

## The Role of Eugenol in the Reduction of Teratogenic Effects of Retinoic Acid on Skeletal Morphology of Mice Embryo

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### Abstract

**Introduction:** Eugenol (Eg), a compound extracted from Clove tree, is known for its anti-anaphylactic, anti-inflammation, antiseptic, antibacterial and analgesic properties as well as chain breaking antioxidant in inhibition of lipid peroxidation. Retinoic acid (RA), one of the synthetic derivatives of vitamin A, has been frequently used for treatment of face acne. In our studies Eg was used to test its probable correcting action on decreasing the toxicity and the teratogenic effects of retinoic acid in female NMRI mice embryo.

**Materials and Methods:** Five groups of 8 weeks old pregnant female mice were fed as follows: 1- an oral dose of 60 mg RA per kg b.w. on day 10 of pregnancy, 2- an oral dose of 100 mg/kg b.w. Eg for ten days (5th to 15th day of pregnancy), 3- olive oil (solvent) for 15 days, 4-Eg plus RA (as in groups 1 and 2) and 5- untreated controls. On day 18 of pregnancy the mice were dissected and the embryos were taken from the uterus. The length of the Crown Rump (CR), Cranio-Facial (CF), hands, feet and tail were measured and the organs were observed for probable defects. ANOVA, Tukey and Chi-square were used for data analysis.

**Results:** A positive relationship was observed between the quantity of RA taken by the animals and the frequency of embryonic organ defects. Simultaneous administration of Eg with RA significantly reduced the frequency and the intensity of embryonic defects. RA could significantly reduce the length of hands, feet, tail, CR and CF ( $p < 0.001$ ). It could also cause embryonic defects such as short lower jaw, spiral tail, foot bending, hand and feet oligodactyly and syndactyly. Simultaneous administration of Eg with RA could significantly correct the length reduction in hands, tail and CF by 16, 18.85, 7.44%, respectively ( $P < 0.001$ ), and CR by 6.57% ( $P < 0.05$ ).

**Conclusion:** The results of this study show that Eg may decrease the teratogenic effects of RA, and might also be suggested as a protective agent to be used with RA in skin therapy during pregnancy.

**Key words:** Teratogenic effect, Eugenol, Retinoic acid, Skeletal, Morphology, Mice embryo.



## Introduction

Today, evidence for an important role for antioxidant nutrients comes from the complete spectrum of biochemical research, animal studies, epidemiologic data and clinical trials.

In these studies, Eg was used for the first time to test its probable protective effects on the teratogenicity of RA which is used by pregnant women for treatment of acne.

Retinoids are a class of compounds consisting of natural and synthetic analogs of vitamin A. They are used therapeutically for skin diseases such as acne, psoriasis and other keratinizing dermatoses (1). Retinoids not only have important physiological functions, but also are used extensively in dermatology (2). In humans, toxicity associated with retinoids is characterized by cheilitis, stomatitis and conjunctivitis, headache and, pain tenderness in bone and joint. In animals, toxicity causes weight loss, desquamation of skin, hair loss, bone fractures, hemorrhage, teratogenicity, reproductive toxicity and fatty liver (3, 4).

Unfortunately, the high teratogenicity of the retinoids is a major drawback to their broad use. There is a characteristic pattern of congenital defects such as craniofacial, cardiac and central nervous system found in human infants exposed to isotretinoin (13-cis-RA) in utero during the first trimester (2). This teratogenic response in women was elicited by a relatively low clinically therapeutic dose (0.5-1.5 mg/kg/day) (2). Although 13-cis-RA is potent human teratogen, this substance is only marginally teratogenic in the mouse at doses 100 times higher than those used in human therapy, and all-trans-isomer, on the other hand, is a potent teratogen in mouse (5, 6). In cell differentiation and embryogenesis, 9-cis and all-trans RA necessarily interact with nuclear retinoid receptors, whereas retinol doesn't (7).

Eg (4-allyl-2-methoxyphenol), a naturally occurring phenolic compound, is a major component of clove oil and exists to a lesser extent in oils of several other plants (8). It has antiseptic and analgesic properties, and finds medicinal application in dental and surgical pastes (8). The joint FAO/WHO committee on Food Additives has permitted an acceptable daily intake of

Eg of 2.5 mg/kg b.w. for humans (8, 9). It is considered non-carcinogenic, non-mutagenic and generally recognised as safe (GRAS) by Food and Drug Administration (10). In traditional medicine, Eg has been used in the treatment of flatulent colic, chronic diarrhea and other gastrointestinal disorders (9). Eg is reported to show a high antibacterial activity (11).

## Materials and Methods

### \* Chemicals

Eg (>99% pure) was obtained from Merck Chemical Company and all-trans RA from Sigma Chemical Corporation.

### \* Animals

Five groups of 8 weeks old pregnant female mice were fed as follows: 1- an oral dose of 60 mg RA per kg b.w on day 10 of pregnancy, 2- an oral dose of 100 mg/kg b.w Eg for ten days (5th to 15th day of pregnancy), 3- olive oil (solvent) for 15 days, 4- Eg plus RA (as in groups 1 and 2) and 5- untreated controls. Six weeks old albino NMRI male mice and 8 weeks old female mice, weighing  $26 \pm 2$  g were housed in individual cages. They were fed pelleted mice diets ad libitum. The animals were maintained under a temperature of  $22 \pm 2^\circ\text{C}$  and acclimated to a 12-hr light-dark cycle.

### \* Teratological Studies

A group of four to six females were caged with a fertile NMRI male between 24 pm and 6 am. The presence of vaginal plug, immediately afterward, was regarded as an evidence for successful mating, and this day was designated as Day 0 of gestation. The dams weighed an average of  $26 \pm 2$  g on the first day of gestation. To make sure that the embryos will have enough Eg to react with RA effects, Eg (100 mg/kg in 2ml of olive oil) was administered orally, five days before RA administration, for 10 days. On the 10th day, beginning of limb embryonic organogenesis, a single dose of RA (60mg/kg in olive oil) was administered by gavage. The doses in these studies were chosen according to the results of our preliminary experiments.

The mice were killed on day 18 of gestation. Upon laparotomy, the fetuses were taken and examined for external malformations. Half of them were fixed by ethanol (80%) and dehydrated by 95% ethanol, and processed for skeletal staining by the Alizarin Red-Alcian Blue method. Control animals were either administrated an appropriate volume of the solvent or left untreated.

### \* Statistical Analysis

The data were subjected to one-way analysis of variance (ANOVA) appropriate to completely randomized design with six mice per group. The means were evaluated by using Tukey's HSD multiple comparison test. Chi-square was applied for analysis of

the statistical significance of fetus malformation differences.

## Results

Oral administration of RA on 10<sup>th</sup> day of gestation was teratogenic to embryos, and most of the exposed embryos were defective (Table 1). RA lowered the embryonic length of hands, feet, tail, CR, and CF by 32.8, 23.7, 26.9, 6.5 and 8.9%, respectively ( $P < 0.001$ ), compared to the solvent or control groups. RA induced many embryonic malformations such as short lower jaw, spiral tail, hand and foot bending, hand oligodactily, hand syndactily, foot syndactily and foot oligodactily by 38.3, 51, 17, 38.2, 46.8, 40, 31.9 and 34%, respectively (Table1).

Table 1: Comparison of teratogenic effects among the experimental groups.

| Treatment         | Number of litters | % Resorbed | Number of live fetuses examined | % of live fetused defects |             |           |           |             |             |               |               |
|-------------------|-------------------|------------|---------------------------------|---------------------------|-------------|-----------|-----------|-------------|-------------|---------------|---------------|
|                   |                   |            |                                 | short lower jaw           | spiral tail | bent foot | bent hand | syndac foot | syndac hand | oligodac foot | oligodac hand |
| Solvent (control) | 6                 | 1          | 54                              | 0                         | 0           | 0         | 0         | 0           | 0           | 0             | 0             |
| RA                | 6                 | 5          | 47                              | 38.3*                     | 51*         | 38.2*     | 17*       | 31.9*       | 40*         | 34*           | 46.8*         |
| Eg                | 5                 | 1          | 47                              | 0                         | 0           | 0         | 0         | 0           | 0           | 0             | 0             |
| Eg+RA             | 7                 | 2          | 52                              | 23.9*                     | 4.3*        | 21.7*     | 15.2*     | 23.9*       | 32.6*       | 32.6*         | 26*           |

\* Significantly different from the control group.

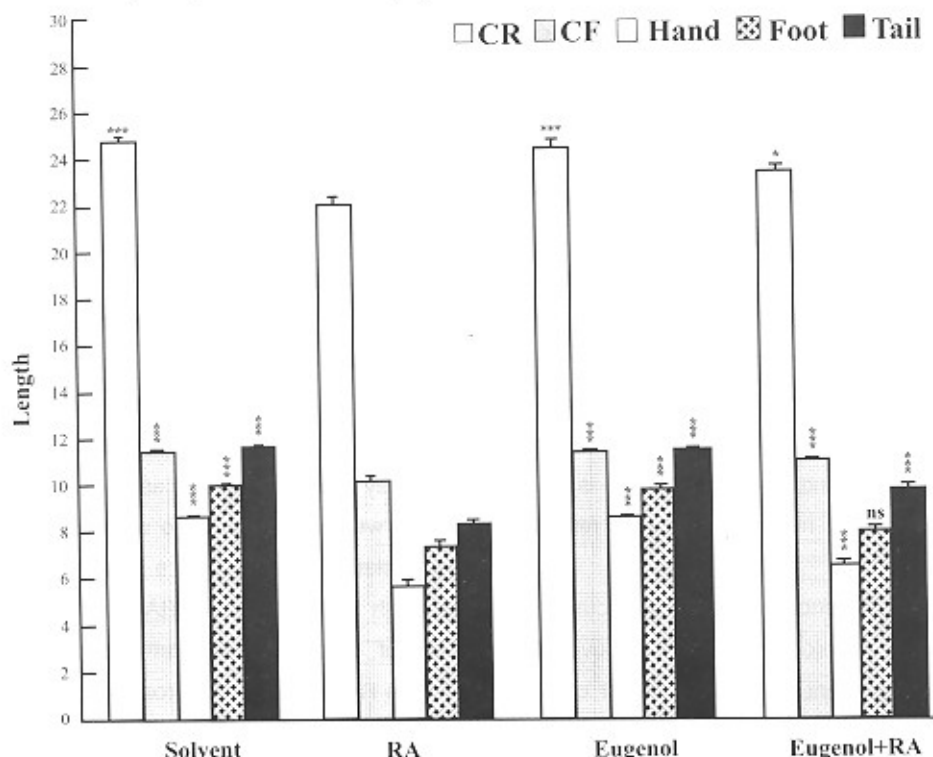
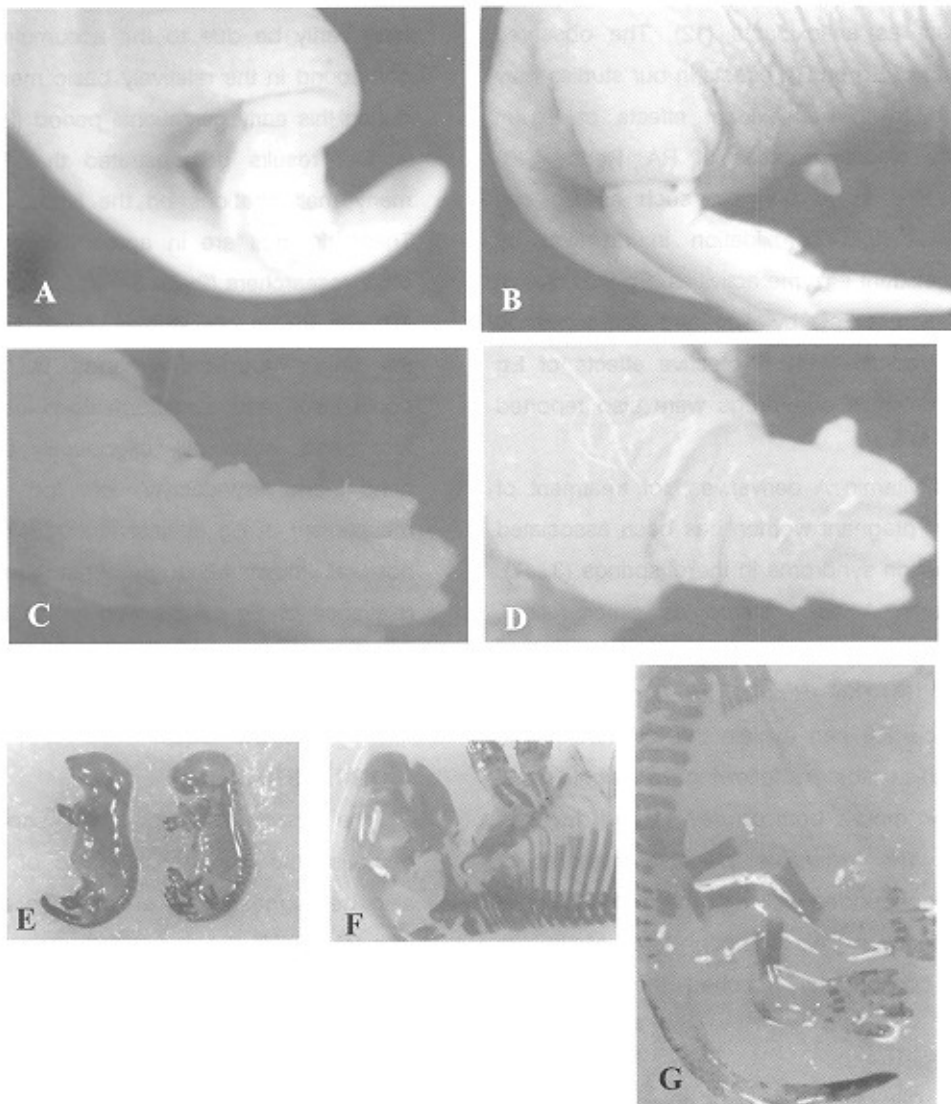


Fig 1: Effects of RA, Eg and RA+ Eg on the leng of fetal organs in the dependent groups. The difference among the groups was determined with respect to the RA. Each bar is Mean±SEM. The data were subjected to the one-way analysis of variance and followed by the protected Tukey's Test for multiple comparison (n=6). \*\*\*=P<0.001, \*\*=P<0.01, \*=P<0.05 ns=not significant.

Since feeding olive oil did not have any significant teratogenic effects on the embryos of pregnant mice compared to those of the control groups, the results of the control and the solvent groups were presented as solvent group (Table 1). Simultaneous administration of Eg with RA could significantly correct the embryonic length reduction in hands, tail and CF by 16, 18.85 and 7.44%, respectively ( $P < 0.001$ ) while CR by 6.57% ( $P < 0.05$ ), but not in foot. Eg could reduce embryonic malformations including, lower Jaw, foot bend, spiral

tail, oligodactyly and syndactyly of hand and synductily of foot 14.4, 16.5, 46.7, 20.8, 7.4 and 8%, respectively, (Table 1), but could not inhibit hands bend and foot oligodactyly (Table1, Fig 2). Statistical analysis revealed that the differences were significant in all dependent groups at a rate of [F<sub>4,239</sub>=10.74,  $P < 0.000001$ ], CF [F<sub>4,239</sub>=32.86,  $P < 0.000001$ ], Hand [F<sub>4,239</sub>=91.64,  $P < 0.000001$ ], Foot [F<sub>4,239</sub>=53.45,  $P < 0.000001$ ] and Tail [F<sub>4,239</sub>=61.81,  $P < 0.000001$ ] (Fig 1).



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Fig 2: A comparison of teratogenic effects of RA and corrective effects of Eg on fetuses.

- A) Hind-limbs of embryos under treatment with RA with shortened foot, oligodactyly digits and shortened tail (B) Hind-limbs of embryos under treatment with RA + Eg, (C) Fore-limbs of embryos under treatment of RA with shortened hand and oligodactyly digits.
- D) Fore-limbs of embryos under treatment with RA and Eg, E, F, G) Alizarin Red and Alcian blue staining of whole body to mark skeletal malformation including (shortened foot, oligodactyly digits and shortened tail) by RA, and was corrected by Eg.

## Discussion

Today, foods which enhance and promote health and prevent disease are attracting the attention of scientists, consumers and industrialists. There is enough evidence to suggest that plant foods decrease the risk of these diseases and obviously the wide variety of protective phytochemicals providing aggregation and synergistic effects appear to be more important than individual nutrients or combination of nutrients for prevention.

The spice principle like eugenol, curcumin, capsaicin and extracts of onion and garlic has the potential to act as antioxidants (12). The observed protective effect of Eg on RA effects in our studies may be contributed to the antioxidant effects of Eg in decreasing the oxidative stress of RA. Reddy (13) demonstrated that spice principle such as Eg can effectively inhibit lipid peroxidation in rat liver by enhancing antioxidant enzyme activities. Eg was shown to inhibit oxidation of  $Fe^{2+}$  by  $H_2O_2$  and also scavenge  $O^{\cdot-}_2$  and  $OH^{\cdot}$  radicals (14). Protective effects of Eg against toxic materials and drugs were also reported (15, 16).

The use of vitamin A derivatives, for treatment of severe acne by pregnant women has been associated with a malformation syndrome in the offsprings (3, 17). Excessive vitamin A administered as either retinol, retinyl ester, or retinoic acid is teratogenic in laboratory animals (2, 18, 19). The extremely low exposure of the embryo to 13-cis RA can explain the low teratogenic potency of this isomer following a single oral administration in mouse both on day 11 and day 9 of gestation (2). The extensive placental transfer of all-trans RA corresponded well with the high teratogenic potency of this compound (2). Metabolite with all-trans configuration reached the embryo in

significant amounts (2). Cusic et al. (20) reported that RA administration on 10<sup>th</sup> day of gestation produced extra-digits which were morphologically more advanced than those in the untreated controls. Lorente et al. (21), also reported RA administration to pregnant rats in doses sufficient to induce 90% incidence of Cleft Palate. Kraft et al. (2) reported that the embryonic concentrations of all-trans RA were higher on day 9 than day 11 of gestation and this might be due to developmental stage dependence on intrinsic sensitivities of embryonic structures. The increased concentration of all-trans RA in the embryos on day 9 may partly be due to the accumulation of an acidic compound in the relatively basic media of the embryo during this early gestational period (2).

Our results demonstrated that RA could induce many malformations on the limbs, tail, CR and CF. These findings are in agreement with the results of other researchers (2, 3). Simultaneous administration of Eg with RA, in our studies, could significantly correct the length reduction in hands, tail, CR and CF. Eg could also, reduce malformations including lower jaw, foot bend, spiral tail, oligodactily and syndactily of hand, and syndactily of foot. The protective mechanism of Eg against teratogenic effects of RA is not well known although literature reviews reveal the relevance of Eg effects with its antioxidant properties (15, 16). Data analysis revealed that the differences were significant in all dependent groups (Fig1).

## Conclusion

The results of this study show that Eg may decrease the teratogenic effects of RA, and it might also be suggested as a protective agent to be used with RA in skin therapy during pregnancy.

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