Evaluation of Acetylsalicylic Acid Effect on Oxidative Stressed Mice

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ABSTRACT
Aspirin (acetylsalicylic acid, ASA) is a commonly prescribed drug with a wide pharmacological spectrum. It is classified as the best non-steroidal anti-inflammatory drugs (NSAIDs). Inflammatory processes may play a pivotal role in several pathogenesis such as osteoarthritis, cerebrovascular injury, hypertension, auto-immune diseases and where oxidative stress plays also a major role. The present study was designed to determine whether a dose of 30 mg/kg of ASA might prevent oxidative damages caused by an intraperitonial injection of peroxide hydrogen (100 mg/kg). Antioxidant enzymatic activities of catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), succinate dehydrogenase (SDH), and index of lipid peroxidation: malodialdehyde (MDA) were determined. Antioxidant biomarkers significantly increased and mortality was noted with peroxide hydrogen (H₂O₂), which was significantly suppressed by treatment with aspirin. These results suggest that ASA may exert protective effects against oxidative stress damages.

Keywords: Acetylsalicylic acid (Aspirin), Oxidative stress, Antioxidant.

INTRODUCTION
Reactive oxygen species (ROS) are generated by normal metabolism through mitochondrial respiration and the cytochrome P450 system as by-products¹. Nevertheless, under several situations, the rate of generation of ROS exceeds the ability of their removal by antioxidants systems and oxidative stress occurs². ROS are involved in a wide spectrum of diseases, including chronic inflammation³, and in a wide variety of cancers⁴,⁵. To protect from these highly reactive intermediates, living organisms possess a defense system consisting of enzymatic (e.g., superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase, catalase) and non-enzymatic (e.g., glutathione (GSH), vitamins C and D) antioxidants that scavenge them⁶.

Aspirin (acetylsalicylic acid, ASA) is an old drug used for its analgesic antipyretic antithrombotic and anti-inflammatory effects. ASA is known to act by directly suppressing the cyclooxygenase enzyme (COX-1 and COX-2), the rate limiting enzyme catalyzing the biosynthesis of prostaglandins, thereby blocking the production of proinflammatory prostaglandins². It is known that inflammation can enhance tissue superoxide anion (O₂⁻) level through numerous mechanisms⁷. Theoretically, anti-inflammatory effects should restore the normal redox balance and thus eliminate the oxidative stress associated to inflammation. The present study aims to investigate the effect of a daily exposure to ASA on oxidant/antioxidant status in stressed mice by H₂O₂.
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. ASA, vitamin C (L-ascorbic acid) and H$_2$O$_2$ were daily administered by intraperitoneal injection during 30 days.

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).

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$_2$O$_2$ in 50 mM potassium phosphate buffer (pH 7) was monitored at 240 nm as indicated in.[11]

Glutathione reductase
The assay of Di ilio et al., (1983) 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., (1986): 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: 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: 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:

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

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

[P]: Protein concentration
Ve: Assay volume

Statistical Analysis
In each assay, all experimental values were expressed as mean ± standard error of mean and the statistical significance between treated and control groups were analyzed by ANOVA. Differences were considered significant at the level p < 0.05.
The analysis was performed with XLSTAT Version 2014.2.02

RESULTS AND DISCUSSION
Figure 1 shows a gain of weight for mice that received ASA and vitamin C as treatment however groups treated with H₂O₂ show a slight decrease of weight when it’s compared to the control.
Stressed mice showed high activities of CAT, SOD, GR and SDH (figures: 2, 3, 4 and 5) in addition to a high level of lipid peroxidation (figure 6). This implicates that all 4 antioxidant enzymes play a massif role in cell detoxification from ROS caused with H₂O₂ treatment. Superoxide dismutase (SOD; EC 1.15.1.1) constitutes the first line of defence against ROS within a cell by catalyzing the conversion of superoxide anion (O₂⁻), oxygen and hydrogen peroxide. Catalase is one of the most efficient enzymes known and cannot be saturated by H₂O₂ at any concentration.
The GSH system is probably the most important cellular defence mechanism that exists in the cell. The system consists of GSH, glutathione peroxidase and glutathione reductase. Glutathione peroxidase catalyses the reduction of H₂O₂ and other peroxidases and converts GSH to its oxidized disulphide form (GSSG).
Succinate dehydrogenase is useful as a marker enzyme for mitochondria. Malondialdehyde is a peroxidation product of polyunsaturated fatty acids and its level correlates with the degree of lipid peroxidation due to oxidative stress in cell membranes and their damage. MDA may be used as a marker of oxidative stress.

According to figure 2, 3, 4, 5 and 6, ASA seems to reduce significantly antioxidant enzymatic biomarkers activity and also lipid peroxidation which may suggest a protection from oxidative damage by aspirin. ASA is a potent antioxidative agent which markedly reduced the vascular production (O₂⁻) by reducing the vascular NAD(P)H oxidase activity in normal and hypertensive rats. In addition Aspirin as an inhibitor of synthesis of prostaglandins, would prevent the generation of oxyradicals. Also anterior studies showed that ASA tended to protect endothelial cells from oxidant damage. Chronic in vivo treatment with aspirin prevented the development of hypertension and reduced insulin resistance significantly. Aspirin seems to produce these effects through its antioxidative properties, since it was found to prevent the increase in aortic (O₂⁻) production observed in chronically glucose-fed rats.

<p>| Table 1: summary of groups treated with ASA, H₂O₂ and Vitamin C |
|-----------------|-----------------|-----------------|-----------------|</p>
<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>
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</tr>
<tr>
<td>2</td>
<td>6</td>
<td>H₂O₂</td>
<td>100 mg/kg</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>ASA</td>
<td>30 mg/kg</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>ASA + H₂O₂</td>
<td>30 mg/kg + 100 mg/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 + H₂O₂</td>
<td>20 mg/kg + 100 mg/kg</td>
</tr>
</tbody>
</table>
Fig. 1: weight gain evolution

![Graph showing weight gain evolution for different groups.]

T: Control, H: H₂O₂; A: acetyl salicylic acid (ASA); A+H: ASA+ H₂O₂; C: Vitamin C; C+H: Vitamin C + H₂O₂.
*significantly different from groups (T and 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: Catalase activity

![Graph showing catalase activity.]

T: Control, H: H₂O₂; A: acetyl salicylic acid (ASA); A+H: ASA+ 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: GR activity

T: Control, H: H₂O₂; A: acetyl salicylic acid (ASA); A+H: ASA+ 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: SOD activity

T: Control, H: H₂O₂; A: acetyl salicylic acid (ASA); A+H: ASA+ 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: SDH activity

T: Control, H: H₂O₂; A: acetyl salicylic acid (ASA); A+H: ASA+ 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: MDA level

T: Control, H: H₂O₂; A: acetyl salicylic acid (ASA); A+H: ASA+ 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 aspirin, an inhibitor of cyclooxygenase. It also showed that that ASA 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.

REFERENCES

