Evaluation of Diclofenac effect on oxidative stressed mice

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INTRODUCTION

Diclofenac, (2-[(2,6-dichlorophenyl) amino]benzene acetic acid) is a non-steroidal anti-inflammatory drug (NSAID) is among the most frequently used medical drug as analgesic, anti-inflammatory and antipyretic effect. This study aims to evaluate if Diclofenac has an antioxidant effect, relative to its analgesic antipyretic and anti-inflammatory activities, or it possesses a cytotoxic potential. Oxidative stress was induced by intraperitoneal injection of peroxide hydrogen (H₂O₂), and then a comparative study is made concerning the activities of the antioxidant enzymes SOD, CAT, GR, SDH as well as MDA lipid peroxidation levels in liver. An decrease in SOD, CAT, GR, SDH activity and MDA lipid peroxidation in mice treated with H₂O₂ accompanied by Diclofenac; compared to the group treated by L-ascorbic acid + H₂O₂ showed that diclofenac show good antioxidant effect. Moreover this study has suggested that acetylsalicylic acid induced anti-inflammatory effects in liver mediated by decrease oxidative stress and restore redox reaction of metabolism.

Key words: Diclofenac, Oxidative stress, Antioxidant.

ABSTRACT

Diclofenac (2-[(2,6-dichlorophenyl)amino]benzene acetic acid) Non-steroidal anti-inflammatory drug (NSAID) is among the most frequently used medical drug as analgesic, anti-inflammatory and antipyretic effect. This study aims to evaluate if Diclofenac has an antioxidant effect, relative to its analgesic antipyretic and anti-inflammatory activities, or it possesses a cytotoxic potential. Oxidative stress was induced by intraperitoneal injection of peroxide hydrogen (H₂O₂), and then a comparative study is made concerning the activities of the antioxidant enzymes SOD, CAT, GR, SDH as well as MDA lipid peroxidation levels in liver. An decrease in SOD, CAT, GR, SDH activity and MDA lipid peroxidation in mice treated with H₂O₂ accompanied by Diclofenac; compared to the group treated by L-ascorbic acid + H₂O₂ showed that diclofenac show good antioxidant effect. Moreover this study has suggested that acetylsalicylic acid induced anti-inflammatory effects in liver mediated by decrease oxidative stress and restore redox reaction of metabolism.

Key words: Diclofenac, Oxidative stress, Antioxidant.
COX-2 is an attractive target for medicinal chemists as it is expressed only in few normal tissues and is greatly up-regulated in inflamed tissues as well as many premalignant and malignant tumors. Since, most of the NSAID in the market show greater selectivity for COX-1 than COX-2 the Diclofenac moiety has been extensively exploited by the organic and medicinal chemist to synthesize potential therapeutic agents such as antibacterial, antitumor and antituberculosis. Several studies have shown that nonselective COX-2 inhibitors had beneficial effects on atherosclerosis progression inhibiting leukocyte aggregation, decreasing inflammatory cytokines, inhibiting superoxide radicals from macrophages and lowering plasma cholesterol levels.

The objective of the present research was to examine and test precisely antioxidant activity of NSAID Diclofenac and even the protective effect at long-term of treatment in liver of mice, compared and analyzed the activity of increasing stress markers that suits hydrogen peroxide by oxidation, and reduction of the activity of stress markers suitable for treatment by Vitamin C, with the decrease of the activity markers stress in the group treated by Diclofenac.

**MATERIAL AND METHODS**

**Tests:**
The test concerned 66 males adult Swiss albino mice weighting 25-30 grams. They were acclimatized to laboratory conditions before the test and fed *ad libitum*. They were fasted 16 hours prior to the treatment.

All experiments were in accordance with the guidelines provided by the CPCSEA. Animals were divided into 11 groups (n = 6 per group) as it's resumed in table 1. Ibuprofen, vitamin C (L-ascorbic acid) and H\textsubscript{2}O\textsubscript{2} were daily administered by intraperitoneal injection during 30 days.

**Table 1: summary of groups treated with Ibuprofen, H\textsubscript{2}O\textsubscript{2} and Vitamin C**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of mice</th>
<th>Treatment</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>NaCl</td>
<td>0.9 %</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>H\textsubscript{2}O\textsubscript{2}</td>
<td>100 mg/kg</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>Diclofenac</td>
<td>30 mg/kg</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>Diclofenac + H\textsubscript{2}O\textsubscript{2}</td>
<td>30 mg/kg + 1.5 g/kg</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>Vitamin C</td>
<td>20 mg/kg</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>Vitamin C + H\textsubscript{2}O\textsubscript{2}</td>
<td>20 mg/kg + 1.5 g/kg</td>
</tr>
</tbody>
</table>

**Preparation of tissues for analytic procedures**
Livers were rapidly thawed and homogenized using a Potter homogenizer (Elvehjem), in 3 volumes of ice-cold 10 mM HEPES, 1 mM EDTA, 0.25 M Sucrose and 10 mM 2-mercaptoethanol, pH 7.4. All procedures were performed at 4°C. Homogenates were centrifuged at 7000 x g for 15 mn at 4°C (sigma 2-16K) and the resultant supernatants were aliquoted and stored at −20°C for later enzyme assays.

**Biochemical assays**
All assays were conducted at 25°C using Jenway 6405 UV/Visible spectrophotometer (Thermo Electron Corporation, Biomate 3, USA).

**Protein Assay**

Protein content was measured according to the Bradford procedure by using bovine serum albumin (BSA) as standard. Protein reagent was added to protein solutions. The absorbance was measured at 595 nm after 10-15 mn. of incubation in the dark.

**Catalase**
The consumption of 7.5 mM H\textsubscript{2}O\textsubscript{2} in 50 mM potassium phosphate buffer (pH 7) was monitored at 240 nm as indicated in.

**Glutathione reductase**
The assay of Di ilio et al., was used. The assay mixture contained 0.5 mM oxidized glutathione, 1 mM EDTA, 0.1 mM NADPH and 50 mM potassium phosphate buffer (pH 7.4) and NADPH consumption was monitored at 340 nm.
Superoxide dismutase
The enzyme was assayed according to Paoletti et al., \(^{13}\): 5 mM EDTA, 2.5 mM MnCl\(_2\), 0.27mM NADH, 3.9 mM 2-mercaptoethanol in 50 mM potassium phosphate buffer (pH 7), monitored at 340 nm. The decrease in absorbance is measured after the addition of NADH to 0.27 mM as final concentration.

Succinate dehydrogenase
The enzyme was assayed according to King\(^{14}\): 100 mM potassium phosphate buffer (pH 7.4), 0.3 mM EDTA, 0.053 mM DCIP and 100 mg of protein. The mixture was pre-incubated 10 min at 25 °C before adding 50 ml of KCN-Succinate (containing 3.25 mg/ml of KCN in 0.5 M succinate). The measure of activity was done at 625 nm.

Thiobarbituric acid reactive substances
Lipid peroxidation was estimated by the formation of thiobarbituric acid reactive substances (TBARS) and quantified in terms of malondialdehyde (MDA) equivalents according to the method described by Samokyszyn and Marnett\(^{15}\): 1 ml of samples was added to 1 ml solution (0.375% thiobarbituric acid and 15% trichloracetic acid in 0.25 M hydrochloric acid). The tubes were heated at 100°C during 15 min and they were cooled in the ice to stop the reaction. One then carries out a centrifugation with 1000 x g during 10 min. The reading of supernatant was made to 535 nm.

Enzyme activity expression
The specific activity of each enzyme was calculated using the following formula:

\[
\text{AS} = \frac{(\Delta \text{Abs/mn} \times 1000)}{(\varepsilon \times [P] \times Ve)}
\]

\(\Delta \text{Abs/mn}:\) Absorbance variation/minute
\(\varepsilon\): (Extinction coefficient):
\(\varepsilon\) (H\(_2\)O\(_2\)) = 40 M\(^{-1}\).cm\(^{-1}\), for CAT
\(\varepsilon\) (NADH) = 6220 M\(^{-1}\).cm\(^{-1}\), for SOD and GR
\(\varepsilon\) (DCIP) = 19100 M\(^{-1}\).cm\(^{-1}\), for SDH
\(\varepsilon\) (MDA-TBA complex) = 153000 mM\(^{-1}\).cm\(^{-1}\), for MDA

\([P]\): Protein concentration
Ve: Assay volume

RESULTS AND DISCUSSION
Monitoring of Body Weight in Mice:
According to Figure 1, the body weight of all treated mice showed variations significantly different from those of the control, and control of stress. Group treated with H\(_2\)O\(_2\) show a significant weight loss showing a low activity during treatment period shows the presence of oxidative stress. Significant increase in weight of groups (Diclofenac +H\(_2\)O\(_2\)) and (Vitamin C+H\(_2\)O\(_2\)) versus control stress, shows that NSAID Diclofenac and L-ascorbic acid has restored the imbalance between pro-oxidant balance caused by the effect of hydrogen peroxide and antioxidant defense systems that lies at the agency level, compared the results with L-ascorbic acid and Diclofenacin terms of weight compared to control mice and the control of stress, shows the presence of the antioxidant effect of the drug.

The results in Figure (2; 3; 4; 5 and 6) showed that the activity of stress markers (CAT, SOD, GPx, SDH and TBARS) increased significantly in the treated group by hydrogen peroxide as compared with those of the control group. Explains presence of chronic oxidative stress in liver control stress group deduced by the presence of reactive oxygen species (ROS) generate by oxidative power of the hydrogen peroxide. Reactive oxygen species (ROS) produces oxidative stress which has been characterized in liver and includes, among several changes, an increase in the level of catalase, superoxide dismutase, glutathione peroxydase, succinate dehydrogenase and thiobarbituric acid reactive substances (TBARS), indicative of lipid peroxidation\(^ {16}\). The level of these enzyme activities in cells is crucial for determining the steady -state levels of superoxide radicals and hydrogen peroxide, superoxide dismutase converts superoxide radicals to hydrogen peroxide, which is then decomposed by catalase, and succinate dehydrogenase catalyzes the oxidation of succinate to fumarate with the reduction of

Differences were considered significant at the level \(p < 0.05\).
The analysis was performed with XLSTAT Version 2014.2.02
ubiquinone to ubiquinol. This occurs in the inner mitochondrial membrane by coupling the two reactions together. By one-electron reduction of oxygen, it gives rise to reactive oxygen species (ROS) which include free as superoxide or hydroxyl radical (OH\(^{−}\)) anion radicals. Other non-oxygen radical species can be produced, such as hydrogen peroxide (H\(_2\)O\(_2\)) and singlet oxygen (O\(_2\)). The result of an imbalance between the balance of pro-oxidants and antioxidant defense systems\(^{17}\).

As shown in Fig (2; 3; 4; 5 and 6) activity of stress markers (CAT, SOD, GPx, SDH and TBARS) decreases significantly in the treated group by (Diclofenac +H\(_2\)O\(_2\)) and (Vitamin C+H\(_2\)O\(_2\)) as compared to the group treated with hydrogen peroxide. Explains the restoration of balance redox reactions compared to antioxidant defense in the liver, by reducing significantly the production of reactive oxygen species (ROS). also show significant protective effect against reactive oxygen species (ROS) and against their oxidative effect on the molecular level, the significant decrease in the activity of stress marker in treated groups by D and C compared to the control groups showed a protective effect against reactive oxygen species (ROS) by restoring the balance of redox reactions, this protection potential is mainly attributed to the antioxidant capacity in ascorbic acid to scavenge reactive oxygen species (ROS), these results also show that acetyl salicylic acid also has scavenging activity ROS, which explains the antioxidant effect of NSAID Diclofenachide behind the other to already known. However, interpretation of these results becomes easy because administration of only of NSAID namely, Diclofenac in the absence of hydrogen peroxide decreases the amount of ROS in liver compared values of those recorded with hydrogen peroxide alone. The pro-oxidant effects of hydrogen peroxide and the observed protective effect of Diclofenac were explored by decreasing the activity of stress marker in treated groups (Diclofenac +H\(_2\)O\(_2\)) and (Vitamin C+H\(_2\)O\(_2\)) relative to control stress and from Diclofenac and Vitamin C compared to the control.

**Fig. 1:** weight gain in grams during 30 days of treatment. T: Control, H: H\(_2\)O\(_2\); D: Diclofenac; D+H: Diclofenac+ H\(_2\)O\(_2\); C: Vitamin C; C+H: Vitamin C + H\(_2\)O\(_2\). *significantly different from groups (T; H) at P<0.05. **significantly different from control of stress at P<0.05. The number of mice used in each group was 6.
Fig. 2: Evaluated Antioxidant effect of NSAID Diclofenac by CAT activity. T: Control, H: H2O2; D: Diclofenac; D+H: Diclofenac + H2O2; C: Vitamin C; C+H: Vitamin C + H2O2. *significantly different from control at P<0.05; **significantly different from control of stress at P<0.05; the number of mice used in each group was 6.

Fig. 3: Evaluated Antioxidant effect of NSAID Diclofenac evaluated by GR activity. T: Control, H: H2O2; D: Diclofenac; D+H: Diclofenac + H2O2; C: Vitamin C; C+H: Vitamin C + H2O2. *significantly different from control at P<0.05; **significantly different from control of stress at P<0.05; the number of mice used in each group was 6.
Fig. 4: Evaluated Antioxidant effect of NSAID Diclofenac evaluated by SOD activity. T: Control, H: H2O2; D: Diclofenac; D+H: Diclofenac+ H2O2; C: Vitamin C; C+H: Vitamin C + H2O2. *significantly different from control at P<0.05; **significantly different from control of stress at P<0.05; the number of mice used in each group was 6.

Fig. 5: Evaluated Antioxidant effect of NSAID Diclofenac evaluated by SDH activity. T: Control, H: H2O2; D: Diclofenac; D+H: Diclofenac+ H2O2; C: Vitamin C; C+H: Vitamin C + H2O2. *significantly different from control at P<0.05; **significantly different from control of stress at P<0.05; the number of mice used in each group was 6.
Fig. 6: Evaluated Antioxidant effect of NSAID Diclofenac evaluated by MDA activity. T: Control, H: H2O2; D: Diclofenac; D+H: Diclofenac+ H2O2; C: Vitamin C; C+H: Vitamin C + H2O2. *significantly different from control at P<0.05; **significantly different from control of stress at P<0.05; the number of mice used in each group was 6.

CONCLUSION

The present study demonstrates the protection against hydrogen peroxide-mediated liver toxicity by NSAID Diclofenac, an inhibitor of cyclooxygenase. It also showed that that NSAIDDiclofenac is a potent antioxidative agent which markedly reduced oxidative stress damages by establishing the redox homeostasis. This might be useful for the prevention of oxidative stress.

One way to confirm these results at the molecular level, a necessary task, is to examine the effect of these treatments on the gene transcription of the different enzyme as biomarkers of oxidative stress. These kinds of studies are in progress in our laboratory.

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REFERENCES