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Original Research Article

Effect of eugenolon white blood cells and corticosterone in sub-acute restraint stress-induced wistar albino rats

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ABSTRACT

Introduction: The effect of eugenol on white blood cells (WBCs) and corticosteroneis known. However, pharmacological properties and activities of eugenol such as anti-inflammatory, antioxidant, anesthetic, muscle relaxant, improved motor coordination, and increased red blood cells were reported in stress - induced rats.

Objective: To evaluate the effect of eugenol on WBCs in sub -acute restraint stress-induced Wistar albino rats.

Materials and Methods: Female Wistar albino rats (n = 30) weighing (150–220 g) were divided into five groups with six animals in each group, Group I: normal control; II : vehicle polyglycerol treated (PG); III: eugenol treated alone - TA (150 mg/kg/i.p for 15 days); IV : stress -induced (SA-stress alone), and V: stress followed by treated (T/S) with. Blood samples were collected at the end ofthestudyand total leucocyte count (TLC), differentiation leucocyte count (DLC), platelet count (PC) and concentration of corticosterone were estimated.

Results: The TA group showed significant ($p<0.01$) increase in TLC when compared with the control group. The platelet count was significantly less in the SA group as compared to T/ S group ($p<0.001$). The effect of eugenol on DLC did not show a significant difference with respect to neutrophil and lymphocytes count in T/S groups, when compared with other groups, whereas, no significant differences were observed between basophils, monocytes, and eosinophils of T/S when compared with SA group. No significant differences were observed in corticosterone levels in all groups.

Conclusion: Acute restraint stress induced distribution of WBC was not significantly attenuated by eugenol and no changes were observed in corticosterone level. However, eugenol increases platelet aggregation.

1. Introduction

Blood is the medium through which immune cells travel to maintain their normal surveillance and to rapidly reach the site where immune activation is required.[1] Hence the quantity of leukocytes in blood represents the distribution pattern of leukocytes in the body and the activation of the immune system.[2] Stress is defined as an event of stimulus - mediated reaction in the brain that initiates physiological fight-or-flight system in the body. Stress, that ends within minutes to hours, is called acute stress and it causes the maximum distribution of immune cells in the body.[2,3] The hormones such as norepinephrine (NE), epinephrine (EPI), and corticosterone (CORT) play major role in acute stress response.[4,5] NE and EPI, released immediately during stress, mobilize immune cells into blood flow whereas CORT decrease sthe quantity of immune cells in the blood and other tissues.[2]

Eugenol (2-methoxy-4-(Prop-2-en-1-yl) phenol is a volatile phenolic compound of essential oil obtained from clove (*Egeniacaryophyllus)*. Eugenol is used in preparation of skincare products, cosmetics, flavoring agent dental and pharmaceutical products due to its antiseptic and antispasmodic properties.[4] In traditional medicine, eugenol has been used in the treatment of flatulence, cholic, chronic diarrhea, and other gastrointestinal disor-

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ders.[5] The pharmacological activities of eugenol include anti-oxidant,[5] antibacterial,[6,7] anti -inflammatory,[8] and antipyretic effects.[9]

Eugenol has been reported to improve motor coordination and decrease plasma corticosterone level in immobilized stress-induced Wistar rats.[10] Moreover, it was reported that *in vitro* eugenol decreases the migration of leukocytes and aids in inflammatory process.[11] The effect of eugenol (150 mg/kg) on red blood cells (RBC) in restraint stress-induced rats was studied in our previous study which showed raised RBC count, packed cell volume, and hemoglobin level.[12] However, no data was available on the effect of eugenol on the distribution of stress- induced WBCs and corticosterone in rats. Hence, the present study was undertaken to evaluate the se effects.

2. Materials and Methods

2.1. Chemicals

Analytic grade eugenol (C_{10} H_{12} O_2), a clear to pale yellow oily liquid extracted from certain essential oils especially from clove oil and cinnamon, was purchased from Sigma Chemical Industry.

2.2. Animals

Female Wistar albino rats weighing between 150 – 220g were part of in this study, and were housed according to the standard conditions. The study was carried out according to guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India. The study protocol was approved by the Institute's Animal Ethics Committee (IAEC no. 01 / 17 /2015).

2.3. Experimental procedure

Animals were divided into five groups with six animals in each group—Group I (normal control) received standard diet, Group II animals were administrated with vehicle used to emulsify eugenol that is polyglycerol (PG) Intraperitoneal for 15 days and Group III animals were administrated with Eugenol (treated alone - TA) 150 mg /kg/i.p body weight for 15 days. Group IV was subjected to immobilization stress alone (SA) for 15 days (6 hr/day). Group V animals were subjected to stress and were immediately treated with Eugenol 150 mg /kg/i.p body weight for15 days. The dose was selected based on toxicity study.

2.4. Immobilization stress induction procedure[10]

Rats were subjected to restraint stress in a wire mesh restrainer for 6 hours per day for 15 days. The wire mesh restrainer (length : 8 cm, breadth: 4 cm and height: 4 cm) had a wooden base and stainless-steel wire mesh restrainer hinged to the base. A padlock and latch helped to secure the rat in the restrainer.

2.5. Blood sample collection[13]

Blood samples were collected at the end of the study; blood was collected from ventral/ dorsal artery or lateral tail vein by nicking vessel and cannulation was done to minimize contamination of the samples. Precautions were taken to avoid the hemostasis.

2.6. Determination of hematological indices

WBC count was performed by Dacie and Lewis method.[14] Turk's fluid was used for TLC (1:20) and cell count was done by using Neubauer counting chamber under light microscope. DLC was performed using method of Mathers et. al.[15]

2.7. Assay of corticosterone

The assay was carried out with slight modification of Singh and Verman[16] method and is based on the oxidation of corticosteroids with ferric iron (III) in an acidic medium and subsequent complex with ferrous iron (II) and potassium hexacyanoferrate. Plasma samples (0.5 μl) was mixed with appropriate volumes of working solutions of CORT were transferred into a series of 10 ml volumetric flasks. Sulphuric acid (4N, 2ml) and ferric chloride (0.5%, 2ml) were added to each followed by potassium hexacyanoferrate (III) solution (0.5%, 0.5 ml). The mixture was heated in a water - bath and maintained at 70$\pm$2ºC for 30 minutes with occasional shaking and diluted to the 5ml mark with distilled water. The absorbance was measured at 780 nm against the reagent blank.

2.8. Statistical analysis

Data were analyzed by ANOVA and Tukey's multiple comparison tests using SPSS 20 software. $P<0.05$ was considered statistically significant.

3. Results

Table 1: Effect of eugenol on WBC and platelet count

Groups	**WBC ($\times 10^3$ /mm^3)**	**Platelets ($\times 10^6$ /mm ^{3})**
Group I (Control)	9.51 $\pm$ 1.33	267.70 $\pm$ 3.66
Group II (PG)	11.43 $\pm$ 1.30	262.53 $\pm$ 4.06
Group III (TA150mg)	12.28 $\pm$ 1.31	180.15 $\pm$ 1.74
Group IV (SA)	13.38 $\pm$ 1.071	54.25 $\pm$ 3.35
Group V (T/S 150mg)	12.97 $\pm$ 0.81	238.80 $\pm$ 1.63

WBC-White blood cells; PG-polyglycerol, TA - treated alone, SA-stress alone, T/S-stress+treatment,

3.1. Effect of eugenol on leukocyte count

As per the leukocyte count of different groups of animals (Table 1), a significant increase in TLC was observed in SA and T/ S in contrast to the control group($p<0.0001$). However, no significant difference was observed between leukocyte count of T / S and SA groups (Table 2).

3.2. Effect of eugenol on Platelets

The platelets count of different groups of animals (table 1) indicated significantly decreased platelet count in SA ($p<0.0001$) as compared to other groups. Whereas, results of T/ S indicated significantly ($p<0.0001$) increased platelet count when compared with TA and SA (Table 2).

3.3. Effect of eugenol on differentiation leucocyte count

The differential leukocyte count of all groups, as represented in table 3, indicates significantly decreased counts of neutrophils, basophils, eosinophil, and monocytein SA group when compared with control, PG, TA and T / S ($p<0.05$). Similarly, significant decreased lymphocytes count was observed in TA when compared with control, SA ($p<0.05$) and PG ($p<0.001$). However, significant increased monocytes count was observed in TA when compared with control and SA (Table 4).

3.4. Effect of eugenol on CORT

The plasma CORT levels in different groups were—control: 1.85 ± 0.761, PG: 1.81 ± 0.767, TA: 1.67 ± 0.668, T/S: 2.15 ± 0.86, and SA : 2.87 ± 1.15. However, no significant differences were observed in mean plasma CORT concentration of groups when compared with each other.

4. Discussion

Stress can be psychological or physical and causes the release of norepinephrine and epinephrine which in turn elevates the release of blood lymphocytes, monocytes, and neutrophils whereas, CORT decreases immune cells in blood and tissues.[2] The aggregation of leukocytes in the blood causes immune -enhancement by availing maximum leukocytes at the site of activation.[12,17]

The acute stress in human causes increase in immune cell distribution in blood as compared to the resting- state; whereas rodents demonstratedecreased immune cells in blood. This is maybe due to the initial aggregation of immune cells and reflect trafficking of immune cells.[2] The study of Rosenberger *et al*. described that response to stress causes transfer of leukocytes inside the blood within a minute, decreases monocytes, and lymphocyte number, and increases the number of neutrophils.[18] In the present study leukocytes concentration decreases in stress- induced rats which may again be due to initial aggregation and reflect trafficking of immune cells. Whereas, eugenol treated group did not show any significant alteration in leukocyte counts. In contrast, a short- term decrease in blood leukocyte numbers represents trafficking of cells out of the blood to the target organs[2] Similarly, in the present study, monocytes and neutrophils were decreased in the stress-induced animals.

The study of Malyszko *et al.*[19] and Takeda *et al.*[20] explained acute water immersion restraint and acute cold - restraint stress which further causes reduced collagen-induced aggregation in whole blood and ADP-induced aggregation in platelet-rich plasma, respectively. Similarly, in the present study, decreased platelet count in animals by acute restraint stress were observed. However, increased platelets count was observed in eugenol and stress-treated animal groups. Similar results were reported by Hata *et a*l.[21]

The study performed by Pitman *et al.*[22] reported increased basal CORT level inrestrain stress-induced rats on day 2 and 3. Although fromday 4-6, the levels were not significantly increased due to habituation to the stress. Similarly, the study of Sadler *et al.*[23] suggest no significant increase in the CORT level in the mice on 14 day restraint due to habituation to stressor. Hypothalamic-pituitary-adrenal axis is less responsive to the repeated restrain stress after 8 or 14 days of restraint.[24,25] In this study, no significant increase in CORT levels were observed among the groups of animals, which may be due to habituation to the stressor. In contrast with results of the present study, the study of Pandian et al. reported significant increase in plasma CORT level in restraint stress- induced for 15 days for 6 hours among the groups when compared with control group. Whereas, stress and eugenol-treated group shows significantly decreased CORT level when compared with stress -alone group.

In this study the administration of eugenol (150 mg/kg) in T/S and TA for 15 days increased the TLC compared with control group rats whereas, the effect of eugenol on DLC did not show significant difference in neutrophil and lymphocytes count in T/ S groups when compared with other groups and no significant differences were observed between basophils, monocytes eosinophils of T/S when compared with SA group.

The present study showed the effect of eugenol on WBC and CORT in sub-acute restraint stress-induced rats. The blood samples were collected at the end of the study which may be resulted in fluctuated results. Hence, further studies are required to determine the effect of eugenol on WBC and CORT at regular time intervals and effect of eugenol on other stress hormones.

5. Conclusion

Stress-induced distribution of WBCs was not significantly attenuated by eugenol as was observed in stress and

Table 2: Comparison between groups

Groups	WBC		Platelets	
	Difference	P value	Difference	P value
PG-Control	1.916	0.066	-5.170	0.051
SA-Control	3.863	0.000***	-213.446	0.000***
TA-Control	2.771	0.003*	-87.553	0.000***
T/S-Control	3.458	0.000***	-28.900	0.000***
SA-PG	1.946	0.060	-208.276	0.000***
TA-PG	0.855	0.722	-82.383	0.000***
T/S-PG	1.541	0.193	-23.730	0.000***
TA-SA	-1.091	0.513	125.893	0.000***
T/S-SA	-0.405	0.975	184.546	0.000***
T/S-TA	0.686	0.851	58.653	0.000***

WBC-White blood cells; PG-polyglycerol, TA - treated alone, SA-stress alone, T/S - stress+treatment, ' *** ' P<0.000 1, ' ** ' P<0.01, ' * ' P<0.05

Table 3: Effect of eugenol on differential leucocyte count

DLC($\times 10^3$ /mcL)	Group I (Control)	Group II (PG)	Group III (TA 150mg)	Group IV (SA)	Group V (T/S 150mg)
Neutrophils	28.21 ± 3.96	31.52 ± 3.27	30.79 ± 2.58	25.54 ± 2.31	31.07 ± 2.96
Lymphocytes	66.39 ± 4.12	70.68 ± 5.09	57.85 ± 3.72	67.79 ± 6.70	67.73 ± 4.03
Basophils	0.70 ± 0.35	0.36 ± 0.11	0.47 ± 0.27	$0.1 \pm 1.52e^{-17}$	0.46 ± 0.44
Monocytes	2.66 ± 1.16	2.97 ± 1.91	4.24 ± 1.48	1.77 ± 0.42	2.58 ± 1.20
Eosinophils	2.13 ± 0.59	2.77 ± 1.61	2.13 ± 0.62	0.52 ± 0.27	1.13 ± 0.55

DLC-differential leukocyte count; PG-polyglycerol, TA - treated alone, SA-stress alone, T/S - stress+treatment.

Table 4: Comparison between groups

Groups	Neutrophils		Lymphocytes		Basophils		Monocytes		Eosinophils	
	Dif.	P	Dif.	P	Dif.	P	Dif.	P	Dif.	P
PG-Control	3.31	0.36	4.29	0.55	-0.34	0.27	0.30	0.99	0.64	0.70
SA-Control	-2.66	0.57	1.39	0.98	-0.60	0.01*	-0.89	0.77	-1.61	0.02*
TA-Control	2.58	0.59	-8.54	0.03*	-0.22	0.65	1.57	0.27	-0.00	1.00
T/S-Control	2.86	0.50	1.34	0.98	-0.24	0.60	-0.08	0.99	-1.00	0.29
SA-PG	-5.97	0.01*	-2.89	0.83	-0.26	0.53	-1.20	0.53	-2.25	0.001**
TA-PG	-0.72	0.99	-12.83	0.000***	0.11	0.95	1.26	0.48	-0.64	0.70
T/S-PG	-0.44	0.99	-2.95	0.82	0.10	0.97	-0.38	0.98	-1.64	0.02*
TA-SA	5.24	0.04*	-9.93	0.01*	0.37	0.19	2.46	0.02*	1.61	0.02*
T/S-SA	5.53	0.03*	-0.05	1.00	0.36	0.22	0.81	0.82	0.61	0.73
T/S-TA	0.28	0.99	9.88	0.01*	-0.01	0.99	1.65	0.23	-0.99	0.29

Dif.-difference, P-pvalue, PG-polyglycerol, TA - treated alone, SA-stressalone, T/S-stress+ treatment, ' *** 'P<0.001, ' ** 'P<0.01, ' * 'P<0.05

eugenol-treated rats and no changes were observed in CORT in all groups of animals. Therefore, additional studies are required to know the action of eugenol on chemical mediators released by stress which is responsible for WBCs and platelets distribution in blood.

6. Source of funding

None.

7. Conflict of interest

None.

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