Stem Cell Factor Attenuates Formation of Acetaminophen–Glutathione Conjugate in Kidney of Mice Treated With a Toxic Dose of Acetaminophen

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ABSTRACT

In this study the possible effects of stem cell factor treatment on acetaminophen renal toxicity via conjugate formation with cellular glutathione was investigated. Mice treated with stem cell factor (40µg/kg B.W i.p) 30 min following acetaminophen (300 mg/kg B.W i.p) and sacrificed at intervals of 6, 12 and 24 h. The levels of acetaminophen-glutathione conjugate in kidney tissues showed that stem cell factor treatment given 30 min after acetaminophen injection to mice caused a significant decrease acetaminophen-glutathione conjugate formation in 6 and 12 h. Under these circumstances, serum urea and creatinin level reduced (p < 0.05) in mice treated with stem cell factor compared to mice treated with acetaminophen alone. These data show that treatment with stem cell factor reduced acetaminophen-related toxicity. The part played by stem cell factor is probably by inhibition of acetaminophen-glutathione formation and retaining of cellular glutathione.

KEY WORDS: Stem cell factor; Glutathione conjugation; Acetaminophen; Glutathione; In vivo

INTRODUCTION

Acetaminophen (APAP) is a widely used analgesic and antipyretic drug which is safe at therapeutic doses however, in the case of overdose, both liver and kidney injury have been observed in humans and laboratory animals [1-4]. At therapeutic doses, APAP is rapidly excreted by conjugation with glucuronate and sulfate [5]. A portion of APAP also undergoes cytochrome P450 (CYP)-mediated activation which is resulted in the formation of N-acetyl-p-benzoquinon imine (NAPQI), a chemically reactive metabolite, both in liver and kidney [6-8]. NAPQI then reacts with GSH and form an APAP glutathione conjugate (APAP–GSH) [6, 9, 10]. At therapeutic doses NAPQI is cleared by GSH, however at overdose setting, excessive amounts of NAPQI formed, deplete cellular reserve of GSH and then bind to cellular proteins, particularly mitochondrial proteins, which leads to toxicity [11-14]. NAPQI production is estimated in vitro by measuring the formation of APAP-GSH. Previous works has shown that this is the single product of the reaction between NABQI and GSH in rat both in vivo and in vitro [15, 16]. Although APAP-GSH has been considered as a detoxification product generally [14,17], there is no evidence of deactivation of NABQI by oxidation of GSH [15,16]. Some studies have shown that APAP-GSH can possess a toxic effect in liver of laboratory animals [18, 19]. Some studies also suggest a contributory role for APAP-GSH in the biochemical toxicology of APAP-induced kidney injury in laboratory animals [20, 21]. Possible mechanism of nephrotoxicity under APAP overdoses is activation of APAP to its oxidative metabolite, followed by formation of APAP-GSH and its metabolites, which interrupts GSH homeostasis in kidney and leads to kidney injury [22]. N-Acetylcysteine (NAC) administered orally or intravenously, is a preferred antidote for APAP poisoning [23, 24]. Although NAC treatment can prevent or reverse APAP-induced hepatocellular damage in humans [25, 26], it is not protective against APAP-induced renal damage [27, 28]. The results of both clinical and experimental studies indicate that because NAC may not always be effective in preventing or
reversing APAP-induced nephrotoxicity, it is important to assess other compounds for their potential efficacy against APAP-induced renal and hepatic injury [29]. Stem cell factor (SCF) and its receptor, c-kit, play an important role in cell differentiation, proliferation, chemotaxis, cell adhesion, and apoptosis [30]. SCF is produced in two different forms, soluble and membrane bound. The membrane bound form may be detached from cell surface and is carried in serum toward target tissue. When binding to its receptor (c-kit), SCF exerts its effects by consequential downstream signaling pathways [31]. SCF/c-kit ligand/receptor system plays a role in attenuating liver damage and/or liver regeneration in a murine model of APAP-induced hepatic injury [32]. In addition, SCF-deficient mice have an increased mortality rate compared with wild-type mice after APAP poisoning, and, furthermore, administration of exogenous SCF to normal mice also decreases mortality associated with APAP intoxication [32].

In the present study attempt was made to investigate the SCF effect on APAP-related kidney toxicity. For this purpose APAP-GSH and GSH level in kidney of intoxicated mice were examined.

MATERIALS AND METHODS

Chemicals
APAP was purchased from Sigma Chemical Company (St. Louis, MO, USA). Disodium ethylenediaminetetraacetic acid (Na2EDTA), Hydrochloric acid, S, S Dithio nitro benzoic acid (DTNB), GSH, 2-Amino-2-hydroxymethyl-propane-1,3-diol (Tris base) and Bovine serum albumin (BSA) were purchased from Merck (Darmstadt, Germany). SCF purchased from Invitrogen (USA). Alanyl amino transferase (ALT), Aspartat aminotransferase (AST), creatinin and blood urea nitrogen (BUN) colorimetric Assay Kits were prepared from Applied biosystems (Spain). All other chemicals were of analytical grade and purchased from Sigma (St Louis, MO) or Merck (Darmstadt, Germany).

Animals
Male BALB/cJ mice (6-8 weeks old), were purchased from Pasteur institute of Iran (Tehran, Iran) and maintained in a conventional sanitary facility with the required consistent temperature and relative humidity. All of them had free access to standard diet and water ad libitum for at least 2 weeks prior to the experiments. The experimental protocols were approved by the medical ethics committee of the Tarbiat Modares University.

Dose of administration
In order to select a proper dose of APAP which would be of nephrotoxic effect in mice; different doses of APAP (including 450, 400, 350 and 300 mg/kg B.W) prepared by dissolving in volume of 500μl of normal saline and administrated to groups of mice (n=5) i.p. Mortality associated with different doses of APAP was investigated up to 24 hours and toxicity was evaluated in animals by assessment of ALT and AST as biomarkers of liver toxicity and BUN and creatinin as markers of nephrotoxicity in 24 hour after APAP injection.

Experimental protocol
45 animals were selected randomly and divided into groups with 5 members (n = 5). To study the time dependent changes, experiments were conducted in three interval times of 6, 12 and 24 hours after APAP injection. In every interval results of experiments were compared among three differently treated groups including:

- APAP- treated group (APAP): 300 mg/kg B.W of APAP was dissolved in 500μl of normal saline, and administrated via i.p injection.
- APAP plus SCF- treated group (APAP+SCF): 30 minutes after administration of 300 mg/kg B.W of APAP (dissolved in 500 μl of normal saline), 40 μg/kg B.W of SCF, dissolved in 100 ml distilled water, injected i.p.
- Saline- treated group (vehicle): 500 ml of normal saline injected to mice.

Groups of animals were killed by cervical dislocation under diethyl ether anesthesia at different pre-mentioned intervals. Blood was drawn from the heart into heparinized syringes and centrifuged under 800×g. The serum was used for assessment of ALT, AST, creatinin and BUN. Upon blood collecting, kidneys were excised and rinsed in saline. A sagital section from kidney of animals in interval time of 24 hours were prepared and placed in 10% phosphate-buffered formalin to be used for hematoxyline and eosine staining (H&E) and pathological analysis. The remaining tissues were stored at ~80 °C.

Biochemical parameters
Concentration of BUN and creatinin were determined with Colorimetric Assay Kits (Applied biosystems, Spain).

Reduced Glutathione (GSH)
GSH concentrations were measured in kidney homogenates applying the Ellman,s reagent, as described previously [33]. Briefly, 100 mg of frozen tissue were homogenized at 0°C in 4 ml 0.2mM disodium ethylenediaminetetraacetic acid (Na2EDTA). 5 ml of homogenate was mixed with 4 ml distilled water and
1 ml 50% TCA. The mixture was vortexed for 15 min and centrifuged at 3000×g for 15 minutes to precipitate the tissue protein. 2 ml of supernatant was diluted with 4 ml of 0.4 M tris containing 0.2 mM EDTA, pH 8.9. Samples were assayed using 0.1 ml of 0.01 M dithionitrobenzoic acid (DTNB) at 412 nm with Nanodrop 2000C (Thermo scientific, USA). All data were expressed in µg/mg kidney GSH, using a GSH standard curve prepared by applying an authentic GSH standard.

**HPLC analysis**

To identify APAP-GSH in samples, hyper performance liquid chromatography (HPLC) analysis was performed using the method previously described [34]. Briefly, kidney tissues were homogenized (1:5, w/v) in 10mM sodium acetate, pH 6.5, and then centrifuged at 16,000 × g for 20 min at 4 °C. then 100 µl of supernatant was mixed with 100 µl of 20% ice-cold trichloroacetic acid for 15 min, and then centrifuged for 5 min at 16,000 × g. Fifty µl of supernatant injected and assayed using reverse-phase Agilent Technologies HPLC and ZORBAX Eclipse-C18 (5µm, 4.6 mm, 150 mm) column with Agilent DAD (1200 series) detection component using wavelength of 254 nm.

Mobile phase consisted of HPLC graded methanol and water (Merck, Germany) with proportion of 10: 90, injection volume was 50 µl and flow rate adjusted to 1.25 ml/min.

Data were reported in µg/mg kidney using a calibration curve set for marker (APAP), where peak areas were correlated to the corresponding concentrations. According to the retention time, marker was identified from the extract again and the relative concentrations were further estimated according to their corresponding peak areas. The occurrence of APAP-GSH conjugate as a metabolite of APAP was confirmed by mass spectrometer. APAP-GSH was quantified by use of the APAP standard curve, as the molar extinction coefficient of APAP and its conjugated metabolites at 254 nm are essentially the same [35]. Calculated results were expressed as mean standard derivation obtained from three independent experiments.

**Statistical analysis**

Differences between groups analyzed using Kuriskal Walis test followed by Man Whitney test, by statistical software package SPSS 16, which are applied for analysis of data which are not normally distributed. A P value of 0.05 was considered significant for all analyses.

**RESULTS**

**APAP-induced nephrotoxicity**

Preliminary studies (data not shown) for determining of an appropriated nephrotoxic dose to use in this study showed that, except for dosage of 300 mg/kg B.W of APAP, injection of all other doses of APAP, followed by mortality in animals before 24h. Administration of 300 mg/kg B.W of APAP led to significant increase (P < 0.05) in serum levels of ALT, AST, BUN and creatinine which is indicative of toxicity (hepatic and renal toxicity, respectively) in mice, however it did not leave any mortality in mice by 24 hours after administration. Therefore, 300mg/kg B.W of APAP was selected and used as a nephrotoxic dose in this study.

**Histopathological examination of kidney sections**

Fig. 1 compares the effects of APAP treatment with or without SCF treatment on kidney of mice 24 hours after ip administration of 300 mg/kg B.W of APAP. Results show that APAP dosage of 300 mg/kg B.W did not lead to so extensive injury of kidney tissue that would be recognizable by morphological light
microscopy examinations both in APAP and APAP+SCF treated animals. However, as results reveal, there are extensive necrotic areas around central veins of mice treated with 300 mg/kg B.W of APAP.

**Effect of SCF treatment on biochemical parameters change in APAP challenge**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>APAP</th>
<th>APAP+SCF</th>
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<tbody>
<tr>
<td>ALT (IU/L)(^1)</td>
<td>56 ± 3.7</td>
<td>603.4 ± 26 *</td>
<td>283 ± 48 **</td>
</tr>
<tr>
<td>AST (IU/L)(^2)</td>
<td>97.4 ± 4.4</td>
<td>410 ± 27.1 *</td>
<td>356 ± 20.8 *</td>
</tr>
<tr>
<td>BUN (mg/dl)(^3)</td>
<td>17.65 ± 1.3</td>
<td>45 ± 2.7 *</td>
<td>33.76 ± 3.2 **</td>
</tr>
<tr>
<td>Creatinin (mg/dl)</td>
<td>0.23 ± 0.13</td>
<td>0.32 ± 0.11 *</td>
<td>0.28 ± 0.18 **</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD for five mice in each group. \(^1\)ALT: Alanine transaminase, \(^2\)AST: aspartate aminotransferase, \(^3\)BUN: blood urea nitrogen. * means significant difference from control. ** means significant difference from both control and APAP groups. P value of 0.05 was considered significant for all analyses.

Table 1 shows that biochemical indicators of kidney injury containing BUN and creatinine in APAP or APAP+SCF groups rose to a significantly (P < 0.05) higher level compared to control group in 24 hours after administration of 300 mg/kg B.W. APAP+SCF group showed a significantly (P < 0.05) decreased level of BUN and creatinine concentration compared to APAP group. Assessment of parameters of liver injury in the sera of the same mice showed that activity of ALT enzyme in APAP and APAP+SCF treated groups was significantly (P < 0.05) higher than control group. APAP+SCF group showed a significantly (P < 0.05) decreased level of ALT activity compared to APAP group. In addition, AST enzyme activity in APAP and APAP+SCF groups rose to a significantly (P < 0.05) higher levels in comparison to control group however, no significant difference was observed in this parameter between APAP and APAP+SCF groups.

**Effects of SCF treatment on APAP-induced GSH depletion**

As it is shown in Fig. 2 GSH level in kidney of mice under 300 mg/kg B.W of APAP administration has no significant difference between APAP treated and APAP+SCF treated mice by 6 hours after APAP administration. However, 12 hours after APAP injection, SCF treated animals showed a significant (P < 0.05) increased level of renal GSH in comparison to mice were not treated with SCF following APAP (300 mg/kg B.W) administration. Results from experiment using APAP dosage of 300 mg/kg B.W showed that 24 hours after APAP injection, GSH levels in kidney of mice treated with neither SCF nor APAP+SCF differed significantly from vehicle and also there was no significant difference between GSH level of APAP and APAP+SCF groups.

**Effects of SCF treatment on APAP-GSH conjugate formation in kidney of mice under APAP treatment.**

Table 2 and Fig. 3 illustrate the results of HPLC analysis of the APAP–GSH conjugate formation in kidney of mice under administration of 300 mg/kg B.W of APAP, with or without SCF treatment. As resulted
chromatograms from HPLC analysis of kidney specimens in Fig.4 depicts, two distinct peaks of the conjugate and free APAP with retention times of approximately 5.7 and 6.5 min, respectively, are detectable.

Table 2 shows that administration of 300 mg/kg B.W of APAP in mice leads to formation of hepato-renal APAP-GSH conjugate both in APAP and APAP+SCF groups after APAP administration. Administration of 300 mg/kg B.W of APAP increased APAP-GSH conjugate of kidney level to significantly (P < 0.05) high values in groups decapitated 6 and 12 hours after APAP administration. Administration of 300 mg/kg B.W of APAP in mice with or without SCF treatment, At least under the condition of this study did not led to formation of detectable levels of APAP-GSH conjugate in kidney specimens of mice 24 hours after APAP administration.

Table 2 HPLC analysis of samples.

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Sacrifice time (h) (μg/gr kidney)</th>
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<tbody>
<tr>
<td></td>
<td>6</td>
</tr>
<tr>
<td>APAP</td>
<td>9.6 ± 0.3</td>
</tr>
<tr>
<td>APAP+SCF</td>
<td>4.7 ± 0.9*</td>
</tr>
</tbody>
</table>

Values (μg/gr kidney) are expressed as mean ± SD for five mice in each group.

GSH conjugate of APAP (APAP-GSH) was detected in mice kidneys after APAP dosing with or without SCF treatment. APAP treated groups treated with 300 mg/kg B.W APAP i.p. APAP+SCF groups received 1μg/100 μl SCF i.p. 30 min following 300 mg/kg B.W APAP i.p. * indicates statistically significant difference for APAP-GSH levels in APAP+SCF treated mice compared to APAP treated mice in two groups with a similar sacrifice time. ND = not detected. P value of 0.05 was considered significant for all analyses.

**Figure. 3** Representative high performance liquid chromatogram.
A) APAP (50 μl, 20 μg/ml); as a marker for calculating APAP-GSH concentration in kidney of mice with approximate retention time of 6.5 min , B) control; resulted from kidney of a mouse one hour after 500 μl PBS i.p injection, C) APAP and GSH conjugate of APAP with approximate retention time of 5.7 min, resulted from kidney of a mouse 6 hour after treatment with 300 mg/ kg B.W APAP , i.p. To produce the chromatograms weigh length of 254 nm was used.

**DISSCUSSION**

The SCF has been first introduced as an effective growth factor involved in the development and function of germ cells [36], melanocytes, for review see Yoshida et al., [37] and also haematopoisis [38].
Previous studies showed that SCF administration to animals may contribute to reducing liver damage and may also improve tissue regeneration which may help decrease mortality associated with APAP toxicity [31, 32]. However, the mechanism(s) responsible for these effects has not been investigated. The pathway of NAPQI conjugation with cellular GSH (APAP-GSH formation pathways) is an important pathway in APAP metabolism. APAP-GSH is a toxic metabolite for liver and kidney by itself [18-22] and compounds which lead to decrease in APAP-GSH formation can prevent or attenuate hepato-renal injuries induced by APAP toxicity. Hence, it is important to know if SCF can decrease GSH conjugation of NAPQI. In the present study APAP-GSH conjugate formation was measured in kidney of mice as a representative of NAPQI toxicity.

The possible mechanism of SCF effect on APAP toxicity was observed by showing that the APAP-related depletion of cellular GSH is readily reversed by SCF treatment. It is important to note that cellular GSH was unaffected due to SCF treatment to normal mice (untreated with APAP) (data not shown).

There are also evidences which show that SCF can suppress Cyp2E1 expression which is responsible of APAP metabolism [31]. The consequence of Cyp2E1 suppression by SCF was associated with modulation of APAP toxicity [31].

The evidences presented in this paper show that SCF treatment to mice following APAP intoxication could attenuate APAP-related nephrotoxicity. For instance, the kidney damage markers such as serum BUN and creatinin level which were significantly elevated in APAP group were reversed due to SCF treatment.

Administration of SCF (40µg/kg B.W) to a group of APAP (300mg/kg B.W) treated mice inhibited BUN and creatinin levels which were initially elevated as a result of kidney damage. Despite the changes in kidney tissue such as GSH depletion and APAP-GSH conjugation which occurred as results of APAP (300mg/kg B.W) administration to mice, there were no obvious changes in kidney tissues in 24h after APAP injection. However, back to preliminary studies for determining a toxic dose of APAP, under the circumstances of this study the dosage used (300mg/kg B.W) in this study was the highest one which was bearable for mice and did not lead to mortality by 24h, and other higher doses were so lethal that would not allow us to compare toxicity between APAP and APAP+SCF treated mice after 24h.

Therefore, to measure up our purpose, we used a properly milder dose of APAP mainly; 300mg/kg B.W. It appears that the treated protocol required the time more that 24h to reveal the histopathological evidences of nephrotoxicity.

CONCLUSION

In conclusion our data suggest that SCF treatment following APAP-intoxication can reduce renal tissue toxicity by decreasing the formation of APAP-GSH conjugate. The part played by SCF is probably by inhibition of NAPQI formation and consequent retaining of the cellular GSH. Therefore SCF can be considered as a alternative remedy to help recovery of APAP-related nephrotoxicity.

REFERENCES


Citation of This Article