 ± 1.61
Hematocrit (%)				
Male rats				
Day 0	69.96 ± 3.05	59.54 ± 4.48	51.84 ± 4.13	56.30 ± 5.93
Day 45	72.20 ± 3.39	72.18 ± 1.27*	58.28 ± 8.34	60.22 ± 7.51
Day 90	54.35 ± 3.04*	72.98 ± 9.23	57.96 ± 4.31	74.00 ± 4.54
Female rats				
Day 0	53.30 ± 0.99	46.54 ± 2.46	54.72 ± 5.91	48.02 ± 7.92
Day 45	60.04 ± 3.70	55.92 ± 8.34	52.54 ± 2.20	67.88 ± 10.34**
Day 90	50.32 ± 1.67*	63.18 ± 9.08	59.86 ± 3.96	70.20 ± 8.84

*$p < 0.05$ with reference to initial values in the concerned group (paired t test).
**$p < 0.01$ with reference to initial values in the concerned group (paired t test).
***$p < 0.001$ with reference to initial values in the concerned group (paired t test).
RBC = red blood cell.

revertant colonies were observed, at all concentrations of menaquinone-7, in tester strains with or without the presence of a metabolic activation system. Bacterial growth pattern in the presence of metabolic activation was comparable to that observed in the vehicle control groups. The incorporation of menaquinone-7 did not produce any change in microbial growth patterns, with the number of revertant colonies indicating the tested preparation as nonmutagenic.

4. Discussion

In recent years, vitamin K, especially vitamin K2, was found to have several important physiological functions in addition to its traditional role in the formation of coagulation factors in the liver. Data on the safety profile of phylloquinone, vitamin K1, menatetrenone, and menaquinone-4 are available, but there are no safety data on menaquinone-7 that is commonly used in nutritional supplements. Epidemiological data point to the inadequate intake of menaquinone-7 leading to many chronic diseases, including coronary heart disease, cancer, and osteoporosis [5,20]. The length of the side chain in menaquinones is considered to play an additional role in their bioavailability. Menaquinones with medium-length side chains (e.g., MK-7) are better absorbed as compared to those with short (MK-4) or long (e.g., MK-8 and MK-9) side chains [21–23]. The different pharmacokinetic properties of MK-7 may result in different biological properties, distinct from other menaquinones. In view of the growing use of MK-7 in nutritional and food supplements, it is important to test MK-7 in systematic toxicity studies.

The mean intake of total vitamin K in European countries and USA is estimated to be 60–250 μg/day in adults (Expert Group on Vitamins and Minerals). In Japan, a traditional preparation of fermented beans, a rich source of vitamin MK2–7 known as natto, is sold in a size that usually contains 40–100 g of fermented beans, preferably served at breakfast. A typical serving pack of natto may contain approximately 350 μg menaquinone-7. We have, therefore, assumed that the dietary intake of menaquinone-7 may vary from 60 μg/day to 350 μg/day. This amount is equivalent to 5.4–31.5 μg of MK2-7 per kg per day in rats. The concentrations used in our 90-day subchronic toxicity study (0.1 mg/kg/day, 0.5 mg/kg/day, and 1 mg/kg/day) are approximately 3–200 times higher than those calculated from the estimated dietary intake in humans.

The outcome of daily oral administration of menaquinone-7 at three concentrations (0.1 mg/kg, 0.5 mg/kg, and 1 mg/kg) for 90 days to albino Wistar rats of both sexes was evaluated by assessing changes in behavior, body weight, blood biochemistry and morphology, urine analysis, and gross and histopathology of selected organs. Change in the animal body weight patterns during the course of the 90-day study is a sensitive indicator of the overall toxicity potential of a test compound. Any drug that produces significant toxicity to the

vital organs or degenerative changes in tissues, or interferes with the nutrient absorption and/or availability is likely to cause a decrease in the animal body weight. In the 90-day regimen with menaquinone-7, Wistar albino rats (of both sexes) showed a nonsignificant body weight gain in comparison to the control rats. This finding indicates that subchronic administration of menaquinone-7 in doses of up to 200 times higher than that calculated from daily human dose does not produce body organ involution and/or does not interfere with the metabolic utilization of food-derived nutrients. There was a statistically significant increase in the average liver weight in the experimental group of male rats receiving menaquinone-7 at a concentration of 0.1 mg/kg. Female rats orally treated with menaquinone-7 at doses of 0.5 mg/kg and 1 mg/kg showed a significantly decreased liver weight. Moreover, a statistically significant decrease in the average heart weight was observed in female animals receiving 0.5 mg/kg of menaquinone-7. These findings describe statistically significant changes in body organ weight that were not dose dependent and occurred in one sex, and not both sexes, of animals with normal gross and histopathology of the body organs.

The following factors were taken into consideration while evaluating biochemical and hematological parameters in the toxicological study of menaquinone-7. Each study parameter was measured three times at the baseline on Day 45 and Day 90. It was further evaluated whether the statistically significant changes occurred in a dose-dependent manner. If the changes were dose dependent and the differences were statistically significant from the control group values, then the changes were considered statistically significant from a toxicological point of view. The results of blood biochemistry parameters and morphology did not show any statistically significant differences between the control and menaquinone-7-receiving groups. External examination of the control and experimental animals, including examination of the body, head, limbs, and discharges from natural orifices such as mouth, nose, vagina, anus, and penis at autopsy did not reveal any significant macroscopic changes in any tested animals.

During the microscopic examination, no histopathological lesions or changes were found in any of the organs studied, except for the following changes in reproductive organs. The only observation of note is the observation of features of ovarian, uterus, and testicular stimulation at higher dose levels (0.5 mg/kg and 1 mg/kg). In the ovaries, an increase in size and number of mature follicles was observed in groups receiving menaquinone-7 at concentrations of 0.5 mg/kg and 1 mg/kg. In the uterus, an increase in size of the muscular layer and proliferation of the epithelial layer were observed in groups receiving menaquinone-7 at concentrations of 0.5 mg/kg and 1 mg/kg. In testis, the evidence of increased spermatogenesis was observed in groups receiving menaquinone-7 at concentrations of 0.5 mg/kg and 1 mg/kg. These effects in reproductive organs were not significant in groups receiving menaquinone-7 at 0.1 mg/kg dose level.

The potential genotoxicity of menaquinone-7 was assessed by employing two assay systems, Ames and micronucleus test. The results indicate that menaquinone-7 does not have genotoxic and mutagenic potentials. Interestingly, a statistically significant increase in the proportion of polychromatic cell population was observed in the menaquinone-7 group (compared to the control group), which might have contributed to increased hematopoietic activity of bone marrow in the menaquinone-7 group. Typically, compounds affecting bone marrow adversely, such as cytotoxic drugs, decrease polychromatic cell population.

In conclusion, in this study, various toxicological evaluations confirmed the safety of oral intake of menaquinone-7. Acute and subacute toxicity studies demonstrated oral administration of menaquinone-7 with no adverse effects in both sex of animals. These results indicate that menaquinone-7, in the MenaquinGold form, was very well tolerated by the experimental animals, and did not show any preclinical or clinical toxicity at the dose levels that are several times higher than the average estimated dietary intake of menaquinone-7.

Conflicts of interest

All authors declare no conflicts of interest.

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