Beneficial Effects of Vitamin E against Indomethacin-induced Glutathione Depletion, Acetylcholinesterase Activity, (Na⁺,K⁺)-ATPase Activity and Osmotic Fragility of Erythrocyte

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Abstract

Background: Indomethacin, is a well-known non-steroidal anti-inflammatory drug (NSAID), a cyclooxygenase inhibitor and extensively used as a medicine to reduce pain, fever, stiffness, swelling or inflammatory responses. Indomethacin inhibits both COX-1 and COX-2, which in turn, inhibits the production of prostaglandins in the gastrointestinal tract and disrupts the mucosal lining in the gastrointestinal tract leading to intestinal bleeding and perforation.

Methods: The protective ability of α-tocopheryl acetate (Vitamin E, Vit E) was assessed against indomethacin-induced glutathione depletion, increase in acetylcholinesterase (AChE), (Na⁺,K⁺)-ATPase activities and decrease in erythrocyte osmotic fragility in the red blood cells sample isolated from male albino rats.

Results: Indomethacin significantly inhibited reduced glutathione (GSH), which was dramatically protected by Vit E. However, no detrimental activities of indomethacin were observed on glutathione reductase (GR) or glutathione peroxidase (GPx). A significant increase in the of AChE activity of red blood cells was observed following treatment with indomethacin, however, Vit E treatment didn't bring the AChE activity to normal and further studies are needed. A small, but non-significant, elevation in the (Na⁺,K⁺)-ATPase activity was observed following treatment with indomethacin 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.

Conclusion: Thus, Vit E may serve as an adjunct preventative agent during the therapeutic treatment with indomethacin.

Keywords: Indomethacin; Vitamin E; Tocopherol; α-Tocopherol; Glutathione; (Na⁺,K⁺)-ATPase Activity; Acetylcholinesterase; AChE; Erythrocyte Osmotic Fragility; Red Cell Membrane

Introduction

Overdose of indomethacin, a well-recognized nonsteroidal anti-inflammatory drug (NSAID), has been demonstrated to induce gastric mucosal injury leading to abdominal pain, bleeding, black tarry stools, weakness, dizziness, ulceration, orthostatic hypotension and perforation in the gastrointestinal tissues. Furthermore, indomethacin reduces the ability of blood to clot, restricts the blood flow to the kidneys thereby cause nephrological dysfunctions and congestive heart failure [1-5].

It has been demonstrated that a deficiency of endogenous prostaglandins due to inhibition of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) by indomethacin is involved in gastrointestinal mucosal injury, macrovascular injury, ulcer leading to gastrointestinal mucosal injury and gastric bleeding [3,5-7]. A significant number of individuals are allergic to NSAIDs and in turn, develop shortness of breath, while asthma patients have higher risk of serious allergic reactions to NSAIDs [3,5].

In this study, we focused to determine the effect of indomethacin on glutathione depletion, acetylcholinesterase (AChE) and (Na⁺,K⁺)-ATPase activities, and to evaluate the efficacy of vitamin E in protecting against indomethacin-induced cellular injury.
We also determined the efficacy of vitamin E in indomethacin-induced toxicity on red blood cells.

Vitamin E, a well-established fat soluble antioxidant and a member of tocopherols and tocotrienol family, intervenes the propagation of free radicals and reactive oxygen species that spread through biological membranes or lipid peroxidation [8-10]. In short, vitamin E provides marvelous protection against oxidative stress in both in vitro and in vivo models. The deactivation of protein kinase C is mediated by vitamin E to intervene smooth muscle growth [11,12]. Vitamin E has been demonstrated to downregulate the expression of the CD36 scavenger receptor gene, which in turn modulate the expression of the connective tissue growth factor [13,14].

Normal human red blood cells have been exhibited to exert a low basal permeability to the cations, which is highly temperature dependent. This effect is constantly corrected by (Na⁺,K⁺)-ATPase [15], while diverse stimulatory conditions, including oxidative burst or chemical insult, aggravate the incidence of cation leaks from red blood cells. The protective efficacy of vitamin E against indomethacin-induced pathophysiology was assessed.

To determine the erythrocyte resistance to hemolysis, it is a common practice to conduct osmotic fragility test. Basically, this test diagnoses the involvement of (a) thalassemia and/or (b) hereditary spherocytosis [16]. Overall, this experiment will evaluate whether following treatment with indomethacin, the characteristics of hemoglobin has been compromised. Red blood cells carry oxygen with the help of hemoglobin and performs diverse biochemical and pathophysiological functions, and any perturbation or malfunctions lead to anemia [17,18].

This study will assess whether indomethacin compromises the ability of hemoglobin. As it is known 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. This study will conduct erythrocyte osmotic fragility (% hemolysis) following treatment with indomethacin and determine possible protective ability of vitamin E.

This investigation is extremely important as indomethacin is extensively used worldwide and often time consumed in high doses by the patients, so this study will evaluate whether co-administration of vitamin E may provide some protection against indomethacin-induced gastrointestinal injury.

Materials and Methods

Chemicals and Reagents

All reagents were of the highest purity available. Reduced glutathione (GSH), Glutathione reductase, NADPH, 5, S′-dithiobis-(2-nitrobenzoic) acid (DTNB), Adenosine triphosphate disodium (ATP-Na⁺), Trizma (Tris-HCl) were purchased from Sigma-Aldrich, St. Louis, MO, USA. Imidazole was purchased from Fluka AG Buchs, Switzerland and Ouabain from E. Merck, Germany. Indomethacin 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 0.42 mg indomethacin/kg body weight p.o. daily. The Group III rats were administered indomethacin 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 [19] after five days of continuous treatments on the sixth day. The osmotic fragility (O.F.) was determined [20] and O.F. profiles were obtained by plotting percent (%) hemolysis against the corresponding NaCl concentration (%) in the range of 0.1 to 0.9 %.

Estimation of Reduced Glutathione (GSH)

The method of Beutler et al. [14] was adapted for erythrocyte reduced glutathione (GSH) estimation, using 5, S′-dithiobis-(2-nitrobenzoic) acid as the reaction agent. 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. [21]. The activity of GR enzyme was
measured following the oxidation of NADPH at 340 nm. 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 GPx 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, and 30 µL of 1:20 hemolysate.

Acetylcholinesterase (AChE) Activity

AChE activity was determined following the method of Beutler et al. [22]. 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. The final assay mixture contained 100 mM Tris-HCl, 0.5 mM EDTA (pH 8.0), 0.025 mM DTNB 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 3500 rpm, 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 leucocyte contamination. The red cell membranes were prepared following the method of Marchesi and Palade [23] and the entire procedure was carried out at 4 °C. The activity of the enzyme (Na⁺,K⁺)-ATPase was assayed following the method of Lukacovic et al. [24].

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

Hb estimation was performed following the method of Beutler et al. [21,22], while erythrocyte osmotic fragility (% hemolysis) tests were performed following the method of Dacie and Lewis [20]. 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 2000 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 Indomethacin (0.42 mg/kg body weight) over a period of five days, however, vitamin E (6.7mg/kg body weight) provided significant protection (p <0.001). Incidentally, co-treatment of the red blood cells with indomethacin and vitamin E provided greater than 90% protection. However, no significant changes were observed in glutathione reductase (GR) or glutathione peroxidase (GPx) levels following treatment of the red blood cell sample with indomethacin or indomethacin + Vitamin E (Table 1).

<table>
<thead>
<tr>
<th>Rat Blood Cell Sample</th>
<th>Reduced Glutathione (GSH)</th>
<th>Glutathione Reductase (GR)</th>
<th>Glutathione Peroxidase (GPx)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/100mL RBC</td>
<td>mg/g Hb</td>
<td>IU/g of Hb</td>
</tr>
<tr>
<td>Control</td>
<td>24.42 ±1.18</td>
<td>1.58 ±0.121</td>
<td>7.34 ±0.34</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>20.44 ±0.90*</td>
<td>1.426 ±0.007*</td>
<td>7.38 ±0.39</td>
</tr>
<tr>
<td>Indomethacin + Vitamin E</td>
<td>23.54 ±0.30*</td>
<td>1.487 ±0.045</td>
<td>7.85 ±0.40</td>
</tr>
</tbody>
</table>

* 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 following treatment of the red blood cell sample with indomethacin or indomethacin + Vitamin E (Table 1).

Acetylcholinesterase (AChE) And (Na⁺,K⁺)-ATPase Activities In Red Blood Cells

Indomethacin (0.42 mg/kg body weight)-induced a significant increase in AChE activity, while indomethacin and vitamin E co-treatment caused no reversal of this effect (Table 2). A slight, but non-significant, increase in (Na⁺,K⁺)-ATPase activity was observed following treatment with indomethacin (0.42 mg/kg body weight) while indomethacin and vitamin E co-treatment exhibited a marginal decline (Table 2).
Following oral administration, indomethacin is rapidly absorbed in the gastrointestinal tract and reported to be virtually 100% bioavailable with peak plasma concentrations after oral administration of a single dose, between 0.9 + 0.4 and 1.5 + 0.8 hours in an empty stomach [26,27]. However, indomethacin bioavailability and absorption is significantly reduced and delayed when taken with meal, while the maximal effect has been reported when taken after a high protein diet, or high-lipid diet. Furthermore, it is interesting to note that advancing age doesn't affect indomethacin absorption or bioavailability [26,27].

In the 1960s, a number of acetic acid derivatives were developed into indomethacin, diclofenac, and sulindac, and propionic derivatives into ibuprofen, naproxen, and ketoprofen [29]. Indomethacin exhibited the most potent activity of all these derivatives. Since then, indomethacin is used in treatment of migraine, headaches and pain alleviation, and became renowned as a headache reliever, or more so, as “indomethacin-responsive” headache disorders [28,29].

Indomethacin is a well-established NSAID and widely used as an effective anti-pyretic, analgesic and anti-inflammatory drug. Structurally, it is a methylated indole derivative having analgesic-antipyretic properties similar to other analgesic antipyretic drugs. Indomethacin inhibits prostaglandin synthesis by inhibiting prostaglandin-forming cyclooxygenase and is one of the most potent inhibitors of cyclooxygenase [25,26]. After oral administration, indomethacin is absorbed rapidly and almost completely from the gastrointestinal tract. The peak plasma concentration reaches within 3 hours after administration and ninety percent of the indomethacin becomes bound to plasma protein. Indomethacin is metabolized in liver by hepatic microsomal enzymes and has been reported to be largely converted to inactive metabolites [27,28].

It has been repeatedly demonstrated that acute renal failure caused by the NSAIDs administered at therapeutic doses is an underestimated complication as it is usually mild, transitory and non-anuric [26,27,30]. Literature exhibits that NSAIDs including

**Table 2:** Red cell membrane Acetylcholinesterase (AChE) and (Na+,K+)-ATPase Activities of Control, Indomethacin treated and Indomethacin with Vitamin E Treated Rats

<table>
<thead>
<tr>
<th>Rat Blood Cell Sample</th>
<th>Acetylcholinesterase (AChE) Activity*IU/g of Hb</th>
<th>(Na+,K+)-ATPase Activity*µmole Pi/mg Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16.55 ±1.27</td>
<td>0.162 ±0.010</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>29.42 ±0.96</td>
<td>0.168 ±0.008</td>
</tr>
<tr>
<td>Indomethacin + Vitamin E</td>
<td>27.06 ±1.71</td>
<td>0.166 ±0.006</td>
</tr>
</tbody>
</table>

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

**Table 3:** Erythrocyte Osmotic Fragility (% Hemolysis) of Control, Indomethacin treated and Indomethacin with Vitamin E Treated Rats

<table>
<thead>
<tr>
<th>% Hemolysis</th>
<th>% NaCl</th>
<th>Control (No Drug Treatment)</th>
<th>Indomethacin</th>
<th>Indomethacin + Vitamin E</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1</td>
<td>98.91 ±0.78</td>
<td>98.29 ±1.38</td>
<td>98.74 ±0.70</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>97.23 ±2.23</td>
<td>94.16 ±1.59</td>
<td>94.53 ±0.52</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>100 ±0.00</td>
<td>100 ±0.00</td>
<td>100 ±0.00</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>97.64 ±2.05</td>
<td>97.83 ±0.96</td>
<td>96.89 ±0.80</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>96.27 ±1.16</td>
<td>96.18 ±2.04</td>
<td>94.98 ±1.77</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>92.2 ±1.64</td>
<td>92.69 ±2.84</td>
<td>92.84 ±2.68</td>
</tr>
<tr>
<td></td>
<td>0.65</td>
<td>92.27 ±3.54</td>
<td>85.86 ±3.17</td>
<td>86.96 ±3.59</td>
</tr>
<tr>
<td></td>
<td>0.7</td>
<td>76.39 ±3.94</td>
<td>59.49 ±2.59</td>
<td>66.07 ±15.19</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>43.80 ±7.87</td>
<td>24.05 ±3.88</td>
<td>36.29 ±9.00</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>14.22 ±4.98</td>
<td>6.78 ±0.77</td>
<td>8.65 ±1.36</td>
</tr>
<tr>
<td></td>
<td>0.85</td>
<td>4.34 ±0.97</td>
<td>1.43 ±0.96</td>
<td>2.79 ±0.47</td>
</tr>
<tr>
<td></td>
<td>0.9</td>
<td>0.00 ±0.00</td>
<td>0.00 ±0.00</td>
<td>0.00 ±0.00</td>
</tr>
</tbody>
</table>

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

**Discussion**

Indomethacin-induced erythrocyte osmotic fragility or percentage of hemolysis of the red blood cells was determined to assess the protective ability of vitamin E. Erythrocyte osmotic fragility (% Hemolysis) of control, indomethacin-treated and indomethacin + vitamin E-treated rats are shown in (Table 3). Indomethacin treatment caused no significant changes in erythrocyte osmotic fragility, when compared with control, and vitamin E treated samples.
chronic and overdose use of indomethacin causes renal toxicity by inhibiting the enzyme cyclooxygenase in the glomerulus, producing vasconstriction. Especially in premature neonates, it has been estimated that therapeutic doses of indomethacin give rise to transitory renal toxicity in 24% of cases [26,27]. Moreover, there is a higher risk of drug-induced kidney toxicity and failure in critically ill patients due to their pre-existing renal complications including renal failure, presence of secondary hypovolemia, hypertension, cardiac failure, hyperaluminaemia, electrolyte disturbances, hepatic disorders affecting metabolism, and co-administration of other prescribed drugs may interfere with the metabolism and/or potentiate the nephrotoxicity [26,27,30].

Since, indomethacin is extensively used popular drug and is associated with adverse reactions and toxicity; we undertook this project to find out whether vitamin E can provide protection against indomethacin-induced toxicity. We demonstrated the effect of indomethacin on glutathione depletion, acetylcholinesterase (AChE) and (Na⁺,K⁺)-ATPase activities in red blood cells and evaluated the efficacy of vitamin E in protecting against indomethacin-induced cellular injury. We also determined the efficacy of vitamin E in indomethacin-induced erythrocyte osmotic fragility. Earlier, we studied the effect of another NSAID, acetylsalicylic acid on red cell osmotic fragility, reduced glutathione level, AChE and (Na⁺,K⁺)-ATPase activities [31].

Our study demonstrated a significant reduction in GSH level following treatment with indomethacin, however, vitamin E treatment provided a significant protection (p<0.001) in GSH level, but no changes were observed in glutathione reductase (GR) or glutathione peroxidase (GPx) levels. Indomethacin (0.42 mg/kg body weight)-induced a significant increase in AChE activity, while indomethacin and vitamin E co-treatment caused no reversal of this effect. We need to do further investigation to unveil this mechanism of action.

A small, but non-significant, increase in (Na⁺,K⁺)-ATPase activity was observed following treatment with indomethacin, while indomethacin and vitamin E co-treatment exhibited a marginal decline. Indomethacin treatment caused a significant decrease in erythrocyte osmotic fragility, when compared with control, while vitamin E showed protection.

Taken together, this investigation exhibits that vitamin E may serve as a novel adjunct therapy in conjunction with indomethacin therapy.

References