A 90 - DAY REPEATED DOSE ORAL GAVAGE TOXICITY EVALUATION STUDY OF THE COMBINED MYO-INOSITOL PRODUCT S-FEMME IN *WISTAR* RATS

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In the present study, a sub-chronic oral toxicity study was designed to assess the safety of S-FEMME powder in Wistar rats. The 90-day toxicity study was conducted by oral administration at doses of 0 (control), 0.12, and 0.36 sachets/kg/day for a continuous period of 90 days in Wistar rats. The evaluated parameters included body weight, clinical observations, hematological and biochemical analyses, gross necropsy, and histopathological assessment. The findings demonstrated that oral exposure to S-FEMME at both dose levels did not induce any clinical sign, no mortality, toxicologically significant changes in hematology, clinical biochemistry, macroscopic findings, or histopathological examination. In summary, the no-observed-adverse-effect-level (NOAEL) of S-FEMME is greater than 0.36 sachets/kg/day when administered orally for 90 consecutive days at two doses in Wistar rats.

Keywords: S-FEMME, repeated dose toxicity, experimental animals.

I. INTRODUCTION

Infertility is a major global reproductive health concern, affecting approximately 10-15% of couples of reproductive age worldwide.[1] The World Health Organization defines infertility as the failure to achieve a clinical pregnancy after at least 12 months of regular unprotected sexual intercourse.[2] Among the causes of female infertility, ovulatory disorders and diminished ovarian reserve are considered key contributors, reflecting underlying endocrine and reproductive dysfunctions.[3] Therefore, early diagnosis and timely intervention are critical to improving treatment outcomes.

Ovarian stimulation is widely used in infertility treatment to increase follicular development and the likelihood of conception, employing agents such as clomiphene citrate, letrozole, gonadotropins, and assisted reproductive technologies, including in vitro fertilization.[4] However, high-dose stimulation is often associated with suboptimal oocyte and embryo quality, as well as adverse effects such as ovarian hyperstimulation syndrome and metabolic disturbances. Consequently, strategies aimed at improving ovarian function and oocyte quality prior to ovulation induction have gained increasing attention.

Recent evidence highlights the beneficial role of bioactive compounds, particularly myo-inositol, in improving insulin sensitivity, endocrine balance, oocyte quality, and ovarian responsiveness, thereby enhancing assisted reproductive outcomes.[5] In addition, antioxidants and metabolic cofactors such as L-carnitine, vitamin E, pantothenic acid, biotin, and folic acid contribute to oxidative stress reduction and support cellular metabolism during folliculogenesis. Oxidative stress is now recognized as a critical factor in female infertility, with antioxidant capacity being positively associated with pregnancy outcomes.[6]

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S-FEMME is a formulation combining myo-inositol with antioxidant and metabolic supporting components to enhance follicular development and female reproductive potential. Although the individual ingredients have been widely reported to be safe, their combined and repeated administration necessitates a thorough safety evaluation.[7-11] Therefore, repeated-dose toxicity studies are essential to assess tolerability and ensure safety for long-term use. This study was conducted to evaluate the repeated-dose toxicity of S-FEMME in an experimental animal model, providing scientific evidence supporting its safety for further development and clinical application.

II. MATERIALS AND METHODS

1. Preparation of S-FEMME

S-FEMME was manufactured by Fortex Nutraceuticals Ltd, Bulgaria, in compliance with GMP-WHO standards. S-FEMME is formulated as a powder in sachets, with each sachet containing 1000 mg myo-inositol, 300 mg L-carnitine, 20 mg α-TE of vitamin E (DL-α-tocopheryl acetate), 10 mg pantothenic acid, 500 mcg biotin, and 400 mcg folic acid, along with other excipients sufficient to one sachet.

The recommended human dose is one sachet per day, dissolved in 250 mL of water and administered orally. S-FEMME is intended to support female hormonal balance and is recommended for women planning pregnancy.

Animals and housing conditions

Adult *Wistar* rats of both sexes, weighing 170 - 250 g, were used in the repeated-dose toxicity study. Animals were housed under standard laboratory conditions at 25 ± 1°C, appropriate humidity, and a 12-hour light/dark cycle. Rats were acclimatized for 5 - 7 days prior to the experiment and maintained throughout the study at the Department of Pharmacology, Hanoi Medical University. Standard laboratory feed was provided, and water was available ad libitum.

2. Methods

The repeated-dose oral toxicity study of S-FEMME was conducted in accordance with OECD guidelines. 408.[12]

A total of 30 *Wistar* rats were randomly divided into three groups, with 10 animals per group:

- Group 1 (control group, n=10): rats were administered orally distilled water 10 ml/kg/day

- Group 2 (n=10): rats were administered S-FEMME orally at 0.12 sachet/kg/day, equivalent to the recommended human dose (conversion factor 6).

- Group 3 (n=10): rats were administered S-FEMME orally at 0.36 sachet/kg/day, corresponding to three times the equivalent human dose.

Animals were administered the test substance or vehicle once daily by the oral route for 90 consecutive days. The signs and parameters were checked during the study:

Experimental observations: General condition, body weight changes of animals;

Clinical pathology (hematology and serum chemistry:

- Evaluation of hematopoietic function through red blood cell count (RBC), mean corpuscular volume (MCV), hemoglobin concentration (Hb), hematocrit (Hct), total white blood cell count (WBC), differential WBC count, and platelet count (PLT).

- Evaluation of liver function and damage assessment through serum biochemical parameters: AST, ALT, total cholesterol (TC), albumin (ALB), and total bilirubin (TBIL).

- Evaluation of kidney function through serum creatinine (CREA) levels.

Parameters were checked before treatment and at 30, 60, and 90 days after treatment.

Gross necropsy and histopathology:

At the end of the experiment, rats were euthanized after blood collection, and the internal organs (heart, liver, spleen, kidney, and lungs) were removed and observed for any gross lesions of potential toxicological significance.

Histological examination was performed on the preserved organs and tissues of the main test animals from both the control and high-dose groups. The livers and kidneys of 30% of the animals in each group were preserved in 10% buffered formaldehyde solution for histopathological studies. Histopathological lesions were evaluated in the liver and kidney tissues according to predefined criteria. The fixed tissues were trimmed, processed, embedded in paraffin, sectioned with a microtome, placed on glass microscope slides, stained with hematoxylin and eosin, and examined by light microscopy. Liver lesions included activated Kupffer cells, sinusoidal dilatation, cytoplasmic vacuolation, hydropic degeneration, karyolysis, and karyorrhexis, while kidney lesions comprised granular casts, cellular casts, protein casts, pycnotic cells, and hydropic degeneration. The severity of histopathological damage was graded on a semi-quantitative scale as follows: 0 = normal (no change); 1 = mild (1 - 30%); 2 = moderate (31 - 70%); and 3 = severe (>70%).[13,14]

Data analysis

Data were collected and processed using SPSS version 20.0 and Microsoft Excel 2020. Differences among the study groups were analyzed using one-way ANOVA followed by the Turkey post hoc test, as well as the Kruskal-Wallis test" Results are presented as mean ± standard deviation (SD). Statistical significance was considered at $p < 0.05$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with the control group. $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$, $^{\Delta\Delta\Delta}p < 0.001$ compared with the time point "before treatment".

III. RESULTS

1. General clinical observations

The general condition, food, and water consumption were assessed. There were no mortality, and SFEMME-related clinical observations (cage-side or detailed clinical observations) for any main test animal during the study. Animals had normal locomotor activities and good feedings.

2. Body weight changes

Figure 1 showed that there were no change in mean body weight or body weight gain attributable to SFEMME administration. Mean weekly body weights for rats in Groups 2 and 3 were comparable to their respective controls throughout the study. No significant difference was observed at 30, 60, and 90 days of treatment; body weight in all rats increased substantially compared with body weight "before treatment".

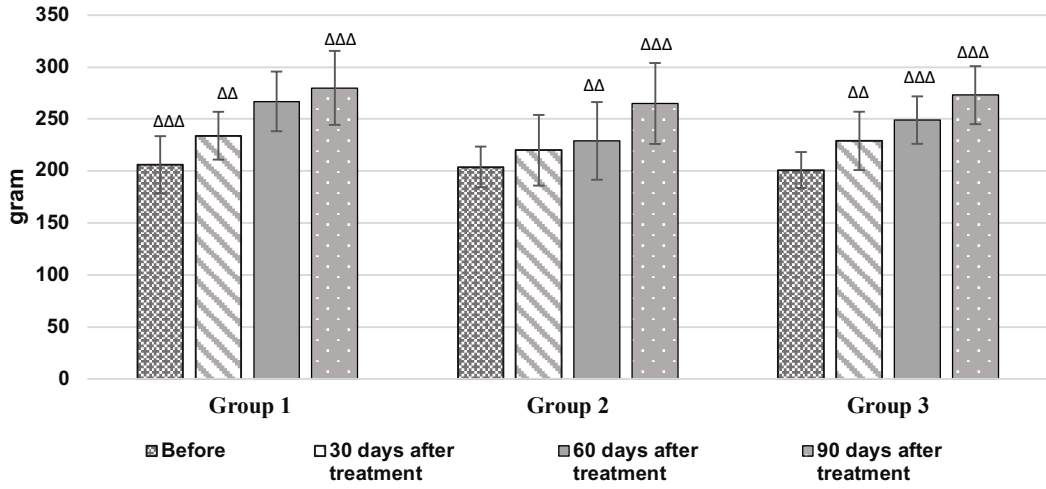


Figure 1. The effect of S-FEMME on body weight changes

$^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$, $^{\Delta\Delta\Delta}p < 0.001$ compared with the time point "before treatment."

3. Effect of S-FEMME on hematology and serum chemistry

There were no significant difference in red blood cell count, hematocrit, hemoglobin level, platelet count, total WBC count, and WBC between S-FEMME groups and the control group (p>0.05) (shown in **Table 1**).

Table 1. Effect of SFEMME on hematological parameters

Parameters	Group	Before treatment	30 days after treatment	60 days after treatment	90 days after treatment
RBC (G/L)	Group 1	8.95 ± 1.47	8.55 ± 0.94	8.44 ± 1.01	9.46 ± 1.61
	Group 2	9.03 ± 0.93	8.23 ± 1.20	8.85 ± 1.05	9.28 ± 1.57
	Group 3	8.66 ± 1.03	8.01 ± 1.16	8.11 ± 1.44	8.71 ± 1.03
Hb (g/dL)	Group 1	12.40 ± 1.67	11.03 ± 1.40	11.23 ± 1.06	11.98 ± 1.49
	Group 2	11.71 ± 1.34	10.73 ± 1.21	10.95 ± 1.26	11.85 ± 1.86
	Group 3	11.22 ± 0.93	10.29 ± 1.48	10.59 ± 1.79	11.06 ± 1.20
Hct (%)	Group 1	45.83 ± 7.95	43.69 ± 3.53	42.91 ± 4.56	44.35 ± 6.51
	Group 2	44.13 ± 3.33	41.02 ± 4.52	43.10 ± 4.67	45.75 ± 7.34
	Group 3	43.99 ± 3.30	44.32 ± 3.59	40.44 ± 4.61	43.44 ± 4.19
MCV (fL)	Group 1	47.30 ± 3.77	48.80 ± 1.62	48.00 ± 1.05	47.80 ± 1.81
	Group 2	49.20 ± 3.55	47.60 ± 1.26	47.10 ± 1.20	49.60 ± 2.95
	Group 3	49.70 ± 3.50	48.80 ± 1.23	49.00 ± 1.41	48.40 ± 3.63

Parameters	Group	Before treatment	30 days after treatment	60 days after treatment	90 days after treatment
WBC (G/L)	Group 1	9.84 ± 2.23	8.97 ± 1.38	9.64 ± 1.48	10.52 ± 2.39
	Group 2	9.49 ± 2.04	8.39 ± 1.58	9.80 ± 1.62	9.70 ± 2.80
	Group 3	10.35 ± 1.68	8.44 ± 1.90	9.85 ± 2.02	9.89 ± 1.17
Lym (%)	Group 1	72.69 ± 4.62	71.13 ± 3.75	70.14 ± 6.69	71.14 ± 5.70
	Group 2	70.98 ± 6.36	72.37 ± 6.30	73.15 ± 6.42	71.48 ± 5.99
	Group 3	71.33 ± 7.53	71.21 ± 6.05	73.54 ± 7.73	74.46 ± 5.80
Neut (%)	Group 1	13.48 ± 3.03	14.81 ± 3.60	15.43 ± 4.51	14.91 ± 4.38
	Group 2	15.02 ± 3.69	13.72 ± 3.91	13.96 ± 4.31	14.70 ± 4.70
	Group 3	15.00 ± 3.92	14.86 ± 4.62	13.81 ± 4.22	11.60 ± 3.10
PLTs (G/L)	Group 1	557.40 ± 97.23	516.10 ± 91.29	557.10 ± 78.62	602.20 ± 100.74
	Group 2	532.50 ± 114.09	511.60 ± 115.51	572.60 ± 91.14	572.80 ± 84.55
	Group 3	538.90 ± 118.55	507.10 ± 99.31	498.10 ± 76.98	583.00 ± 119.21

4. Effect of S-FEMME on liver and kidney functions

Table 2 shows that after 30, 60, and 90 days of treatment, S-FEMME administered at doses of 0.12 and 0.36 sachets/kg/day did not result in statistically significant difference in AST, ALT, total bilirubin, albumin, total cholesterol, or creatinine levels compared with the control group ($p > 0.05$).

Table 2. The effect of S-FEMME on liver and kidney functions

Parameters	Group	Before treatment	30 days after treatment	60 days after treatment	90 days after treatment
AST (UI/L)	Group 1	69.70 ± 13.46	71.00 ± 14.34	60.10 ± 15.32	57.70 ± 12.84
	Group 2	64.70 ± 13.06	62.30 ± 16.77	58.40 ± 15.66	58.30 ± 14.83
	Group 3	64.00 ± 13.75	61.10 ± 15.69	58.20 ± 17.01	60.40 ± 14.51
ALT (UI/L)	Group 1	41.30 ± 8.29	42.40 ± 10.28	36.30 ± 10.46	40.70 ± 8.06
	Group 2	42.30 ± 10.86	39.40 ± 9.51	37.80 ± 10.39	39.70 ± 8.17
	Group 3	44.80 ± 8.94	41.10 ± 8.58	38.60 ± 8.97	40.10 ± 8.46
Total Bilirubin (mmol/L)	Group 1	6.92 ± 0.54	7.23 ± 1.21	7.00 ± 0.72	7.20 ± 1.08
	Group 2	6.79 ± 0.53	7.07 ± 0.87	7.64 ± 1.21	7.25 ± 0.84
	Group 3	6.97 ± 0.70	7.64 ± 1.08	7.49 ± 1.31	7.43 ± 1.14

Parameters	Group	Before treatment	30 days after treatment	60 days after treatment	90 days after treatment
Albumin (g/dL)	Group 1	2.46 ± 0.22	2.26 ± 0.13	2.60 ± 0.09	2.58 ± 0.24
	Group 2	2.40 ± 0.19	2.28 ± 0.35	2.57 ± 0.28	2.54 ± 0.20
	Group 3	2.28 ± 0.22	2.16 ± 0.42	2.49 ± 0.18	2.44 ± 0.25
Total cholesterol (mmol/dL)	Group 1	45.41 ± 9.75	46.07 ± 3.91	45.26 ± 5.36	44.07 ± 5.82
	Group 2	44.52 ± 4.49	42.23 ± 4.55	43.02 ± 5.34	41.72 ± 5.49
	Group 3	42.49 ± 5.80	43.01 ± 4.81	43.94 ± 5.94	41.62 ± 4.94
Creatinin (µmol/L)	Group 1	66.00 ± 5.25	68.00 ± 7.97	67.80 ± 8.16	70.30 ± 6.75
	Group 2	62.00 ± 4.85	67.40 ± 11.80	67.70 ± 12.10	65.00 ± 7.38
	Group 3	62.80 ± 5.09	66.80 ± 5.85	69.50 ± 7.11	67.30 ± 4.14

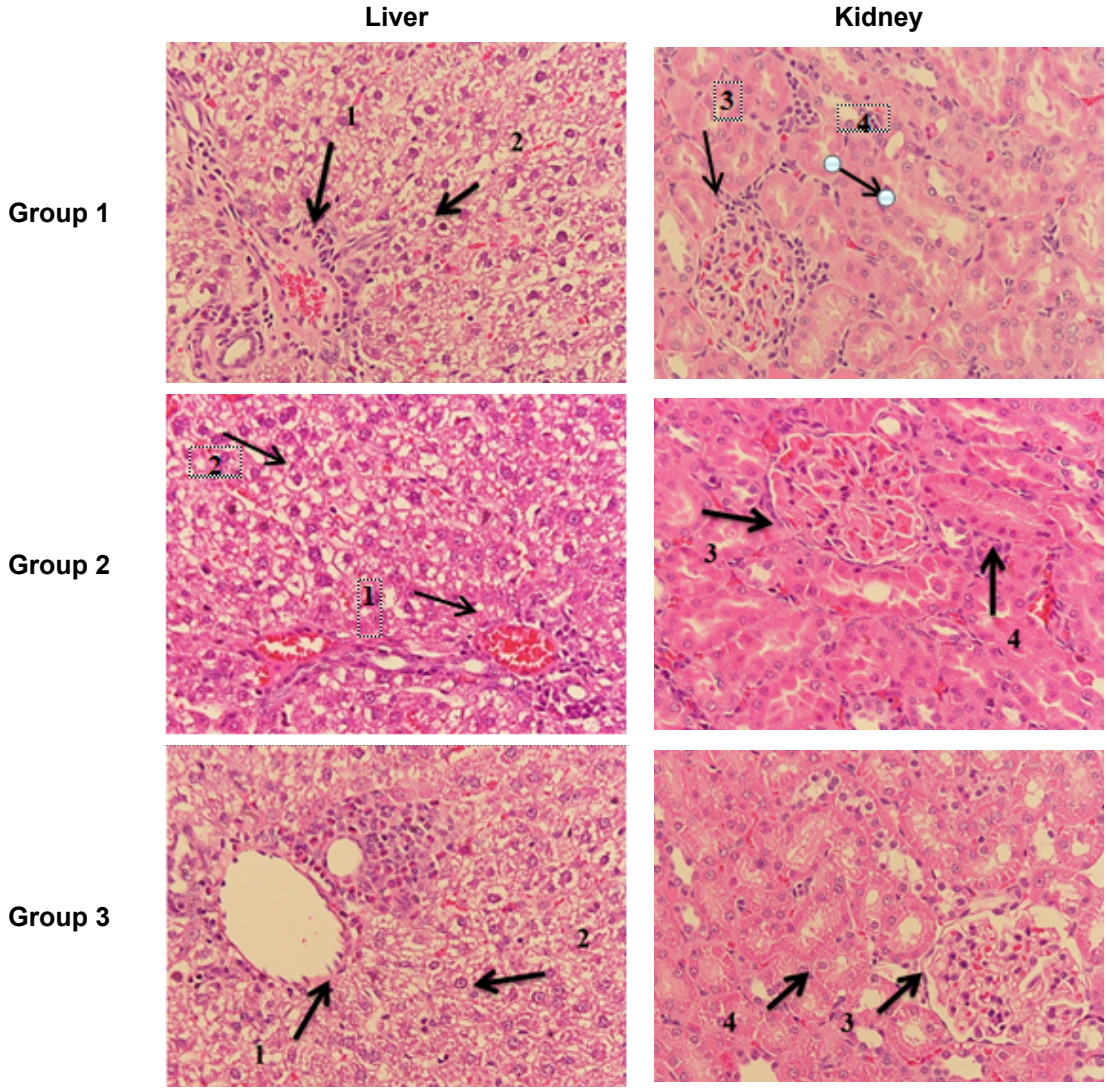
5. Histopathological examination

Gross findings: In all experimental mice (the control group and the two treatment groups), no gross pathological change was noted in the organs, including the heart, lungs, liver, spleen, pancreas, kidneys, and the gastrointestinal system. There were no microscopic finding directly related to SFEMME administration.

Histopathological findings of the liver and kidneys: There were no statistically significant difference in the degree of microscopic lesions between the two treatment groups and the biological control group ($p > 0.05$, Kruskal-Wallis test) (**Table 3 and Figure 2).**

Table 3. Results of scoring of lesions on the liver and kidneys of rats

Organ	Lesions	Control	Group 1	Group 2	p (Kruskal-Wallis test)
Liver	Activated Kupffer cells	0	0	0	1.00
	Sinusoidal dilatation	0	0	0	1.00
	Cytoplasmic vacuolation	0	0	0	1.00
	Hydropic degeneration	0	0	0	1.00
	Karyolysis	0	0	0	1.00
	Karyorrhexis	0	0	0	1.00
Kidney	Granular casts	0	0	0	1.00
	Cellular casts	0	0	0	1.00
	Protein casts	0	0	0	1.00
	Pycnotic cells	0	0	0	1.00
	Hydropic degeneration	0	0	0	1.00



1: Central vein; 2: Hepatocytes 3: Bowman's capsule; 4: Renal tubules

Figure 2. Histopathological morphology of livers and kidneys (HE × 400)

IV. DISCUSSION

Toxicity is defined as the occurrence of adverse effects on biological systems resulting from exposure to a substance. Accurate assessment of toxicity requires the selection of appropriate experimental models, as unsuitable systems may fail to reveal potential toxic effects. The toxicological profile of a compound is influenced by multiple factors, including the route and duration of exposure, physicochemical characteristics, and the specific organ systems involved. Repeated-dose toxicity studies are therefore essential to evaluate the potential adverse effects associated with continuous or repeated administration of chemically defined compounds over a defined period of time in experimental animals, such as rodents.[15] Changes in body weight represent one of the most fundamental indicators of general health

status and reflect the overall physiological impact of xenobiotics on the organism.[12,16] In the present study, animals receiving S-FEMME showed normal body weight gain throughout the experimental period. Furthermore, no macroscopic or gross pathological abnormality was observed in any treated groups compared with the control group. These findings indicate that repeated oral administration of S-FEMME did not disrupt normal metabolic processes in the experimental animals, as evidenced by the absence of significant differences relative to controls.

The circulatory system plays a vital role in maintaining physiological homeostasis, and the hematopoietic system is recognized as one of the most sensitive targets of toxic substances.[12,16] Alterations in hematological parameters are therefore considered reliable indicators of both physiological and pathological conditions in experimental animals and are of particular relevance in toxicological risk assessment due to their high predictive value for potential human toxicity. In the present study, no statistically significant difference was observed in red blood cell count, hematocrit, hemoglobin concentration, platelet count, total white blood cell count, or differential leukocyte profiles between S-FEMME-treated groups and the control group following 90 days of repeated oral administration. These findings indicate that S-FEMME did not exert adverse effects on the hematological system under the experimental conditions.

Evaluation of hepatic and renal function is a critical component of toxicity assessment, as the liver and kidneys play essential roles in metabolism, detoxification, and excretion necessary for organism survival. Serum biochemical analyses were therefore conducted to investigate potential treatment-related alterations in liver and kidney function following repeated administration.[17] Parameters such as total bilirubin, albumin, and total cholesterol were assessed as indicators of hepatic excretory and synthetic function, while ALT and AST were measured as sensitive markers of hepatocellular injury. Elevations in circulating AST and ALT levels are commonly associated with hepatic inflammation or necrosis. In addition, serum creatinine concentration was evaluated as an indicator of renal functional integrity. In the present study, no statistically significant change in AST or ALT levels was observed in either male or female rats treated with S-FEMME at any tested dose. Furthermore, serum biochemical parameters in S-FEMME-treated groups remained comparable to those of the control group, indicating the absence of treatment-related effects on hepatic and renal function. Consistent with these biochemical findings, histopathological examination of the liver and kidney revealed no treatment-related morphological alteration in any of the treated groups compared with controls. Collectively, these results demonstrate that repeated oral administration of S-FEMME for 90 days did not adversely affect liver or kidney structure or function under the experimental conditions. The results of this study indicate that no significant difference was observed in hematological parameters, biochemical indices, or histopathological findings of liver and kidney tissues between the S-FEMME-treated groups and the control group. Our findings are consistent with previously reported studies on the toxicity of the individual components of S-FEMME.

Myo-inositol is the main component of S-FEMME. An international safety review on myo-inositol indicates that its direct toxicity has not been extensively investigated. However, a

study by G. Carlomagno and V. Unfer reported that only at very high doses, exceeding 12 g/day in humans, were mild gastrointestinal disturbances observed, while available data have not demonstrated any serious organ toxicity in animal experiments or nutritional studies.[15] A 45-day study demonstrated that oral administration at ≤ 5000 mg/kg/day did not produce any evident toxic effects in rats.[7] Meanwhile, the myo-inositol content in S-FEMME is 1000 mg, which is at least five times lower than the doses used in the reported toxicity studies. In addition, Bellamine A. and Durkee S. (Lonza Consumer Health Inc.) conducted studies to evaluate the genotoxicity and repeated-dose oral toxicity of L-carnitine and L-carnitine L-tartrate in experimental animal models. In particular, a 90-day repeated-dose oral toxicity study was carried out in rats using different dose levels of L-carnitine and L-carnitine L-tartrate. Throughout the study period, no statistically significant difference was observed between the treated groups and the control group in terms of mortality, clinical signs, body weight changes, food consumption, hematological parameters, or serum biochemical indices. Furthermore, no gross or microscopic lesion was detected in the examined organs upon histopathological evaluation. Similarly, biotin is considered a vitamin with very low toxicity in animals. Experimental studies have shown no evident oral toxic effects even at doses far exceeding nutritional requirements. Available data indicate that both acute and subchronic toxicity studies did not reveal any significant toxic signs following high-dose biotin administration.[8] Hiromi Sawamura and colleagues reported that biotin did not induce toxicity in a 28-day feeding study in rats at a dose equivalent to 79.2 mg/kg body weight/day, and no significant pathological change was observed in the liver or kidneys.[9] Regarding vitamin E, to date, its toxicity has not been fully elucidated and some inconsistencies remain. However, experimental studies evaluating the toxicity of vitamin E have been well documented in animals. Subchronic toxicity studies conducted in rodents have demonstrated that adverse effects occur at high doses, specifically at 2000 mg/kg/day. Specifically, a 13-week toxicity study of d-alpha-tocopheryl acetate in rats showed that toxic effects occurred only at very high doses (2,000 mg/kg), manifested by alterations in hematological parameters and certain tissue lesions.[10] In addition, pantothenic acid exhibits very low toxicity in animals, and no evident adverse effects have been reported in long-term toxicity studies. In a 190-day study in rats administered doses of approximately 50-200 mg/day, no sign of toxicity or histopathological alterations in the liver and kidneys was observed.[11] In the present study, repeated oral administration of S-FEMME at 0.12 and 0.36 sachets/kg/day for 90 consecutive days did not produce any observable sign of toxicity in Wistar rats. According to standard toxicological principles, the no-observed-adverse-effect level (NOAEL) is defined as the highest tested dose at which no adverse effects are observed under specified experimental conditions. In this study, since no adverse effect was detected even at the highest administered dose (0.36 sachets/kg/day), the NOAEL is considered to be greater than this level. This is consistent with previous evidence indicating the safety of its main component, myo-inositol, as well as other accompanying ingredients, which have been widely used and reported to be well tolerated. However, it should be noted that the exact NOAEL could not be definitively established in this study, as no adverse effect was observed at the highest tested dose. Therefore, the true NOAEL may lie above 0.36 sachets/kg/day. In

line with OECD Guideline 408, the selected dose levels were considered appropriate for evaluating subchronic toxicity; nevertheless, further studies using higher dose levels would be necessary to determine the threshold at which adverse effects may occur.

In summary, these observations suggest that S-FEMME does not induce repeated dose toxicity in experimental animals.

V. CONCLUSION

Repeated oral administration of S-FEMME at 0.12 and 0.36 sachets/kg/day over a 90-day period was well tolerated in rodents, with no evidence of toxic manifestation or adverse effect on body weight. Hematological and biochemical indices remained comparable to those of the control group. Furthermore, histopathological analysis demonstrated no significant treatment-related alteration in the examined tissues. Therefore, the no-observed-adverse-effect-level (NOAEL) of S-FEMME is greater than 0.36 sachets/kg/day when administered orally for 90 consecutive days at two doses in Wistar rats.

LIMITATION

This study is limited by its adherence to OECD guidelines, which focus on standard toxicity endpoints and exclude more detailed analyses (e.g., molecular, hormonal, and oxidative stress markers). Additionally, the 3R principle resulted in a limited sample size, potentially reducing the ability to detect subtle or rare effects.

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