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Effects of kaurenoic acid, a bioactive diterpene on embryo implantation and pregnancy outcome in mice

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ABSTRACT

The diterpene kaurenoic acid (KA) isolated from the oleoresin of Amazonas traditional medicinal plant *Copaifera langsdorffii* demonstrates interesting biological properties such as the anti-inflammatory, hypotensive, hypoglycemic and diuretic effects *in vivo* and the antimicrobial, smooth muscle relaxant, and cytotoxic actions *in vitro*. In COX-enzyme assays, KA exhibits a non-selective inhibition of cyclooxygenase (COX) isoforms, COX-1 and COX-2. Since both selective and non-selective COX-inhibitors were shown to be anti-inflammatory and exert prenatal toxicity, the present study was aimed to assess the reproductive toxicity potential of KA in mice. Mice were treated by oral gavage with KA (25 and 50 mg/kg) from Day 1 to Day 7 or Day 8 to Day 15 of pregnancy. Necroscopies were performed on Day 10 to note the number of implantation sites and the proportion of animals pregnant, or Day 20 for gestational outcome and the live fetuses were examined for gender, external, visceral and skeletal malformation and variations. The results show that KA at the highest dose tested (50 mg/kg, p.o.), interferes with the nidation process and also cause post-implantation loss, without the signs of maternal toxicity. In addition, an increased frequency of fetal skeleton variations was seen but did not cause embryo deaths or other malformations at any dose level. Our results indicate that kaurenoic acid at a dose of 50 mg/kg impairs fertility in mice, possibly through a COX-inhibitory mechanism.

KEYWORDS: kaurenoic acid, plant diterpene, implantation loss, pregnancy outcome, fertility, mouse

ABBREVIATIONS

COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; $cPLA_2$, cytosolic phospholipase A2; DMSO, dimethylsulfoxide; fMLP, N-formyl-methionine-leucine phenylalanine; GD, gestation day; iNOS, inducible nitric oxide synthase; KA, kaurenoic acid; LPS, lipopolysaccharide; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; PG, prostaglandin; PGE_2, prostaglandin E_2; TNF-α, tumor necrosis factor-alpha

INTRODUCTION

Kaurenoic acid (KA) is one of the intermediate compounds involved in biosynthesis of diverse kaurane diterpenes that include gibberellins, which function as growth hormones in higher plants as well as in some fungi and bacteria [1]. Plants that belong to *Copaifera*, *Annona*, *Mikania* and *Xylopia* spp are rich in kaurenoic acid (KA), which was shown to possess uterotonic [2] anti-parasitic and anti-microbial [3-6] properties. Studies also show that KA is genotoxic and mutagenic in human peripheral blood leukocytes, yeast (*S. cerevisiae*) and in mice (bone marrow, liver

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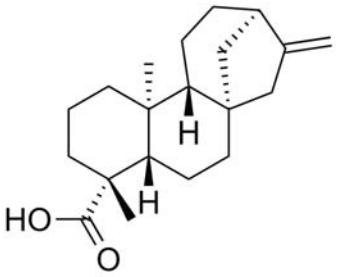

Fig. 1. Chemical structure of Kaurenoic acid (ent-16-en-19-oic acid).

and kidney) due to the generation of DNA double-strand breaks and/or inhibition of topoisomerase I [7], and when tested on human sperm, KA induced sperm immobilization but caused only a weak or negligible capacity of killing sperms [8].

In the present investigation, KA (Fig. 1) was isolated from the oleo-resin of *Copaifera langsdorffii* Desf (Leguminosae), a popular medicinal plant that grows abundantly in Amazon region of Brazil. This oleoresin (copaiba oil) is a reputed folk remedy in its natural form for the treatment of sore throat, urinary and pulmonary infections and to promote healing of ulcers and wounds. Also, there have been reports on the diuretic, vasorelaxant, hypoglycemic and anti-inflammatory effects of its major diterpene constituent KA [9-12]. Besides, KA demonstrated an anti-proliferative action in tumor cell cultures and hemolytic effect against mouse and human erythrocytes [13]. It was reported that KA has an inhibitory potential on COX enzyme activity with no selectivity to the isoforms and inhibits the prostaglandins (PGs) generation [14]. The processes of ovulation and implantation are considered analogous to "pro-inflammatory" responses and the participation of PGs in these processes has been speculated because of their vasoactive, mitogenic and differentiating properties [15-17]. Both isoenzymes have been detected in placenta and fetal tissue during gestation [18] and their selective or non-selective inhibition may be toxic to rodent reproduction when administered in a high dose [19]. In the light of these potential therapeutic and toxicological properties, it was of interest in this study to evaluate the possible effects of KA on embryo implantation and gestational outcome in mice.

MATERIALS AND METHODS

Chemical Agents

Kaurenoic acid (Fig. 1) was isolated from the oleoresin of *Copaifera langsdorffii* Desf (Leguminaceae) as previously described [13]. KA was dissolved in 5% DMSO in normal saline. Alizarin Red S was purchased from Sigma-Aldrich, Brazil. All other chemicals used in the study were of analytical grade.

Animals

Swiss albino mice (20-25g body weight) obtained from the Central Animal House of this Institute was used for the investigation. They were housed in polypropylene cages with stainless steel wire lids and wood shavings as bedding. Animal room was maintained at 22 ± 2^0C, a relative humidity of 55±5%, and a 12-h light/dark cycle. Food (Purina pellet feed) and water were provided *ad libitum* throughout experimental period. The experimental protocols using animals were approved by Institutional Animal Ethical Committee of the Federal University of Ceara in accordance with the guidelines of National Institute of Health, Bethseda, USA.

Mating procedure and treatments

Nulliparous females in pro-estrus phase of cycle were caged overnight with adult males in the proportion of 3:1. Successful mating was ascertained in the following morning by the presence of vaginal sperm plug, and was designated as Day 1 of gestation (GD 1). Mated animals were randomly assigned to six treatment groups (n = 12-17). Groups 1 and 2 were treated orally with drinking water (normal control) or vehicle (5% DMSO in water) in a volume of 10 mL/kg on GD 1-7 (for implantation studies) or GD 8-15 (for post implantation studies). While the third and fourth groups were similarly treated with KA 25 and 50 mg/kg, respectively, by the same route during GD 1-7, the fifth and sixth groups received KA in similar doses on GD 8-15. The dose selection for KA was based on our previous studies that show *in vivo* biological activity at 25- 50 mg/kg. The dams were weighed on GD 1, GD 10 and GD 20, and on Day 10 or 20 of pregnancy, the dams were sacrificed and necropsied by cervical dislocation to note the number of uterine implantation sites,

resorptions and the number of corpora lutea in ovaries. In addition to these, GD 20 animals were also observed for the number of live and dead fetuses. Each one of the live fetuses was weighed and after fixation in Bouin's solution, the fetuses were examined for visceral alterations [20] and stained with alizarin red S for skeleton variation [21]. Conception rate and fetal losses were also calculated.

Statistical analysis

Data on quantitative parameters were presented as mean±S.E.M. Body weights, the implantation sites and live fetuses were analysed by one-way analysis of variance (ANOVA), followed by Dunnett`s test for any significant differences. The percentages of post-implantation loss and resorptions were evaluated by the Kruskall Wallis test, followed by the Mann Whitney test where appropriate. $P < 0.05$ was considered as the level of statistical significance.

RESULTS

Treatment effects of kaurenoic acid (KA) on embryo-implantations and maternal weight gains are shown in Table 1. The group of mice treated with KA (50 mg/kg) during GD 1-7 presented no implantation sites in 10 out of 12 animals mated showing significantly decreased conception rate (83.3%), when compared to water, vehicle or low-dose KA (25 mg/kg)-treated groups of mice, which manifested a decrease by 8.3%, 1.8%, and 16.7%, respectively. Similarly, high-dose KA (50 mg/kg) significantly suppressed the number of implantation sites, which corresponded with the number of corpora lutea. Maternal weight gains were also less at 25 mg/kg and appeared more severe at 50 mg/kg treatment (3.67±0.50 g and 2.25±0.43 g, respectively, as against 5.00±0.70 g of vehicle control).

The effects of KA on pregnancy outcome in mice treated on GD 8-15 are shown in Table 2. Mice treated with KA on GD 8-15 and examined on GD 20 presented total absence of fetuses in 3 out of 13 at 25 mg/kg and 14 out of 17 animals at 50 mg/kg. The maternal weight gain in animal group treated with 50 mg/kg KA was significantly less as compared to other groups. The fetal loss due to resorption in this group was upto a level of 88% as against 17, 20 and 14%, respectively, in groups treated with water, vehicle or 25 mg/kg KA (Table 2). The individual pup weight or placental weight, and the percentages of male/female proportions were not significantly altered by any

Table 1. Effects of kaurenoic acid (KA) on implantation and maternal parameters in mice treated on GD 1-7.

	Control		KA 25 mg/kg	KA 50 mg/Kg
	Normal (Water)	Vehicle (DMSO 5%)		
Number of females				
Mated	12	8	12	12
Not conceived*	1	0	2	10
Maternal weight gain (g)				
Total (Final-Initial)	5.40±0.51	5.00±0.70	3.67±0.50	2.25±0.43[a,c]
Treatment period	3.00±0.30	3.25±0.25	2.00±0.28[b]	1.60±0.26[a,b]
N° of corpora lutei	10.00±1.1	9.75±0.65	7.80±1.17	1.70±1.13[a,b,c]
N° of implantation sites	6.60±1.32	8.00±1.00	6.90±1.30	1.70±1.13[a, b, c]
Conception rate (%)	8.33	1.80	16.66	83.33[a,b,c]

*Mice with no implantation sites. Data are expressed as mean±S.E.M. [a]$p<0.05$ vs. control, [b]$p<0.05$ vs. DMSO, [c]$p<0.05$ vs. KA 25.

Table 2. Effects of kaurenoic acid (KA) on pregnancy outcome in mice treated on GD 8-15.

	Control		KA 25 mg/Kg	KA 50 mg/Kg
	Normal (water)	Vehicle (DMSO 5%)		
Number of females				
Mated	12	10	13	17
Not conceived*	0	0	3	14
Maternal weight gain(g)				
Total (Final-Initial)	16.75±1.14	19.9±2.44	14.62±1.9	2.8±0.8 [a,b,c]
Treatment period	7.25±0.54	8.6±0.9	5.9±1.1[b]	2.8±0.8 [a,b,c]
N° of live fetuses	106±0	79±0	81±0	25±0
N° of corpora lutei	9.17±0.67	9.9±0.55	7.00±1.15	1.53±0.84[a,b,c]
N° of implantation sites	8.83±0.72	8.1±1.00	6.39±1.12	1.53±0.84[a,b,c]
Fetal loss (%)	4.17±3.4	17.85±8.20	29.4±11.5	88.20±8.05[a,b,c]
Pup weight (g)	1.50±0.02	1.38±0.02	1.37±0.02	1.54±0.02
Placental weight (g)	0.27±0.01	0.29±0.01	0.26±0.01[b]	ND
Skeleton variations (%)	0 (0/106)	0 (0/79)	5 (4/79)	32 (8/25)
Males/Females (%)	44.76/55.24	44.4/55.6	47.16/52.84	53.8/46.20

*Mice with total absence of fetuses on GD 20. Data are expressed as mean±S.E.M. ND = not determined. [a]$p<0.05$ vs. control, [b]$p<0.05$ vs. DMSO, [c]$p<0.05$ vs. KA 25.

one of these treatments. No skeleton malformations were observed in live fetuses that received water or vehicle (DMSO). However, fetuses from mice that received KA on GD 8-15 and examined on day 20 showed retarded ossification of sternebra and metacarpals, and skeleton variations in cervical ribs, thoracic vertebra (mostly asymmetry or splitting) and limb defects to the extent of 5% at 25 mg/kg and 32% at the dose of 50 mg/kg (Table 2).

DISCUSSION

The reported non-selective COX enzyme inhibitory and the DNA damage inducing effects of kaurenoic acid (KA) [14, 22] led us to investigate the reproductive outcome in Swiss mice. In reproductive outcome studies, our first objective was to verify the effect of KA on mouse uterine implantation. The KA was given to dams by oral gavage at doses of 25 and 50 mg/kg body weight from GD 1-7. While KA (25 mg/kg) at a smaller dose caused no adverse effect on embryo implantation, the higher dose (50 mg/kg) impaired the uterine implantation. It is generally agreed that sex steroids oestradiol and progesterone, which have pro- and anti-inflammatory activities respectively, play a major role in successful reproductive function and pregnancy success, and in this regard, the paracrine effects of the sex steroids on the ovary are similar to the endocrine effects on the uterus. Ovarian leukocyte recruitment and cytokine release are central to follicle development, ovulation and corpus luteum function. At the uterine level, the cyclical changes in sex steroids regulate the number and distribution of endometrial and decidual reaction allowing blastocyst implantation [23]. Thus the processes of ovulation and implantation are considered analogous to a pro-inflammatory reaction largely mediated by inflammatory mediators that include superoxygen (O_2^-), NO, PGs, $cPLA_2$ and cytokines such as TNFα [15, 18, 19, 24]. Past studies indicate that KA can cause a significant

increase in the generation of O_2^- in human neutrophils stimulated by fMLP and a mild inhibitory effect on NO generation in LPS stimulated NR8383 macrophages and could inhibit nitric oxide (NO) production, prostaglandin E_2 (PGE_2) release, cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) expression in LPS-induced RAW264.7 macrophages [25-27]. The first evidence for a functional role of NADPH-dependent O_2^- production in the ovary and uterus of cycling and pregnant mice was provided by Jain *et al.* [28]. Further, it was reported that KA has an inhibitory potential on COX enzyme activity with no selectivity to the isoforms at an IC_{50} (the half maximal inhibitory concentration) of 332 and 592 μM, respectively, for COX-1 and COX-2 [14].

Very recently the anti-inflammatory activity of KA has been firmly established through studies *in vitro* and *in vivo* [27] and it is suggested that the inhibition of iNOS and COX-2 expression might be one of the mechanisms responsible for its anti-inflammatory properties. Thus in this study, the anti-inflammatory effect of KA at ovarian and uterine sites presumably contributed for the impaired reproductive outcome when dams were treated at a dose of 50 mg/kg either on GD 1-7 or GD 8-15 that resulted in implantation failure and in an increase of resorptions.

Increased developmental skeletal variation occurred in 32% of the KA (50 mg/kg)-exposed fetuses that were examined on GD 20. On the other hand, no malformations were observed in water or vehicle treated control fetuses. This observation was consistant with a previous study that noted an increased developmental skeletal variation in rats treated at higher doses of non-selective COX inhibitors like indomethacin, tolmetin, ibuprofen, or piroxicam [19, 29].

KA is the major diterpene compound present in Copaiba oil (oleoresin) of *Copaifera langsdorfii*, a reputed folk remedy in its natural form for the treatment of sore throat, urinary and pulmonary infections and to promote healing of ulcers and wounds. The prevalence of herbal medicine use during pregnancy is not uncommon [30] and therefore the importance of knowing their potential dangers in women of childbearing age. Although KA exhibits interesting therapeutic properties that including anti-inflammatory, and antinociceptive effects, the present study with mice reveal that a high dose use of KA (50 mg/kg) would cause fertility impairment. Therefore, the levels of KA need to be limited in commercial preparations of copaiba oil for therapeutic safety.

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