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Evaluation of Camphor Mutagenicity in Somatic Cells of Pregnant Rats

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Abstract: Camphor mutagenicity in pregnant rats has been evaluated in bone marrow cells at three different intervals of pregnancy period by using the chromosomal aberrations and micronucleus analysis. One hundred and thirty pregnant rats were used. Four experimental groups were used for this study, each group consist of 30 pregnant females received treatments orally on the 1st day of gestation, first group given corn oil, the other three groups have given camphor at the levels of 5, 10 and 20 mg kg^{-1} b.wt. Control group consists of ten females received a single intraperitoneal injection of 25 mg kg^{-1} b.wt. cyclophosphamide. Pregnant females of treated and control groups were sacrificed at 7, 14 and 20 days of gestation. At the beginning, the oral administration of camphor to pregnant female rats induced an increase in the percentage (abortion) and this increase corresponds to the dose of camphor they received further. Camphor caused an increase in the frequencies of individuals and total chromosomal aberrations as compared to control at the three different doses and intervals, but these increase were non significant. Data of micronucleus analysis showed that there was no significant differences in the frequencies of micro nucleated polychromatic erythrocytes (MNPCEs) induced by camphor different doses at the three intervals and those of control.It is concluded that camphor has no mutagenic activity and the abortion of treated pregnant rats not due to the mutagenicity of camphor but may be due to hormonal disturbance exhibited by camphor.

Key words: Camphor, pregnant female rat, bone marrow, chromosome aberrations, micronucleus analysis

INTRODUCTION

During the past decade, traditional systems of medicine have become a topic of global importance. Current estimates suggest that, in many developing countries, a large proportion of the population relies heavily on traditional practitioners and medicinal plants to meet primary health care needs. Although, modern medicine may be available in these countries, herbal medicines (phytomedicines) have often maintained popularity for historical and cultural reasons. Concurrently, many people in developed countries have begun to turn to alternative or complementary therapies, including medicinal herbs.

Camphor is a waxy, white or transparent solid with a strong, aromatic odor. It is a terpenoid with the chemical formula $C_{10}H_{16}O$. It is originally obtained by distillation of the bark from the camphor tree, *Cinnamomum camphora*. Today, it is produced synthetically from turpentine oil. Camphor is highly lipophilic, which accounts for its rapid movement across mucous membranes and its large volume of distribution. After absorption, camphor oxidized to camphorol which is conjugated to the glucuronide form. Active metabolites are stored in fat deposits and ultimately excreted in the urine (Völkel, 2006). Camphor used topically to relieve pain and treat warts, cold sores, hemorrhoids and osteoarthritis and is a common ingredient in a wide variety of the-counter topical products. It is shown to be safe when used topically in low concentrations for short periods. Concentrations ranging from

0.1 to 11% seem to be safe for short-term topical use on intact skin. However, camphor is likely unsafe when used topically on broken or injured skin, as it can result in systemic absorption and toxicity (McGuffin *et al*., 1997; Cohen *et al*., 2003). Since 1983, the Food and Drug Administration (FDA) has regulated that over-the-counter products may not contain concentrations of camphor which exceed 11%. Camphor is unsafe when used orally, especially in children. Ingestion of camphor can cause significant toxicity, including death (Gouin and Patel, 1996; Bhaya and Beniwal, 2007).

Camphor has been used historically as an aphrodisiac, contraceptive, abortifacient, analeptic, lactation suppressant, cardiac and central nervous system stimulant, cold remedy, muscle and joint liniment, substance of abuse and rodent repellant (Goldfrank *et al*., 2002).

Camphor can easily cross the skin, the mucous membranes and the placental barrier and it can cause significant hepatoneurotoxicity (Jimenez *et al*., 1983; Fajardo *et al*., 2008; Gohel and Nagori, 2009; Hayashi *et al*., 2009), Ingestion of even small doses of camphor can cause fatal poisoning in small children (Siegel and Wason, 1986; Lahoud *et al*., 1997; Bhaya and Beniwal, 2007; Khine *et al*., 2009). Camphor used medicinally, in perfumes, insecticide and to make celluloid and as a wood preservative. It can put in shoes to cure perspiring feet (probably by acting as a deodorant rather than preventing perspiration (Kunkel, 1984). Considering these findings as well as the wide use of camphor in human therapy and their related chemical structure, it was aimed in this study to investigate their mutagenicity in bone marrow cells of pregnant rats at different intervals of pregnancy period. Two endpoints used for evaluating the mutagenicity of camphor such as chromosomal aberrations and micronucleus test.

MATERIALS AND METHODS

Animals And Husbandry

Adult male (body weight, 150-200 g) and virgin female (body weight, 120-150 g) rat obtained from Laboratory Animal house of king Fahd Research Center at King Abulaziz University and maintained in constant temperature control rooms (22±2°C). The research project was conducted from 1/3/2008 to 1/3/2009. Animals received food and water then maintained on a 12 h light/12 h dark photoperiod. After one week of acclimatization, untreated females and males mated by overnight cohabitation (one male to three females). Females examined for the presence of a vaginal plug the following morning. The day a dropped copulatory plug found designated as day zero of gestation. The pregnant females divided into four groups according to the recommendations of Jamshidzadeh and Sajedianfard (2006) with slight modifications, each group consists of 30 pregnant females. On the 1st day of gestation, females of the first group given 1/2 mL corn oil daily by oral gavages and considered as control. The pregnant females of the other three groups given 1/2 mL of the different doses of camphor at the levels of 5, 10 and 20 mg kg^{-1} b.wt. by using corn oil as vehicle. Group of ten females were given a single intraperitoneal injection of 25 mg kg^{-1} b.wt. cyclophosphamide and considered as positive control. Pregnant females of the experimental and control groups sacrificed by cervical dislocation at 7, 14 and 20 day of gestation and their abdomens were opened to determine their gravid status.

Bone Marrow Chromosome Preparation

Cytogenetic analysis performed on bone-marrow cells according to the recommendations of Preston *et al*. (1987), with slight modifications. Five pregnant rats of each group at different intervals (7, 14 and 20 day) were injected with 1.0 mL of 0.5% colchicines intraperitoneally, 2 h prior to sacrificing the animals to arrest the mitotic division at metaphase stage. Bone marrow cells collected in saline from the femurs, then pipetting by using Pasteur pipette. The cells sediment by centrifugation

at 1200 rpm for 10 min. Potassium chloride solution 0.56% added to sediment as hypotonic solution and incubated at 37°C for 30 min. Cells fixed in freshly prepared cold fixative (3-1 methanol-glacial acetic acid) for 15 min in refrigerator and then the mixture centrifuged for 10 min. The fixative was changed and left overnight in refrigerator and then centrifugation and fixation repeated three times at an interval of 20 min. The material resuspended in a small volume of the fixative, dropped on a slides which was dipped in 70% ethanol and flame-dried. Slides were stained in 10% phosphate buffered Giemsa (pH 6.8) for 35 min then washed in phosphate buffer solution. One hundred good metaphases for each animal examined microscopically and chromosome aberrations recorded.

Micronucleus Test

A micronucleus (MN) formed during the metaphase/anaphase transition of mitosis (cell division). It may arise from a whole lagging chromosome (aneugenic event leading to chromosome loss) or an acentric chromosome fragment detaching from a chromosome after breakage (clastogenic event) which do not integrate in the daughter nuclei. The bone marrow of five pregnant females of control and treated groups of different intervals were extracted, smear preparations made by using fetal calf serum and stained in 10% phosphate buffered Giemsa (pH 6.8) for 5 min according to Salamone *et al*. (1980). Polychromatic erythrocytes scored for micronuclei under the microscope. All slides, including those of positive and negative controls, should be independently coded before microscopic analysis. At least 2,000 immature erythrocytes per animal scored for the incidence of micronucleated immature erythrocytes.

Statistical Analysis

To evaluate the mutalpgenic effects of camphor the data analyzed using the ANOVA and Duncan's comparison tests. The significance level of ($p \leq 0.05$) adopted to compare data within the same experiment.

RESULTS

In the present study, the oral administration of camphor to pregnant female rats at the first day of gestation induced an increase in the percentage of embryo failure (abortion) with increasing the dose, thus, the percentage of abortion in the dose of 5 mg kg^{-1} b.wt. camphor was 73.3%, in the dose of 10 mg kg^{-1} b.wt. was 80% and in the dose of 20 mg kg^{-1} b.wt. was 93.3%.

The present study conducted in order to identify the possible mutagenicity, which may be caused by camphor in bone marrow of pregnant rats and to demonstrate that there is a relation between the abortion caused by camphor and the incidence of chromosomal aberrations.

The evaluation of mutagenicity was conducted using two methodologies: chromosome aberration and micronucleus test in bone marrow cells of pregnant rats at days 7, 14 and 20 of gestation. The structural chromosomal aberrations that were observed for were chromatid gap, chromatid break, deletions and centromeric attenuations. While, the numerical aberrations that were observed for were hypoploidy and polyploidy.

Chromosomal Analysis

The effects of different doses treatment of camphor on pregnant rat bone marrow at 7, 14 and 20 day of gestation were shown in (Table 1-3). The data represented by Mean±SD. Results showed that camphor causes a moderate increase in the frequencies of individuals and total chromosomal aberrations as compared to control at the three different doses and intervals, but these increase were non significant. In addition, the analysis of the frequencies of total chromosomal aberrations showed

Table 1: Mean value and standers deviations of different types of chromosomal aberrations induced by camphor in bone marrow cells of pregnant female rats at day seven of gestation

Experimental groups	Numerical chromosomal aberrations		Structural chromosomal aberrations				Total chromosomal aberrations
	Polyploidy	Hypoploids	Centromeric attenuations	Deletions	Cht. break	Cht. gap	
Control	0.20±0.45b	1.20±0.84a	2.00±0.55ab	0.00±0.00b	0.60±0.55b	0.80±0.45b	4.60±1.14b
+ ve control	1.00±0.71a	1.60±0.55a	2.80±0.84a	2.00±0.71a	2.20±0.45a	2.40±0.55a	12.00±1.00a
5 mg kg^{-1} camphor	0.00±0.00b	1.60±0.55a	1.20±0.84b	0.40±0.55b	0.60±0.55b	1.40±0.55b	5.20±0.45b
10 mgkg^{-1} camphor	0.00±0.00b	1.00±0.71a	1.60±0.55b	0.40±0.55b	0.80±0.84b	1.40±0.55b	5.20±0.45b
20 mg kg^{-1} camphor	0.00±0.00b	1.00±0.84a	2.00±0.45ab	0.60±0.55b	0.60±0.55b	1.20±0.84b	5.40±0.45b

Table 2: Mean value and standers deviations of different types of chromosomal aberrations induced by camphor in bone marrow cells of pregnant female rats at day fourteen of gestation

Experimental groups	Numerical chromosomal aberrations		Structural chromosomal aberrations				Total chromosomal aberrations
	Polyploidy	Hypoploids	Centromeric attenuations	Deletions	Cht. break	Cht. gap	
Control	0.20±0.45b	1.20±0.45ab	1.80±0.45ab	0.00±0.00b	0.60±0.55b	1.00±0.71b	4.80±0.45b
+ ve control	1.00±0.71a	1.60±0.55ab	2.80±0.84a	2.00±0.71a	2.40±0.55a	2.40±0.55a	12.20±0.84a
5 mg kg^{-1} camphor	0.00±0.00b	1.80±0.45a	1.80±1.30ab	0.60±0.55b	0.40±0.55b	1.00±0.71b	5.60±0.89b
10 mg kg^{-1} camphor	0.00±0.00b	1.00±0.00b	1.80±0.45ab	0.40±0.55b	0.80±0.45b	1.20±0.45b	5.20±0.45b
20 mg kg^{-1} camphor	0.00±0.00b	1.20b±0.84ab	1.60±0.55b	0.60±0.55b	0.60±0.55b	1.20±0.45b	5.20±0.45b

Each value represents the mean and standard deviations of five animals. Cht. means chromatid Means with different letters within each column are significant at 5% level

Table 3: Mean value and standers deviations of different types of chromosomal aberrations induced by camphor in bone marrow cells of pregnant female rats at day twenty of gestation

Experimental groups	Numerical chromosomal aberrations		Structural chromosomal aberrations				Total chromosomal aberrations
	Polyploidy	Hypoploids	Centromeric attenuations	Deletions	Cht. break	Cht. gap	
Control	0.20±0.45b	1.00±0.71a	1.60±0.55b	0.00±0.00b	0.60±0.55b	0.80±0.45b	4.20±0.84b
+ ve control	1.00±0.71a	1.60±0.55a	2.80±0.84a	2.00±0.71a	2.40±0.55a	2.40±0.55a	12.20±0.84a
5 mg kg^{-1} camphor	0.00±0.00b	1.40±0.55a	1.20±0.84b	0.40±0.55b	0.20±0.45b	1.20±0.45b	4.40±0.55b
10 mg kg^{-1} camphor	0.00±0.00b	0.80±0.45a	1.80±0.45b	0.40±0.55b	0.80±0.45b	1.00±0.00b	5.00±0.45b
20 mg kg^{-1} camphor	0.00±0.00b	1.20±0.84a	1.60±0.55b	0.20±0.45b	0.60±0.55b	1.00±0.00b	4.60±0.55b

Each value represents the mean and standard deviations of five animals. Cht. means chromatid Means with different letters within each column are significant at 5% level

Table 4: Frequencies of micronuclei in pregnant female rat bone marrow of all experimental groups

Experimental group	No. of animals	No. of examined cells	7th day of gestation	14th day of gestation	20 day of gestation
Control	5	10 000	3.80±0.84b	3.60±0.55b	3.60±0.89b
Positive	5	10 000	25.40±3.65a	25.40±3.65a	25.40±3.65a
5 mg kg^{-1} camphor	5	10 000	3.60±0.55b	3.00±0.71b	3.00±0.71b
10 mg kg^{-1} camphor	5	10 000	4.20±0.45b	3.60±0.55b	3.20±0.45b
20 mg kg^{-1} camphor	5	10 000	4.40±0.55b	3.80±0.45b	3.60±0.55b

Means with different letters within each column are significant at 5% level

that there were non-significant increases in the mean values of total chromosomal aberrations of camphor doses at day seven and fourteen of gestation than control. While, at gestation day 20, there were no significant differences between camphor doses and control (Table 1-3).

Micronucleus Analysis

Results of micronucleus test in bone marrow cells of pregnant female rats of all experimental groups are summarized in Table 4. Data indicated that the dose of 5 mg kg^{-1} b.wt. camphor induced non significant decrease in the frequencies of micro nucleated polychromatic erythrocytes (MNPCEs) at three intervals compared to control. However, doses of 10 and 20 mg kg^{-1} b.wt. exhibited a non-significant increase in the frequency of MNPCEs at seven day of gestation when compared to negative control. While, there was no significant differences at fourteen and twenty days of gestation (Table 4).

Camphor against aflatoxin B1 in S. typhimurium TA100. From the previous finding, we can conclude that camphor at the concentrations tested do not has mutagenic activity and the abortion of treated pregnant rats are not due to the mutagenicity of camphor.

DISCUSSION

The essential oils and their monoterpenoid constituents have been widely used as fragrances in cosmetics, as flavouring food additives, as scenting agents in a variety of household products, as active ingredients in some old drugs and as intermediates in the synthesis of perfume chemicals (Gomes-Carneiro *et al*., 1998). Camphor is a pleasant- smelling cyclic ketone of the hydroaromatic terpene group. Its history dates to ancient Chinese medicine. Camphor was highly regarded as a circulatory and cardiac stimulant in the late 19th and early 20th centuries. Traditional uses have been as an abortifacient, contraceptive (Vasey and Karayannopoulos, 1972), cold remedy, aphrodisiac, antiaphrodisiac, suppressor of lactation and antiseptic (Compadre *et al*., 1986; Goldfrank *et al*., 2002).

In the present study, the oral administration of camphor to pregnant female rats induced an increase of embryo loss and the rate of embryo loses increases with an increasing dose of Camphor. Such result is in accordance with previous reports (Riggs *et al*., 1965; Weiss and Catalano, 1973) which showed that camphor could easily pass placental barrier and affect fetal development. Camphor ingestion might lead to abortion because camphor crosses the placenta and foetuses lack the enzymes to hydroxylate and conjugate with glucuronic acid (Rabl *et al*., 1997). In addition, 4-methyl-benzylidene camphor (4-MBC) exhibits estrogenic activity, is a preferential Estrogen Receptor (ER)-β ligand and interferes with development of female reproductive organs and brain of both sexes in rats (Seidlová-Wuttke *et al*., 2006; Durrer *et al*., 2007). 4-methylbenzylidene Camphor exert their effects on endocrine function disrupting including uterotrophic that is a preferential Estrogen Receptor (ER)-β ligand and interferes with development of female reproductive organs and brain of both sexes in rats.

In the present study, results show that camphor does not increase the frequencies of total chromosomal aberrations and number of micronuclei in bone marrow cells of pregnant rats. These results indicate that there was no evidence of mutagenicity of camphor at any of the concentrations tested. This is in agreement with earlier studies (Gomes-Carneiro *et al*., 1998; Kneževic-Vukcevic *et al*., 2006) in which Camphor did not exhibit mutagenic activity in the Ames test, by using the Salmonella/microsome assay (TA97a, TA98, TA100 and TA102 tester strains), without and with addition of an extrinsic metabolic activation system (lyophilized rat liver S9 fraction induced by Aroclor 1254). In addition, Chang *et al*. (1998, 2000) found that the camphorated phenolic compounds used in clinical dentistry as sedatives for the dental pulp did not cause genotoxic effects on cultured human pulp fibroblasts *in vitro*.

Micronucleus test result indicated that camphor 5 mg kg^{-1} b.wt. dose showed non-significant decrease in the frequencies of micronuclei compare to those of control. This result may interprets by (Kneževic-Vukcevic *et al*., 2006) they showed that essential oil of sage and its fractions can be considered to contain substances with antimutagenic potential against UV-induced and possibly spontaneous mutations. In view of the composition of sage oil and fractions, obvious candidates are α-thujone, 1, 8-cineole, camphor and α-humulene. Meanwhile, Goel *et al*. (1989) demonstrated that camphor antagonized γ-radiation-induced increase in SCE frequency in mice bone marrow cells. In addition, Kim *et al*. (1992) reported an antimutagenic effect of cineole and disturbance exhibited by camphor oils. This latest depending on the test organism used, the effect could also be attributed to certain molecules that are involved in those oils. Therefore, further antimutagenesis and anticarcinogenesis studies are recommend.

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