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IN VITRO EVALUATION OF ANTI INFLAMMATORY ACTIVITY OF VITAMIN E BY MEMBRANE STABILIZATION TEST

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ABSTRACT

Objective: To evaluate the anti-inflammatory activity of α-tocopherol(Vitamin-E) by its membrane stabilising action on Human RBC's using invitro method. Methodology: Membrane disruption in RBC's is induced by adding Prostaglandin $F_{2\alpha}$ (PG $F_{2\alpha}$), which is induced by generating free oxygen radicals. The cell membrane damage was assessed by measuring the haemoglobin level in the supernatant solution using Photoelectric Colorimeter and the histological examination of RBC suspension was done under high power microscope. Result: Marked inhibition of hemolysis was observed in Vitamin-E treated RBC's which increased with increasing the dose. Histological examination of RBC's showed the presence of Spherocytes and Crenated RBC's in the control, whereas in Vitamin-E treated RBC's there was a decrease in the presence of Spherocytes and Crenated RBC's, with maximum inhibition observed with 40µg of Vitamin-E. Conclusion: This study provides evidence that PG $F_{2\alpha}$ causes cell membrane damage and Vitamin-E protects the cell membrane from free radical injury, thereby establishing its anti-inflammatory action.

INTRODUCTION

Eicosanoids play a major role in the inflammatory and immune responses. They are archidonate metabolites, including Prostaglandins(PGs), prostacyclin(PGI2), Thromboxane A2 (TXA2), Leukotrienes (LTS),are produced by most of the cells, when a variety of physical,chemical and hormonal stimuli activate acyl hydrolases that make archidonate available(1). In addition to enzymatic formation of eicosanoids, several families of eicosanoids isomers are generated at significant concentrations in vivo by non-enzymatic free radical catalysed oxidation of arachidonic acid(AA) . The best characterized of these isoeicosanoids are the F_2 Isoprostanes (F_2-ISO Ps). Unlike PGs, these compounds are initially formed esterified in phospholipids, after which they are hydrolysed to their free form by phospholipase, including PAF acetylhydrolase, which then circulate and are metabolized and excerted into urine(1).

Their production is not inhibited in-vivo by inhibitors of cox-1 or cox-2, but their formation is suppressed by anti-oxidants. The PG-$F_{2\alpha}$ isomer,8-iso- PG-$F_{2\alpha}$,was the first F_2-ISO P to be identified(2). Measuring levels of these compounds in plasma and urine(3) is considered the most accurate method to asses oxidative stress status in-vivo and increased levels has been associated with Acute Coronary Syndrome(4), Atherosclerois(5), Hypercholesterolemia (6), Diabetes (7). Vitamin-E is a phenolic anti-oxidant, which readily donate the hydrogen from the hydroxyl group on the ring structure to free radicals, which then become unreactive(8).It has a major biological role in protecting polyunsaturated fats and other components of cell membrane from oxidation by free radicals preventing lipid perioxidation and is therefore, primarily located within the phospholipid bilayer of cell membrane(9). Vitamin-E deficiency causes damage to cell membrane and leakage of cell contents, resulting in myopathies, neuropathies, liver necrosis, and increased erythrocyte hemolysis(10).

Membrane stabilization of RBC is an in vitro method to test the anti-inflammatory activity of a drug, where the membrane destruction is prevented by drugs or extracts which have free radical scavenging property(11).Prostaglandins cause cell membrane damage and isoprostanes which are prostaglandin like molecules can cause membrane disruption by generating free oxygen radicals(12). So in this study prostaglandinF2α was used to produce membrane damage of red blood cells and Vitamin-E (dl-α tocopherol acetate) to protect the RBC's from damage. In this study, an attempt has been made to study the stability of erythrocytes biochemically. Erythrocytes were chosen for the study because of their ready availability and relative simplicity. They have been used as model system to study the effect

of toxic substances on erythrocytes membrane by measuring haemoglobin leakage and histological changes in RBC (13).

MATERIALS & METHODS

Objective:

To demonstrate the anti-inflammatory activity of Vitamin-E by membrane stabilization test, an in-vitro method by using human RBC.

Study design:

In vitro method using human RBC's

Sample:

3ml of blood from 2 healthy Volunteers.

Study centre:

Institute of pharmacology, Madras Medical College, Chennai

Dept of clinical pathology, Rajiv Gandhi Government General Hospital, Chennai

HRBC Membrane Stablization Method:-

This method involves the stabilization of Human Red Blood cell (HRBC) membrane by Vitamin-E from Prostaglandin $F_{2\alpha}$ induced membrane lysis.

Reagents:-

-HRBC suspension 10%

-EDTA (Ethylene diamine tetra acetic acid)

-15-methyl $PGF_{2\alpha}$ (Carboprost) 25µg/ml

- dl- α- tocopherol acetate(Evion paediatric suspension)

(10mg,20mg,30mg,40mg,50mg,60mg)

Preparation of 0.5ml of 10% HRBC Suspension:-

6ml of Blood

↓

Centrifuge at 3000rpm for 10 min

↓

Packed cells were washed 3 times with Normal saline

↓

10% v/v suspension of the packed cells was made with normal saline.

Procedure-1:-

Fresh whole human blood of 3ml from two healthy volunteers of same blood group was collected and transferred to test tubes containing EDTA .The tubes are centrifuged at 3000rpm for 10 minutes and packed cells obtained were washed 3times with equal volumes of normal saline. The volume of blood was measured and reconstituted as 10%v/v suspension with normal saline.

Solutions of 6 different concentrations of Vitamin-E emulsion were prepared by reconstituting with normal saline. Assay mixture (3.5ml) contained 0.5ml of RBC, suspension, 1ml of Vit-E,1ml of normal saline and 1ml of Prostaglandin (25mg/ml). Control solution was prepared as above with 1ml of normal saline instead of Vitamin-E

All the tubes were incubated at 37^0C for 30 min. They were centrifuged and the hemoglobin content in the supernatant layer was estimated using photo electric colorimeter at 560nm. The percentage inhibition of hemolysis was calculated using below formula.

% inhibition of hemolysis = 100 X $\{OD_1 - OD_2/OD\}$

Where,

OD1= Optical density of control solution

OD2= Optical density of test sample

RESULTS

Effect of α-tocopherol on prostaglandin $F_{2\alpha}$ induced Human RBC lysis

SAMPLE	CONCENTRATION µg/ml	OPTICAL DENSITY	% INHIBITION OF HEMOLYSIS
PG with Normal saline		0.34	_
PG with Vit-E	10	0.18	47.06
PG with Vit-E	20	0.14	58.82
PG with Vit-E	30	0.12	64.71
PG with Vit-E	40	0.11	67.65
PG with Vit-E	50	0.09	73.53
PG with Vit-E	60	0.07	79.41

PG- Prostaglandin

Inhibition of hemolysis was observed significantly starting from 30µg of Vitamin-E, which increased with increasing doses of Vitamin-E.

Procedure-2:

A drop of RBC suspension taken from the above assay mixture was placed on a glass slide, followed by a cover slip over the drop. The slide was immediately examined under High Power Microscope and the morphology of RBC was studied. The changes in size and shape of RBC's were observed in comparison with normal RBC.

RESULT

Effect of α-tocopherol on $PGF_{2\alpha}$ induced histological changes in Human RBC's

	Slides	Normal RBC	Spherocytes	Crenated RBC
1	Normal RBC	++++	-	-
2	Prostaglandin(PG)	+	+	++
3	PG+Vit-E 10µg	+	++	++
4	PG+Vit-E 20 µg	++	+	+
5	PG+Vit-E 30 µg	+++	+	-
6	PG+Vit-E 40 µg	++++	-	-
7	PG+Vit-E 50 µg	++++	-	-
8	PG+Vit-E 60 µg	++++	-	-

25% of cells per Hpf → +

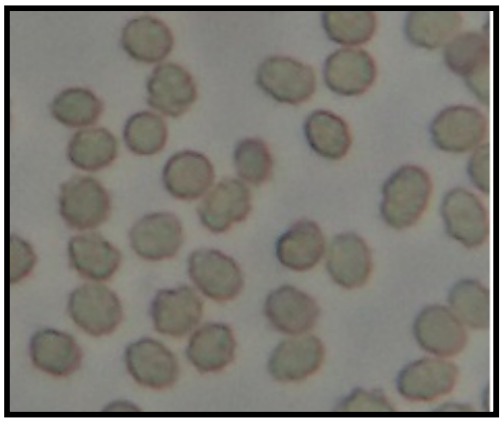
50% of cells per Hpf → ++

75% of cells per Hpf → +++

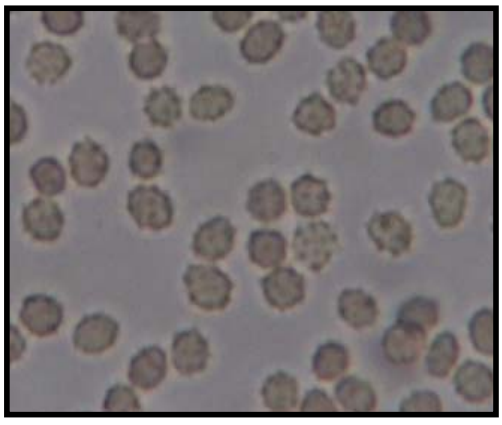
100% of cells per Hpf → ++++

Spherocytes → Spheroidal and less disc like cells than normal RBC's(14)

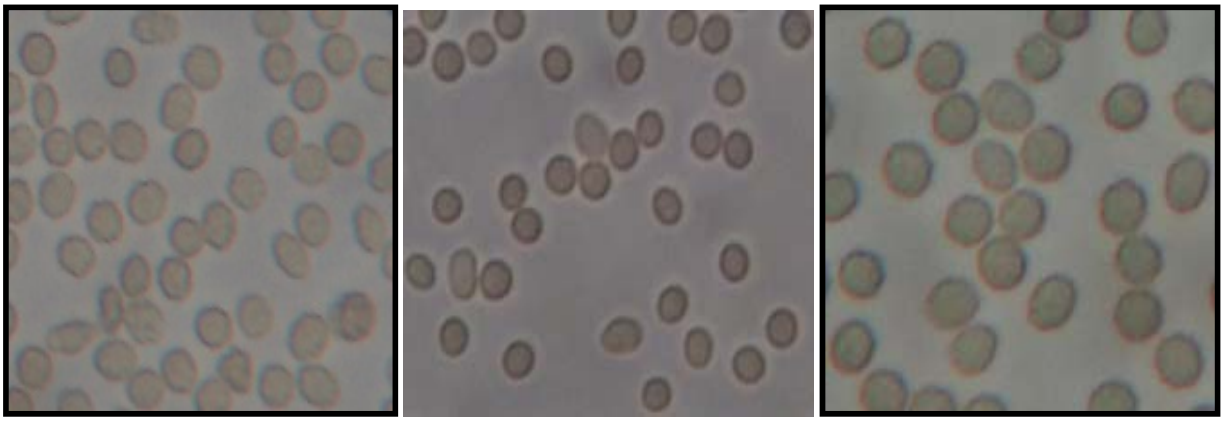
Crenated RBC's → Cells with contracted & irregular margin(15)



Normal RBC without cell lysis



With PG's Membrane Disruption and Cell Lysis



With VITAMIN E 30 µg With VIT-E 40µg With VITAMIN E 60µg

DISCUSSION

Increased prostaglandin synthesis causes cell membrane damage by generating free oxygen radicals(12),which is mainly suppressed by anti-oxidants.(1)In this study $PGF_{2\alpha}$ was used to produce cell membrane lysis in Human RBC's and α-tocopherol was added to protect the RBC's from oxidative damage. Vitamin-E has a major biological role in protecting cell membranes from lipid peroxidation, as it is primarily located within the phospholipid bilayer of cell membranes(9). Assay mixture containing Vitamin-E showed significant inhibition of hemolysis, starting from 30µg, which increased with increase in the dose of Vitamin-E. The maximum inhibition of hemolysis was observed with 60µg of Vitamin-E.

The histological changes induced by $PGF_{2\alpha}$ in RBC's include spherocytes and crenated RBC's. Presence of spherocytes and crenated RBC's was gradually reduced on increasing the doses of Vitamin-E. A dose of 40µg of Vitamin-E showed complete protection of membrane damage of RBC's induced by $PGF_{2\alpha}$.The response was sustained with increase in dose of Vitamin-E upto 60µg.Histological examination of RBC's for its size and shape can be considered as one of the most accurate method to asses oxidative stress status in-vivo. The results of the study showed that α-tocopherol protects cell membrane from oxidative damage. Therefore has membrane stabilising property and thereby inhibit the early stage of inflammatory process.

CONCLUSION

Free radical injury plays a major role in many chronic inflammatory diseases like Osteoarthritis(16), Rheumatoid arthritis(17) and the role of NSAID's in these disorders is only symptomatic. Anti-oxidants like Vitamin-E are very effective in controlling inflammation especially induced by free radicals (18). They not only provide symptomatic relief but also have disease modifying action in these disorders. The anti-inflammatory effect

of Vitamin-E has been confirmed by this in-vitro study. Therefore α-tocopherol can be included as a first line of drug in many chronic inflammatory diseases.

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