Evaluation of Acetaminophen Effect on Oxidative Stressed Mice by Peroxide Hydrogen

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Abstract

Acetaminophen (Paracetamol) is among the most commonly used analgesic and antipyretic drugs worldwide, it’s often, but anomalously, classified as non-steroidal anti-inflammatory drugs (NSAIDs) in textbooks of pharmacology [1,2]. This study aims to evaluate if paracetamol has an antioxidant effect, relative to its analgesic antipyretic and weak 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, and GR as well as lipid peroxidation levels in liver. An increase in SOD, CAT, GR activity and lipid peroxidation in mice treated with H2O2 accompanied by paracetamol; compared to the group treated by vitamin C + H2O2 showed that acetaminophen doesn’t show any antioxidant effect. Moreover this study has suggested that acetaminophen induced cytotoxicity in liver mediated by increased oxidative stress and altered redox metabolism.

Keywords: acetaminophen (paracetamol), peroxide hydrogen, oxidative stress

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1. Introduction

Acetaminophen (paracetamol) is an effective, cheap, and widely available analgesic; it’s classified as non steroidal anti-inflammatory drugs (NSAIDs), because it possesses analgesic activity against pain of mild to moderate severity but has few anti-inflammatory properties.

Acetaminophen, unlike NSAIDs, has only a weak inhibitory effect on purified preparations of cyclooxygenase (COX)-1 and COX-2 at therapeutic concentrations, it has been believed that the target of acetaminophen is a “brainspecific” COX enzyme that produces prostaglandins in the central nervous system thereby initiating fever and pain [3]. Thus it is a major cause of liver failure; it has been shown that high doses of the analgesic drug acetaminophen (APAP) produce centrilobular liver necrosis in human and other susceptible species [4,5,6]. Although the precise mechanism is still not determined, oxidative stress has been suggested to be involved in hepatotoxicity by APAP.

Oxidative stress is known as a disturbance of homeostasis between rate of antioxidant defenses and the rate of reactive oxygen species (ROS). These reactive species are products of regular cell metabolism (‘O2: singlet oxygen, H2O2: hydrogen peroxide, OH− hydroxyl radical, O2−: superoxide radical). However in low levels they are indispensable in many biochemical processes, including intracellular messaging in such us: apoptosis [7] immunity [8], and defense against micro-organisms [9,10]. A number of enzymes are known to have major antioxidant activity, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione reductase (GR). Antioxidants can be defined as any substance that when present at low concentrations compared to that of an oxidizable substrate, significantly delays or inhibits the oxidation of that substrate [11,12]. Therefore SOD detoxified O2−, CAT reduces H2O2 and GR catalyzes the NADPH-dependent regeneration of glutathione (GSH) from the oxidized form (GSSG) generated by GPX.

In view of the widespread availability of paracetamol we sought to examine the effect of paracetamol in a stressed mouse. We hypothesised that administration of this drug following stress induction by hydrogen peroxide would reduce or increase liver oxidant stress

2. Materials and Methods

2.1. Tests

The test concerned 30 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 [13]. All experiments were in accordance with the guidelines provided by the CPCSEA.

Animals were divided into 6 groups as it’s resumed in Table 1. Paracetamol was first administered intraperitoneally for a month, then according to [14] H₂O₂ was injected 30 mn before sacrifice, where we achieved higher levels of ROS generated by H₂O₂ treatment during that incubation time.

| Groups | Number of mice | Treatment               | Dose         |
|--------|----------------|-------------------------|--------------|
| 1      | 4              | NaCl                    | 0.9 %        |
| 2      | 4              | Paracetamol             | 30 mg/kg     |
| 3      | 4              | Paracetamol + H₂O₂      | 30 mg/kg +1.5 g/kg |
| 4      | 4              | Vitamin C               | 500 mg/kg    |
| 5      | 4              | Vitamin C + H₂O₂        | 500 mg/kg + 1.5 g/kg |
| 6      | 4              | H₂O₂                    | 1.5 g/kg     |

### 2.2. Tissue Preparation for Analytic Procedures

Livers were rapidly thawed and manually homogenized, using a Potter homogenizer (Elvehjem) with a glass pestle, 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 on ice. Homogenates were centrifuged at 2000 x g for 60 mn at 4°C (sigma 2-16K) and the resultant supernatants were aliquoted and stored at – 20°C for later enzyme assays.

### 2.3. Chemicals

All biochemicals were obtained from Sigma (St. Louis, MO), Roche Diagnostics (Mannheim, Germany), or Bio-Rad Laboratories (Hercules, CA). All the other chemicals were purchased from Merck (Darmstadt, Germany) and all other chemicals were of analytical grade.

### 2.4. Biochemical Assays

All assays were conducted at 25°C using a spectrophotometer (Thermo electron corporation, Biomate 3).

#### 2.4.1. Catalase

The consumption of 7.5 mM H₂O₂ in 50 mM potassium phosphate buffer (pH 7) was monitored at 240 nm as indicated in [15].

#### 2.4.2. Glutathione Reductase

The assay of [16] 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.

#### 2.4.3. Superoxide Dismutase

The enzyme was assayed according to [17] with assay conditions: 5 mM EDTA, 2.5 mM MnCl₂, 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.

#### 2.4.4. Thiobarbutiric 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 [18]: 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 mn and they were cooled in the ice to stop the reaction. One then carries out a centrifugation with 1000 xg during 10 mn. The reading of supernatant was made to 535 nm.

#### 2.4.5. Protein Assay

Protein content was measured according to the Bradford procedure [19] 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.

#### 2.4.6. Enzyme Activity Expression

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

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

\[\Delta Abs/mn: \text{absorbance variation/minute}\]
\[\varepsilon: \text{extinction coefficient}\]
\[\varepsilon (H₂O₂) = 40 \text{ M}^{-1} \text{cm}^{-1}, \text{ for CAT}\]
\[\varepsilon (NADH) = 6220 \text{ M}^{-1} \text{cm}^{-1}, \text{ for SOD and GR}\]
\[\varepsilon (MDA-TBA complex) = 153000 \text{ M}^{-1} \text{cm}^{-1}, \text{ for MDA}\]

\([P]: \text{protein concentration}\]

\[Ve: \text{assay volume}\]

## 2.5. Statistical Analysis:

All values were expressed as mean ± standard error of mean and the statistical significance between treated and control groups were analyzed by means of Student’s t-test. P<0.05 was considered significant.

### 3. Results and Discussion

#### 3.1. Monitoring of Body Weight in Mice

According to Figure 1, the body weight of all treated mice showed slight variations but weren’t significantly different from those of the control, except mice treated with H₂O₂. They show a significant weight loss showing a low activity during treatment period. Many toxicity studies (repeated dose of H₂O₂) indicated a decreased body weight gain in animal studies (i.e., at dose levels of 50-500 mg/kg/day in rats) as well as changes in blood chemistry parameters [20,21].
Figure 1. weight gain in grams during 30 days of treatment: H: H₂O₂, C: Vitamin C, C+H: Vitamin C + H₂O₂, P: Paracetamol, P+H: Paracetamol + H₂O₂ *significantly different from control at P < 0.05. The number of mice used in each group was 4

3.2. Determination of the Enzymatic Activity of Antioxidants

According to Figure 2, Figure 3, Figure 4 and Figure 5 catalase, superoxide dismutase, glutathione reductase activity and the MDA level were found to be significantly raised (P<0.05) in group 6. This indicates that these indicators of oxidative stress status were reactive inside the liver cell and were potentially utilized for counteracting the harmful effects of H₂O₂.

Superoxide dismutases (SOD) are a group of metalloenzymes that protect cells from superoxide radicals by catalyzing the dismutation of the superoxide radical to molecular O₂ and H₂O₂. The main function of SOD is to scavenge O₂ radicals generated in various physiological processes, thus preventing the oxidation of biological molecules, either by the radicals themselves, or by their derivatives [22]. In the present study, the highest activity was seen in mice treated with H₂O₂ (group 6) followed by P+H (group 3) (Figure 3). Elevated SOD activity indicates the production of higher levels of ROS generated by H₂O₂ and paracetamol treatment which in turn triggered the elevated SOD expression in the cells. Control, paracetamol, vitamin C and vitamin C + H₂O₂ showed comparatively lower activity. In the case of vitamin C + H₂O₂ the activity was much lower which suggest that vitamin C was able to counter the H₂O₂ effects with reduced need for higher SOD expression.

To counter the potential hazards of intracellular H₂O₂, which freely diffuses into cells and oxidizes cell membranes, proteins, and DNA [23], most organisms possess catalase (CAT), a high-molecular-weight, heme-containing protein whose primary function is to counter H₂O₂, leaving O₂ and water as by-products [24]. In the present research the highest activity was observed in the H₂O₂ treatment, followed by Paracetamol + H₂O₂. This indicates the active role of CAT in modulating the harmful effects of H₂O₂. Interestingly, this also directly correlated to high SOD activity. Lowest CAT activity was observed in the case of vitamin C, while Vitamin C + H₂O₂ was
much lower (Figure 5) indicating again the beneficial role of vitamin C in quenching free radicals.

Malondialdehyde (MDA) is one of the most frequently used indicators of lipid peroxidation [25]. The production of hydroxyl radical and other powerful radicals can initiate a chain reaction of lipid peroxidation in which polyunsaturated fatty acids are converted into lipid peroxides. Increased MDA can be interpreted as resulting from cellular membrane damage initially caused by increased formation of radicals [26]. In the present study, higher MDA content was observed at groups treated with paracetamol + H₂O₂ indicating higher levels of lipid peroxidation. However the other groups showed lower MDA content indicating the normal membrane deterioration process (Figure 4). Given that MDA is considered as a valuable indicator of oxidative damage of cellular components, our results suggest that paracetamol and H₂O₂ treatment enhanced reactive oxygen species generation in the liver and that antioxidant defenses were not totally able to effectively scavenge them which was the case in group treated with vitamin C.

The role of GR is fundamental for GPX activity, maintaining the cytosolic concentration of reduced glutathione [27,28], and therefore involved in detoxification reactive oxygen. Peroxidases decompose hydrogen peroxide and organic hydroperoxides produced during normal metabolism and also prevents peroxide-induced DNA damage, lipid peroxidation, and protein degradation [29]. In this work, Mice treated with Paracetamol and H₂O₂ showed the highest activity of glutathione reductase followed by H₂O₂ alone treatment. However other groups showed lower activity (Figure 2).

Results obtained in this study can be explained by the fact that APAP (N-acetyl-p-aminophenol) by CYP (cytochrome P450) leads to the formation of N-acetyl-pbenzoquinoneimine (NAPQI), a highly reactive intermediate metabolite [30], which is normally detoxified by conjugation with reduced glutathione (GSH). This may explain the highest activity of GR observed in mice treated with paracetamol + H₂O₂ (Figure 2).

Oxidative stress is also considered to be involved in the induction of hepatotoxicity by APAP. The one electron oxidation of APAP by CYPs may generate reactive oxygen species (ROS). Hydrogen peroxide and superoxide are produced during metabolic activation of APAP in the mixed function oxidase system [31,32,33].

These results have clearly suggested that acetaminophen induced hepatotoxicity by increased oxidative stress demonstrated by the high pool of antioxidant enzymes (CAT, GR and SOD), plus MDA level. Therefore paracetamol didn’t show any protective effect when combined with H₂O₂ (group3), however the redox imbalance was more altered, indicated by pronounced peroxidation and higher rate of antioxidant enzymes activity. These data also confirmed the protective effect of vitamin C, a known antioxidant that acts by scavenging free radicals molecules with one or more unpaired electrons and transform them to stable molecules. In conclusion acetaminophen should be used carefully in order to prevent hepatotoxicity, induced by oxidative damage and redox imbalance. This toxicity can be initiated by the metabolic activation of APAP to NAPQI, a reactive metabolite, ROS generation and subsequent lipid peroxidation during APAP biotransformation.

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