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Protective Ability of Vitamin E Against Acetylsalicylic Acid-Induced Glutathione Depletion, Acetylcholinesterase and (Na^+,K^+)-ATPase Activities, and Erythrocyte Osmotic Fragility

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Abstract

Acetylsalicylic acid (ASA), a well-recognized non-steroidal anti-inflammatory drug (NSAID), is well known for the treatment of fever, pain alleviation and inflammatory conditions, especially for the prevention of cardiovascular complications and disorders. However, ASA has been reported to cause hepato- and gastro-toxicities. We evaluated the protective ability of α-tocopheryl acetate (Vitamin E, Vit E) against ASA-induced glutathione depletion, reduction in acetylcholinesterase (AChE) activity, Na^+,K^+-ATPase activity and erythrocyte osmotic fragility in the red blood cells isolated from male albino rats. ASA significantly inhibited reduced glutathione (GSH) which was dramatically protected by Vit E. However, no detrimental activities of ASA were observed on glutathione reductase (GR) or glutathione peroxidase (GPx). Significant reduction of AChE activity in red blood cells was observed following treatment with ASA and Vit E significantly protected this detrimental effect. A small, but no-significant, elevation in the Na^+,K^+-ATPase activity was observed following treatment with ASA and slight non-significant reduction was observed following treatment with Vit E. Similar results were observed in erythrocyte osmotic fragility experiment demonstrating % hemolysis in these red blood cells. Thus, Vit E may serve an adjunct preventative agent during the therapeutic treatment of ASA.

Keywords: Acetylsalicylic acid; Vitamin E; Glutathione; Na,K-ATPase activity; Acetylcholinesterase; Erythrocyte osmotic fragility; Red cell membrane

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) such as acetylsalicylic acid (aspirin, ASA) have been demonstrated to induce gastrointestinal erosions and ulcer leading to gastrointestinal mucosal injury and gastric bleeding [1-3]. ASA has been demonstrated to interact directly with gastric mucosa leading to mucosal injury, inhibition of protective prostaglandins, enhancement in acid back-diffusion, microvascular injury and neutrophil activation leading to massive production of oxygen free radicals and oxidative injury [1-5]. In the present investigation, we focused to determine the effect of ASA on glutathione depletion, acetylcholinesterase (AChE) and Na^+,K^+-ATPase activities, and to evaluate the efficacy of vitamin E in ameliorating this effect. Parmar *et al.* (2017) have demonstrated that ASA significantly inhibited acetylcholinesterase (AChE) activity in an *in vitro* mechanistic investigation and indicated that a low dose ASA therapeutic strategy may serve subjects suffering from Alzheimer's disease [6]. In this investigation, we evaluated the efficacy of vitamin E in ASA-induced effects on red blood cells.

Vitamin E, an integral member of tocopherols and tocotrienols family, demonstrates an array of distinctive antioxidant activities in vivo [7,8]. Vitamin E is intricately associated with the regulation of gene expression, immunomodulatory activities and cell signaling [7,8]. Furthermore, vitamin E can inhibit NF-kB, a transcription factor involved in cell apoptosis and proliferation. We evaluated the protective efficacy of Vitamin E in this pathophysiology. Vitamin E has demonstrated dramatic protective

abilities against diverse models of oxidative insult [7,8]. It has been well demonstrated that normal human red blood cells exert a significantly low basal permeability to the cations and it is highly temperature dependent, which is constantly corrected by Na,K-ATPase [9]. The incidence of cation leaks from red blood cells is aggravated under various stimulatory conditions, including oxidative burst or chemical insult [9]. In this investigation, we assessed the protective efficacy of vitamin E against ASA-induced aggravation of the incidence.

The osmotic fragility test is conducted to assess the erythrocyte resistance to hemolysis, which is done to help diagnose two conditions (i) thalassemia and (ii) hereditary spherocytosis [10]. Basically, this investigation determines whether the ability of hemoglobin has been comprised following treatment with ASA. As it is well documented that hemoglobin assists red blood cells to carry oxygen and perform the routine biochemical and pathophysiological functions, and any disruption in this pathogenesis will cause anemia. In this investigation, we extensively conducted erythrocyte osmotic fragility (% Hemolysis) following treatment with ASA and evaluated the protective ability of vitamin E in this pathophysiology.

Overall, our investigation was very important because ASA is extensively used worldwide and sometimes ASA is consumed in high dose, so the present investigation is particularly important to evaluate whether co-administration of vitamin E may provide some protection against ASA-induced cellular injury.

Materials and Methods

Chemicals and Reagents

All reagents were of the highest purity available. Reduced glutathione (GSH), Glutathione reductase, NADPH, 5, 5'-dithiobis-(2-nitrobenzoic) acid (DTNB), Adenosine triphosphate disodium (ATP-Na_2), Trizma (Tris-HCl) were purchased from Sigma-Alrdrich, St. Louis, MO, USA. Imidazole was purchased from Fluka AG Buchs, Switzerland and Ouabain from E. Merck, Germany. Acetylsalicylic acid (ASA) was obtained from Bayer-India Ltd (Thane, Maharashtra, India).

Experimental Animals and Treatment

The study protocol was approved by the Institutional Animal Ethics Committee and performed in accordance with the rules and guidelines on animal experimentation. Albino rats, *Charles foster* strain were obtained from the National Laboratory Animal Center (NLAC) of the CSIR- Central Drug Research Institute, Lucknow, UP, India, for breeding and experimental purposes. The animals were maintained on standard hygienic conditions, in the form of an open system of husbandry under natural photoperiod and the average temperature of the animal house was maintained 24±3 °C throughout the year. Young male albino rats (*Charles foster*), weighing 200-250 g, age: 60 days, maintained on commercial rat diet (Lipton Co., India Ltd./Unilever, Mumbai, Maharashtra, India), under standard hygienic conditions, were divided into three groups- Group I, Group II and Group III. The rats of Group I served as Control, were given the diet and water ad libitum. The rats of Group II were administered non-therapeutic dose of 250 mg acetyl salicylic acid (ASA) /kg body weigh p.o. daily [11]. The Group III rats were administered ASA at a dose similar to Group II along with Vitamin E (α-tocopheryl acetate) at a dose of 6.7 mg/kg body weight/day. Since, the significant changes were observed in the case of rats treated for five days, accordingly the rats treated for five days were selected for the experiments.

Blood samples from Group I, Group II and Group III rats were collected from the caudal vein after five days of continuous treatments on the sixth day [12]. The osmotic fragility (O.F.) was determined and O.F., profiles were obtained by plotting percent (%) hemolysis against the corresponding NaCl concentration (%) in the range of 0.1 to 0.9 % [13].

Estimation of Reduced Glutathione (GSH)

The method of Beutler *et al.* was adapted for erythrocyte reduced glutathione (GSH) estimation, using 5, 5'-dithiobis-(2-nitrobenzoic) acid as the reaction agent [14]. The GSH standard 2 µg-100 µg demonstrated the absorbance (412 nm) linear relationship with reduced glutathione concentration.

Glutathione Reductase (GR) and Glutathione Peroxidase (GPx)

The red cell enzyme activities of GR and GPx were assayed as per the method of Beutler *et al.* The activity of GR enzyme was measured following the oxidation of NADPH at 340 nm [15]. The final assay mixture contained 50mM Tris-HCl, 0.25 mM EDTA (pH 8.0), 1 µM FAD, 3 mM GSSG, 0.1 mM NADPH and 30 µl 1:20 hemolysate. The activity of the enzyme GP was assayed by measuring the oxidation of GSH to GSSG by t-butylhydroperoxide, then measuring its rate of formation, using the GR reaction and the oxidation of NADPH was followed at 340 nm. The final assay mixture contained 100 mM Tris-HCl, 0.5 mM EDTA (pH 8.0), 2 mM GSH, 0.3 ml of 10 units/ml of GR, 0.2 mM NADPH, 0.07 mM t-butylhydroperoxide, 30 µl of 1:20 hemolysate.

Acetylcholinesterase (AChE) Activity

AChE activity was determined following the method of Beutler *et al.* In the assay system, acetylthiocholineiodide was used as the substrate. The rate of production of thiocholineiodide by AChE was measured, using 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), which produced yellow color and its rate of formation was recorded at 412 nm [15]. The final assay mixture contained 100 mM Tris-HCl, 0.5 mM EDTA (pH 8.0), 0.025 mM DT%NB in 0.1% Sodium citrate and 50 µl of 1:20 diluted hemolysate.

Preparation of Red Cell Membrane and Assay of (Na^+-K^+)-Atpase Activity

The blood samples were centrifuged for 15 minutes at 1,000 x g, the plasma and buffy coats were removed and the sedimented red cells were washed 4-5 times with 0.154 M (physiological concentration) of NaCl. Precaution was taken to eliminate leukocyte contamination. The red cell membranes were prepared following the method of Marchesi and Palade[16] and the entire procedure was carried out at 4 ºC. The activity of the enzyme (Na^+-K^+)-ATPase was assayed following the method of Lukacovic *et al.* [17].

Hemoglobin (Hb) and Erythrocyte Osmotic Fragility (O.F., % Hemolysis) Assay

Hb estimation was performed following the method of Beutler *et al*, while erythrocyte osmotic fragility (% hemolysis) tests were performed following the method of Dacie and Lewis [15,13]. For hemoglobin estimation, 20 µl of hemolysate was added in 1 ml Drabkin's Solution (Ferricynide-cyanide reagent) and the OD was measured at 540 nm within 5 minutes. For O.F., measurements, heparinized blood was added to hypotonic solutions of varying concentration of NaCl in the range of 0.1% to 0.9% and the tubes were allowed to stand at room temperature for 30 minutes. The tubes were then centrifuged in a clinical centrifuge at 2,000 rpm for 5 minutes and optical density of the supernatant was measured at 440 nm, using supernatant form 0.9% NaCl as blank, having undetectable lysis.

Statistical Analysis

The results are expressed as means ±SD. All the data of different treated groups as compared to the control groups were statistically analyzed, using Student's t-test and values $p<0.05$ were considered significant.

Results

Reduced Glutathione (GSH), Glutathione Reductase (GR) and Glutathione Peroxidase (GPx)

Significant reduction was observed in GSH level following treatment with ASA (250mg/kg body weight) over a period of five days, however, vitamin E (6.7mg/kg body weight) provided remarkable protection (*$p < 0.001$). Incidentally, co-treatment of the red blood cells with ASA and vitamin E provided greater than 96% protection. However, no significant changes were observed following treatment of the blood sample with ASA or ASA+Vitamin E (Table 1).

Rat Blood Sample	Reduced Glutathione (GSH)*		Glutathione Reductase (GR)*b	Glutathione Peroxidase (GPx)*b
	mg/100ml RBC	mg/g Hb	IU/g Hb	IU/g Hb
Control	24.76±0.50	1.618±0.076	7.42±0.37	25.18±1.10
Acetylsalicylic acid (ASA)	10.64±0.07[a]	0.841±0.015[a]	7.48±0.15	26.13±1.32
Acetylsalicylic acid (ASA)+Vitamin E	23.65±0.32	1.544±0.076	7.92±0.32	25.61±1.16

*- Each value is the mean of at least 3-4 experiments ±SD

a- P< 0.001; highly significant when compared with control

b- Enzyme activity is expressed in terms of IU (µmole of NADPH oxidized/minute) per g of hemoglobin at 37 °C

Table 1: Reduced Glutathione (GSH), Glutathione Reductase (GR) and Glutathione Peroxidase (GPx) Levels of Control, Acetylsalicylic acid (ASA) treated and Acetylsalicylic acid (ASA)+Vitamin E Treated Rats

Acetylcholinesterase (AChE) and Na^+,K^+-ATPase Activities

ASA (250 mg/kg body weight)- induced a significant reduction in AChE activity, while ASA and vitamin E co-treatment caused approximately 96% reversal of this effect (Table 2) (*$p < 0.001$). A slight, but non-significant, increase in Na^+,K^+-ATPase activity following treatment with ASA (250mg/kg body weight) while ASA and vitamin E co-treatment exhibited a marginal decline (Table 2).

Rat Blood Sample	Acetylcholinesterase (AChE) Activity*b (IU/g Hb)	Na^+, K^+-ATPase Activity*c (µmole Pi/mg Protein)
Control	18.99 ±1.29	0.163 ±0.023
Acetylsalicylic acid (ASA)	11.35 ±1.04[a]	0.188 ±0.014
Acetylsalicylic acid (ASA)+Vitamin E	18.98 ±2.15	0.182 ±0.006

*- Each value is the mean of at least 3-4 experiments ±SD

a- P< 0.001; highly significant when compared with control

b- Enzyme activity is expressed in terms of IU (µmole of acetyl thiocholine iodide hydrolyzed/minute) per g of hemoglobin at 37 °C

c- Enzyme activity is expressed in terms of µmole Pi liberated per hour per mg of protein.

Table 2: Red cell membrane Acetylcholinesterase (AChE) and Na^+, K^+-ATPase Activities of Control, Acetylsalicylic acid (ASA) treated and Acetylsalicylic acid (ASA)+Vitamin E Treated Rats

Erythrocyte Osmotic Fragility (%Hemolysis of the Red Blood Cells)

ASA-induced erythrocyte osmotic fragility or percentage of hemolysis of the red blood cells were determined and the protective ability of vitamin E was assessed. Erythrocyte osmotic fragility profile of control, ASA-treated and ASA + vitamin E-treated rats

(Figure 1, Table 3). ASA treatment caused an incidence of erythrocyte osmotic fragility (Figure 1), while vitamin E provided significant protection (*p< 0.001).

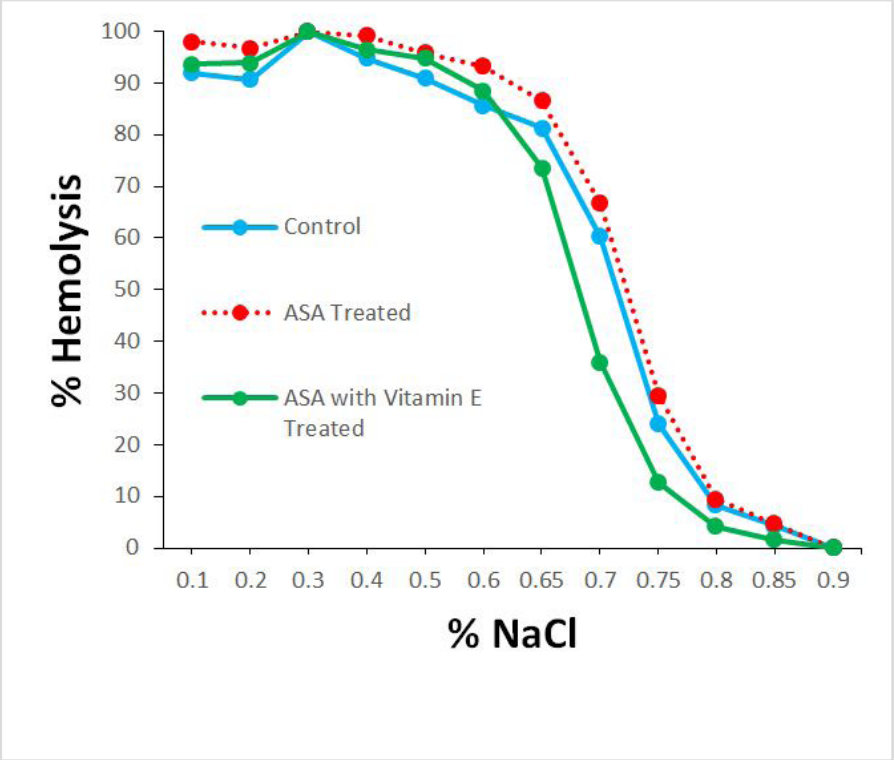

Figure 1: Erythrocyte Osmotic Fragility Profile of Control, Acetylsalicylic acid (ASA) treated and Acetylsalicylic acid (ASA) with Vitamin E Treated Rats

% Hemolysis			
% NaCl	Control	Acetylsalicylic acid (ASA)	Acetylsalicylic acid (ASA)+Vitamin E
0.1	91.95 ±1.63	97.98 ±0.62	93.61 ±2.17
0.2	90.72 ±2.32	96.71 ±1.54	93.90 ±3.94
0.3	100 ±0.00	100 ±0.00	100 ±0.00
0.4	94.77 ±0.33	99.09 ±0.15	96.51 ±0.95
0.5	90.85 ±2.75	95.87 ±0.67	94.75 ±1.91
0.6	85.60 ±0.61	93.15 ±2.65	88.61 ±0.83
0.65	81.30 ±2.04	86.47 ±5.06	73.58 ±12.09
0.7	60.35 ±0.54	66.74 ±5.61	35.97 ±17.07
0.75	23.99 ±0.72	29.58 ±8.98	12.66 ±7.91
0.8	8.29 ±3.29	9.58 ±1.16	4.14 ±2.56
0.85	4.27 ±1.46	4.60 ±1.35	1.47 ±0.83
0.9	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00

Erythrocyte osmotic fragility was assessed as a % Hemolysis of control, ASA-treated and ASA+vitamin E-treated rats. %Hemolysis was assessed at various concentrations of NaCl. See Materials and Methods section for details.

Table 3: Erythrocyte Osmotic Fragility (%Hemolysis) of Control, Acetylsalicylic acid (ASA) treated and Acetylsalicylic acid (ASA)+Vitamin E Treated Rats

Discussion

Incidentally, salicylic acid derivatives are naturally and extensively available in herbs, spices, fruits and vegetables, and it has been reported that serum salicylic acid concentrations are significantly higher in vegetarians as compared to the non-vegetarians [18,19]. In fact, salicylic acid derivatives provide a resistance to pathogens and environmental stressors [20]. Aspirin, a non-steroidal anti-inflammatory drug (NSAID), belongs to the family of salicylates, which was introduced into medical treatment approximately 100 years ago [21-23]. Salicylic acid, the principal metabolite of aspirin, exerts the anti-inflammatory benefits by irreversibly inhibiting cyclooxygenase-1 and -2, the key elements in arachidonic acid metabolism to prostanoids [1,3,5]. Clinical evidence demonstrated a synergism between aspirin and dietary phytochemicals in ameliorating colorectal cancer [24].

Aspirin and salicylates are extensively available as over-the-counter medications and in multiple prescription drugs, including topical preparations recommended for the treatment of pain, warts, and acne [2,3,5,22,23]. However, overdose can cause potential adverse events [25]. Following oral ingestion, aspirin is rapidly converted into salicylic acid, which is then readily absorbed in the stomach and small intestine [1,2]. At therapeutic doses, salicylic acid is metabolized by the liver and eliminated within 2-3 hours [1-3]. However, an overdose of ASA may cause detrimental effects as demonstrated by EM Boyd that it causes acute oral toxicity in dogs, cats and albino rats [26]. He demonstrated the oral median lethal dose of ASA to be 0.92+0.045 gms/kg body weight, which demonstrated an array of adverse side effects including inactivity, impaired response to sound and sight, cataplectic tenseness, anorexia, diarrhea, nose-bleeding, hyperreflexia, convulsions, respiratory failure and deaths [26]. Furthermore, he displayed varying degrees of gastroenteritis, hepatitis, nephritis, pulmonary edema, and lesser toxic changes in the salivary glands, ovaries, skin, adrenals, thymus, mesentery, spleen, cardiac muscle, and skeletal muscle after conducting autopsy. These effects substantiates the importance and rationale of our present investigation.

Basically, salicylates directly or indirectly cause injuries to most organ systems by uncoupling oxidative phosphorylation, inhibiting Krebs cycle enzymes, leading to ketosis and a wide anion-gap metabolic acidosis, increasing lactate production, and inhibiting amino acid and limiting the production of ATP synthesis. Salicylate poisoning has been reported in the central nervous system, cardiovascular, pulmonary, hepatic, and renal metabolic systems [1-6]. Salicylates further stimulate the respiratory center, leading to hyperventilation and respiratory alkalosis [1-6]. Common side effects of salicylate include nausea, vomiting, upset stomach, stomach pain, nervousness, severe headache, facial swelling, trouble sleeping, nose bleeding, lethargy, blood in the urine or feces, heavy menstrual bleeding, asthma attack, coma, neurotoxicity, confusion, hearing loss, etc., [1,2,27]. Salicylate has also been demonstrated to induce non-cardiogenic pulmonary edema in few patients, while hypoxia has been pointed out that it may serve as a major factor [27].

It is quite evident that pain and pain alleviation is a major problem worldwide, and people consume aspirin and salicylic acid formulations extensively. We focused to protect against aspirin-induced toxicity using vitamin E (α-tocopheryl acetate). Earlier clinical investigation by Pohle *et al.* (2001) have demonstrated the involvement of oxygen free radicals and oxidative stress in aspirin-induced gastrointestinal injury in human volunteers, and the remarkable protective ability of vitamin C in this pathogenesis [1]. This study was mainly focused on gastrointestinal injury, however, this study motivated us to conduct this mechanistic investigation to assess the protective ability of vitamin E against aspirin-induced glutathione depletion, and modulation of AChE, Na^+,K^+-ATPase activities and erythrocyte osmotic fragility in red blood samples.

Since, aspirin has the ability to induce multi-organ toxicity, we investigated different biomarkers responsible for organ-specific toxicity. The protective ability of Vitamin E (6.7mg/kg body weight) was determined against aspirin (ASA)-induced glutathione depletion, AChE activity, Na^+,K^+-ATPase activity and erythrocyte osmotic fragility in the red blood sample isolated from male albino rats. ASA significantly inhibited reduced glutathione (GSH) activity, while Vit E provided excellent protection. On the contrary, ASA didn't induce any detrimental effect on glutathione reductase (GR) or glutathione peroxidase (GPx). ASA reduced AChE activity in red blood cells, while Vit E exerted remarkable protective efficacy. A small, but no-significant, elevation in the Na+,K+-ATPase activity was observed following treatment with ASA, while Vit E provided little non-significant reduction. Similar effects were observed in erythrocyte osmotic fragility experiment demonstrating %hemolysis in these red blood cells. Thus, Vit E may serve as an adjunct preventative agent during the therapeutic treatment of ASA.

Overall, our investigation clearly demonstrates that Vitamin E may serve as a potent adjunct therapy in conjunction with ASA during the treatment involved in pain alleviation.

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