In vivo and in vitro genotoxicity studies of Semelil (ANGIPARS™)

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ABSTRACT

Semelil is a novel herbal-based compound formulated for treatment of bed sore and diabetic foot ulcer. The objective of the present preclinical study was to assess the mutagenicity and genotoxicity of Semelil in full compliance with the standard guidelines for testing chemicals.

The potential genotoxicity of Semelil, as part of the safety evaluation process was assessed in three different *in vitro* and *in vivo* tests, including bacterial reverse mutation (Ames test), mammalian bone marrow chromosomal aberration, and rodent dominant lethal assays.

Effects of Semelil was clearly negative at different doses in the Ames test. No statistically significant differences were observed between the levels of chromosomal aberrations in bone marrow cells of mice from the experimental and control groups. The rate of post-implantation losses and thus, the number of lethal mutations in germ cells at different stages of spermatogenesis in male mice treated with a single dose of Semelil did not statistically exceed the control rate. While on the basis of these observations, Semelil can be considered genotoxically safe, further investigations using other bio-assays for mutagenicity studies are warranted.

Keywords: Semelil, ANGIPARS™, *Melilotus officinalis*, Genotoxicity, Mutagenicity

INTROUDUCTION

Melilotus officinalis is the major ingredient of a modified herbal extract Semelil, which contains this extract is a promising candidate drug for wound healing. It has been indicated in several studies that *Melilotus* extract administration by internal or external routes have some beneficial effects in ameliorating skin aging, treatment of pimples, micro-circulation improvement, and anti-inflammatory therapy (1-3). Based on these data, the drug Semelil was recently designed to act as a remedy for healing sub-acute wounds like diabetic foot ulcers which is an important issue in diabetic patient care (4, 5). Hopefully, it could cure the wound by ameliorating the quality of the tissue and efficacious hair growth on the scars.

Generally, before setting of a clinical trial of a novel drug, its safety must be evaluated by toxicity test procedures. The goal of the present study was to assess the mutagenicity and genotoxicity of Semelil in three different *in vitro* and *in vivo* tests. In order to evaluate the ability of the drug to induce mutations in indicator microorganisms, the Ames test was used. The effect of Semelil on the genetic system was weighed up by analysing induced chromosomal aberrations in mice bone marrow cells. Dominant lethal mutations in mice were also studied to evaluate the potential mutagenic properties of Semelil. All these tests were conducted based on recommendation of the OECD guidelines (Nos. 471, 473 and 474) (6).

MATERIALS AND METHODS

Chemicals and reagents

Standardized extract of *Melilotus officinalis* was mixed and processed with other ingredients, Semelil (ANGIPARS™), was prepared and delivered by ParsRoos Co. (Tehran, Iran). Distilled water as a negative control and 4-nitroquinoline 1-oxide (4NQO), 9-aminoacridine (9AA), sodium azide (NaN_3) and 2-aminoanthracene (2AA) as the positive control substances were used in the Ames assay. Cofactor-supplemented post-mitochondrial supernatant of rat-liver homogenate (fraction S9)

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prepared from the livers of 7-week-old male Sprague-Dawley rats treated with Aroclor 1254 was applied as an exogenous metabolic activation system in the Ames test. All chemical reagents were purchased from Sigma Chemical Co. (USA).

Bacterial reverse mutation test (Ames test)
The Ames test (7) was conducted by using *Salmonella typhimurium* strains TA98, TA100, TA1537, which are histidine auxotrophs, as indicator microorganisms (8). In this procedure, bacterial cultures were exposed to the test substance in the presence and absence of the metabolic activation system.
Fresh cultures of bacteria were grown under suitable conditions, at 37°C up to the early stationary phase of growth (approximately 10^9 cells per ml). 0.33 ml of Semelil preparation was diluted in distilled water (final concentration of 10 mg/ml) and in the range from 0.1 to 1000 µg/dish placed on each bacterial plate together with 100 µl of distilled water (negative control) or 100 µl of each positive control. 100 µl of the preparation, 100 µl of bacterial suspension and 100 µl of S9 fraction with or without cofactors were added to the melted agar in the test tubes. The resulting combinations were mixed and semisolid agar was immediately poured on the layer of minimal agar in the Petri dish. After complete solidification of agar, the dishes were put in 37°C incubator. This experiment was triplicated with each dose of the preparation and complete or incomplete microsomal activating mixture (fraction S9), separately. After 48 hours of incubation at 37°C, the number of prototrophic revertant colonies in each plate was counted using a colony counter. The mutagenic activity was expressed as the quotient of the number of revertant colonies over the number of colonies in the negative control. A mutagenic potential of a test substance was assumed if the mutant frequency was 2.0 or higher according to the previous method (7). A possible mutagenic potential could also be supposed if the quotient ranged from 1.7 to 1.9 in combination with a dose-response relationship.

Experimental animals
Hybrid mice $F_1(CBA\times C_{57}Bl/_6)$ weighing 18-20 g were purchased from Stolbovaya animal farm (RAMS, Russia) and assigned to the test following at least 5-day acclimation period. The animals were kept in T-3 type cages at a temperature of 20-22°C and 60-65% humidity under a 12-h light/dark cycle and had free access to food and water supply. Animals were handled in accord with the laboratory animal welfare guidelines. The protocol of the study was approved by the Institute of Animals Ethics Committee.

Chromosomal aberration test
Fifteens mice were divided into three groups, one control (n=5) and two experimental. Semelil was 10-fold diluted in normal saline and injected intramuscularly as a single injection at the dose of 0.7 ml/kg to five mice or repeatedly at 0.07 ml/kg for four consecutive days to other 5 mice. Normal saline was used as negative control and was injected in the same volumes to control group. Five hours after the last injection, the animals were I.P. injected by colchicine (Colcemid®) as a metaphase arresting agent at the dose of 4.8µg/g and one hour later the animals were killed. Immediately after sacrifice, bone marrow was obtained, exposed to hypotonic solution and fixed. Then, the cells were spread on slides and stained with 5% buffered Giemsa (pH 7.0), and metaphase cells were analyzed for chromosome aberrations (9, 10).

Dominant lethal mutations in mice
The study was conducted on 24 male F_1 $(CBA\times C_{57}Bl/_6)$ and 209 virgin female F1 $(CBA\times C_{57}Bl/_6)$ mice. 0.7 ml/kg of Semelil was diluted (1:10) in normal saline and the same volume of diluent were injected intramuscularly into male mice of the test and control groups, respectively. After injection, 3 intact virgin female mice were placed next to each male. Three times every seven days, the females were replaced by new ones. On days of 15-17 of pregnancy, the females were sacrificed and the number of dead and live embryos was counted (11).

Statistical Analysis
All statistical analyses were performed by using SPSS, ver.11.5. The probability levels of significance reported were based on Student's t-test or non-parametric test. All determinations were subjected to analysis of mean and SD for the outcome variables.

RESULTS AND DISCUSION

Bacterial reverse mutation test (Ames test)
A mutagenic potential of the test substance was not observed for all assessed combinations in the presence of S9 mixed either with or without cofactors: all the quotients ranged between 1.0 (or lower) and 1.6 (Table 1). The absence of the dose-effect relationship underlined this statement. So, Semelil did not show any mutagenic activity in the Ames test.

Chromosomal aberration test
Evaluation of chromosomal aberration was

Table 1. Results of bacterial reverse mutation test of Semelil in *Salmonella typhimurium* strains.

The studied substance	Dose µg/Petri dish	TA98 Strain			
		-S9		+S9	
		Mt/Mc	MA	Mt/Mc	MA
H_2O	0		-		-
2AA	20			229.8	+
4NQO	0.5	14.3	+		
SEMELIL	0.1	1.01	-	0.88	-
	1.0	0.88	-	1.07	-
	10.0	0.99	-	0.93	-
	100.0	1.03	-	1.01	-
	1000.0	0.98	-	1.02	-
TA100 strain					
H_2O	0		-		-
2AA	20			21.05	+
NaN_3	2.0	14.3	+		
SEMELIL	0.1	1.01	-	0.92	-
	1.0	1.09	-	0.96	-
	10.0	1.17	-	1.04	-
	100.0	1.10	-	0.93	-
	1000.0	0.97	-	0.95	-
TA1537 strain					
H_2O	0		-		-
2AA	20			136.7	+
9AA	2.0	1891.4	+		
SEMELIL	0.1	1.01	-	0.70	-
	1.0	1.56	-	0.87	-
	10.0	1.41	-	0.95	-
	100.0	0.97	-	0.89	-
	1000.0	0.89	-	1.26	-

2AA= 2-aminoanthracene; 4NQO= 4-nitroquinoline 1-oxide; 9AA= 9-aminoacridine hydrochloride; NaN_3 = sodium azide, Mt/Mc: The ratio of the number revertants in test to the number of revertants in control, MA: Mutagen activity

Table 2. Structural disturbances of chromosomes in the mice bone marrow cells under the effect of Semelil.

Semelil Dose (µl/kg)	No. of animals	No. of cells	No. of cells with aberration (mean ± SD)
700	5	500	1.0± 0.3
70	5	500	0.8±0.4
Normal saline	5	500	1.2±0.5

The data are shown as mean ± SD (standard deviation)

Table 3. The effect of Semelil on the induction of dominant lethal mutations in mice germ cells

Stage of spermatogene	Semelil Dose (µl/kg)	Total number of female	No. of pregnant females	Fertility %	Post-implantation losses
Mature sperm	0	36	29	81	0.053
	700	33	29	88	0.044
Late spermatide	0	36	32	89	0.068
	700	35	28	80	0.066
Early spermatide	0	36	32	89	0.062
	700	33	28	85	0.056

conducted at two dose levels. As it can be seen in the Table 2, no statistically significant induction of aberrations was observed in bone marrow cells of mice that received Semelil compared to control (i.e. no difference in number of broken chromosomes, etc.). There was no significant relation between groups. Thus, Semelil exhibited no signs of genotoxicity in chromosomes of mouse bone marrow cells in both dose groups.
Generally, for genotoxicity determination the appropriate animal organs are selected to be studied in accord with the guidelines proposed by the Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (12). However, supplementary targets may be considered, e.g. some hepatocarcinogens which were activated in liver were negative in the bone marrow micronucleus test (13).

Dominant lethal mutations in mice

The results of the mutagenicity study of Semelil in mice germ cells (Table 3) showed that the level of post-implantation losses in females related to males, which received a single intramuscular injection of Semelil (700 µl/kg) was not more than that in the control female group. So, the drug had no mutagenic effects in this experiment.
In this study, genotoxicity of Semelil was evaluated for the first time. In the Ames test we observed that the drug did not increase the number of revertants in *S. typhimurium* strains neither with nor without metabolic activation system. Therefore, according to results of the Ames test it can be concluded that Semelil does not have mutagenic effects. In the chromosomal aberration test, no statistical significant differences in the level of chromosomal aberrations in bone marrow cells of test mice compared to control were observed. Finally, the mutagenic properties of Semelil were not shown by another test that indicated that Semelil did not increase the number of lethal mutations in mice male germ cells at different stages of spermatogenesis. Taken together, the mutagenic potential of Semelil was undetectable by all three tests. Therefore, the mutagenicity of Semelil was not found in this study.

CONCLUSION

In conclusion, according to results of all three genotoxicity tests, Semelil can be considered safe. Further investigations using other bio-assays for mutagenicity studies may be helpful. Semelil could be recommended for clinical trials in humans. Since antioxidants are believed to be useful in the management of diabetes and its complications (15) and considering antioxidant potential of Semelil, the next step of trials can be focused on diabetic wounds healing.

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