Protective effect of L-carnitine against diclofenac sodium toxicity in mice

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SUMMARY

This study investigated the potential protective effects of L-carnitine against renal and liver damage caused by high doses of diclofenac sodium in mice. A pilot study, designed to determine the highest toxic dose of diclofenac, was conducted on 32 Swiss Albino mice randomly divided into 4 equal groups according to the drug dose: 0 (control), 2.5 (low), 5 (moderate) and 10 mg/kg/day (high dose) for 5 days by the subcutaneous route. Serum biochemical parameters (BUN and creatinine concentrations and AST, ALT and ALP activities) were measured as well as GSH and MDA contents in liver and kidney at the end of the treatment. The 2 highest dosages of diclofenac have induced significant increases of the serum markers and MDA accumulation in tissues whereas the kidney and liver GSH contents were depressed in parallel. Besides, a strong dose-effect relationship was evidenced. In the second experimental step, 4 groups of 8 mice received subcutaneous injections for 5 days of saline solution (NaCl, 20 mL/g body weight/day) (group I), of L-carnitine (500 mg/kg/day) (group II), of diclofenac sodium (10 mg/kg/day) (group III) and of diclofenac (10 mg/kg/day) plus L-carnitine (500 mg/kg/day 3 days before and 2 days during the diclofenac treatment) (group IV) respectively. The diclofenac treatment alone or in combination with L-carnitine induced liver and kidney damage as attested by significant increases of the serum markers and by tissue MDA accumulation. Nevertheless, these variations were significantly reduced in co-treated mice. Whereas the GSH pools in liver and kidney were markedly depressed in the group III, they were significantly enhanced in mice treated with L-carnitine alone, and remained unaffected in co-treated mice (group IV) compared to the controls. These results demonstrated that the diclofenac toxicity is due to lipid peroxidation and impairment of the antioxidant systems in liver and kidney and that a co-treatment with L-carnitine can partially alleviate it by restoring antioxidant capacity.

Keywords: Diclofenac sodium, toxicity, liver, kidney, markers, lipid peroxidation, glutathione.

RÉSUMÉ

Effet protecteur de la L-carnitine à l’égard de la toxicité du diclofénac chez la souris

Cette étude vise à évaluer les effets protecteurs de la L-carnitine contre les dommages rénaux et hépatiques provoqués par de fortes doses de diclofénac de sodium chez la souris. Afin de déterminer la dose la plus toxique de diclofénac, une étude préliminaire a été réalisée sur 32 souris adultes Suisses albinos aléatoirement réparties en 4 groupes égaux en fonction de la dose administrée par voie sous-cutanée pendant 5 jours : 0 (contrôle), 2.5 (faible dose), 5 (dose modérée) et 10 mg/kg (dose élevée). Les paramètres biochimiques (concentrations de BUN et de créatinine, activités des enzymes AST, ALT et PAL) ainsi que les teneurs en GSH et de MDA dans le foie et le rein ont été mesurés à la fin du traitement. Les 2 plus fortes doses de diclofénac ont induit des augmentations significatives des marqueurs sériques et une accumulation de MDA dans le foie et le rein, alors qu’en parallèle, les teneurs en GSH ont été diminuées. De plus, une forte relation "dose effet" a été mise en évidence. Dans une 2ème étape, 4 groupes de 8 souris ont reçu des injections sous-cutanées pendant 5 jours soit de sérum physiologique (NaCl 0.9%, 20 mL/g de poids vif/jour) (groupe I), soit de L-carnitine (500 mg/kg/jour) (groupe II), soit de diclofénac de sodium (10 mg/kg/jour) (groupe III) et soit de diclofénac (10 mg/kg/jour) plus L-carnitine (500 mg/kg/jour 3 jours avant et 2 jours pendant le traitement par le diclofénac) (groupe IV). Le diclofénac seul ou associé à la L-carnitine a induit des lésions hépatiques et rénales se traduisant par des augmentations significatives des marqueurs sériques et l’accumulation tissulaire du MDA. Néanmoins, ces altérations ont été significativement réduites chez les souris co-traitées. Alors que les teneurs en GSH dans le foie et le rein ont été fortement diminuées dans le groupe III, elles ont été significativement augmentées chez les souris traitées par la L-carnitine seule et sont restées identiques chez les souris co-traitées (groupe IV) à celles obtenues chez les contrôles. Ces résultats suggèrent que la toxicité hépatique et rénale du diclofénac est due à un phénomène de peroxydation lipidique associé à une défaillance des systèmes anti-oxydants et qu’un traitement adjuvant par la L-carnitine peut partiellement l’atténuer en restaurant les capacités anti-oxydantes.

Mots-clés : Diclofénac, toxicité, foie, rein, marqueurs, peroxydation lipidique, glutathione.

Introduction

Diclofenac sodium like other non-steroid anti-inflammatory drugs (NSAIDs) expresses its anti-inflammatory, analgesic and antipyretic effects as well as adverse effect through decrease of the prostaglandin synthesis from arachidonic acid by inhibition of the cyclooxygenase enzyme activity [7, 20, 33]. Diclofenac sodium has undesirable side effects such as gastrointestinal ulceration, hepatotoxicity and nephrotoxicity [7, 11, 15, 27, 28, 31, 32]. Recent studies have shown that NSAIDs may play a role in the formation of radical cations co-oxidising GSH or NADH to generate reactive oxygen species (ROS), by becoming oxidised via peroxidase [25, 26]. This suggestion may explain the mechanism involved in gastrointestinal ulceration, hepatotoxicity and nephrotoxicity inflicted by NSAIDs [32, 35]. However information on the oxidative diclofenac sodium effect is scarce.

The L-carnitine plays an important role in the energy production by transferring long chain fatty acids into the mitochondrial matrix where fatty acid beta-oxidation takes place [4, 12, 14, 25]. L-carnitine also prevents potential harmful effects of acyl-CoA accumulation in the mitochondria as a buffer by the formation of acyl carnitine from short chain acyl-CoAs [12]. In addition, L-carnitine has been shown to protect cell against free radical damage and lipid peroxidation [21, 30]. Oxidative stress is considered to play an important role in the pathophysiology of many disease processes. Since the mitochondrion is an important organelle in the energy production and one of the sources of free radical generation, L-carnitine supplementation may be important in protection of some critical organs such as liver and kidneys where many metabolic events take place such as drug metabolism and detoxification [2, 3, 5]. Furthermore, the use of parenteral L-carnitine has been shown to alleviate toxic effects of some chemicals including prevention of doxorubicin-induced cardiomyocyte apoptosis via inhibition of ceramide generation [1, 22] and reducing lipid peroxidation and free radical generation [23, 30, 36].

This study was therefore designed to evaluate the protective effects of L-carnitine against diclofenac sodium adverse side effects in liver and in kidney in mice.

Materials and Methods

ANIMALS

The study was conducted on 64 clinically healthy, 12-14 weeks old, Swiss Albino mice, weighing 25-30 g obtained from Laboratory Animals Unit of the Faculty of Veterinary Science, University of Kafkas, Turkey. The mice were fed with a standard pellet diet and feed and water were provided ad libitum. The animals were treated according to the Animal Care and Use Regulation (European Convention for the protection of Vertebrate Animals used for Experimental and other Scientific Purpose 1996). Animals were acclimatized to laboratory conditions under 12 h daylight / 12 h night cycles at 22-25°C.

STUDY DESIGN

Firstly, a pilot study was made to determine the toxic dose of diclofenac sodium. For this purpose, 32 mice were used and were divided into four equal groups. A saline solution (0.9% NaCl; Baxter, Mediflex, Eczacibasi, Istanbul, Turkey) was subcutaneously (SC) injected (20 mL/g body weight) for 5 days to the control mice. In the 3 other groups, mice subcutaneously received low (2.5 mg/kg at a volume of 25-30 µL), moderate (5 mg/kg at a volume of 50-60 µL) or high (10 mg/kg at a volume of 100-120 µL) dose of diclofenac sodium (Dikloron Ampul®, 75 mg/3ml; Deva Ilac, Istanbul-Turkey) for 5 days.

In the second stage, the potential protective effects of L-carnitine against diclofenac sodium induced toxicity were evaluated. For that, 32 mice were divided into four equal groups as follow; mice of the groups I and II were subcutaneously injected by 0.9% NaCl (20 mL/g body weight) or by L-carnitine (500 mg/kg/day at a volume of 50-75 µL, CARNITINE®, Santa Farma Ilac Sanayii A.S., Istanbul, Turkey) respectively for 5 days whereas mice of the group III received diclofenac sodium at the highest dose reported as toxic in the preliminary pilot study (10 mg/kg/day, SC, for 5 days, at a volume of 100-120 µL) and in the group IV, mice were treated not only with diclofenac sodium (10 mg/kg/day, SC, for 5 days, at a volume of 100-120 µL) but also with L-carnitine (500 mg/kg/day, SC, at a volume of 50-75 µL 3 days before and 2 days during the diclofenac sodium treatment).

Blood samples (approximately 1–1.5 ml) were collected from the heart via cardiac puncture under light ether anaesthesia into plain tubes for determination of serum biochemical parameters. They were centrifuged at 1 200g at 4 ºC for 10 minutes and sera were kept at -25 ºC until analyses. Mice were decapitated 24 hours after the last injection. Liver and kidney samples were collected immediately after decapitation.

BIOCHEMICAL ANALYSIS

Blood BUN (Olympus OSR6134, Olympus Life and Material Sciences, Ireland) and creatinine (Olympus OSR6178, Olympus Life and Material Sciences, Ireland) concentrations as well as ALT (Olympus OSR6107, Olympus Life and Material Sciences, Ireland), AST (Olympus OSR6109, Olympus Life and Material Sciences, Ireland) and ALP (Olympus OSR6103, Olympus Life and Material Sciences, Ireland) activities were determined using commercially available kits on an auto analyser (Olympus Chemistry Analyzer AU 640, Type: 640-03, Japan).

In addition, liver and kidneys were rinsed with ice-cold 0.9% NaCl and then 1g of tissue was homogenised in four fold phosphate buffer in 0.1 M KCl (pH 7.4) in an ice bath. The homogenates were centrifuged at 5000 g for 15 minutes. Tissue GSH and MDA contents were colorimetrically measured by the methods of BEUTLER et al. [8] and of YOSHIKO et al. [37] respectively using a spectrophotometer (UV-1201, Shimadzu, Japan).

STATISTICAL ANALYSIS

Statistical differences between the groups were tested by analysis of variance (ANOVA) and Duncan test using SPSS for Windows version 10.0. Data were presented as mean ± standard errors, and p values less than p<0.05 were considered as significant.

Results

PILOT STUDY

The variations of the serum and tissue biochemical parameters according to the dose of diclofenac sodium are presented in Tables I and II, respectively. Moderate and high dosages of diclofenac sodium have induced significant increases of BUN and creatinine concentrations (p < 0.05).
Serum activities of the hepatic enzymes (AST, ALT and ALP) were also significantly elevated in mice receiving 5 and 10 mg/kg of diclofenac compared to controls (p < 0.05) (Table I). In the same way, significant MDA accumulations were observed in liver and kidneys of mice from these 2 groups (p < 0.05) whereas tissue GSH contents were significantly depressed in parallel (p < 0.05) (Table II). Furthermore, alterations of the serum and tissue biochemical parameters were proportional to the drug dosage: variations of serum BUN and creatinine concentrations, of liver enzyme activities and of tissue GSH and MDA contents observed in mice treated with 10 mg/kg were significantly higher than those observed in mice treated with 5 mg/kg (p < 0.05) (Tables I and II).

**EVALUATION OF THE PROTECTIVE EFFECTS OF L CARNITINE**

The L carnitine treatment alone did not significantly alter serum biochemical parameters (Table III) or tissue MDA contents (Table IV) compared to the controls. Nevertheless, slight but significant increases of the GSH contents in the liver and in the kidney compared to the group I (p < 0.05) were recorded in this group (group II) (Table IV). As in the pilot study, mice treated with diclofenac (10 mg/kg/day) (group III) exhibited marked and significant increases of the serum biochemical makers compared to the controls (p < 0.05) (Table III). Moreover, these changes were associated with high MDA contents (p < 0.05) and with depressed GSH contents (p < 0.05) in liver and in kidney (Table IV). In the group IV, the serum concentrations of the markers of the renal function and the serum activities of the hepatic enzymes were significantly increased compared to the controls (p < 0.05) but these variations were significantly less intense than those evidenced in the group III (p < 0.05) (Table III). In the same way, albeit significantly elevated compared to the controls (p < 0.05), the MDA contents measured in liver and in kidney from animals receiving diclofenac and L carnitine were significantly lower than those observed in animals treated with diclofenac alone (group III) (p < 0.05) (Table IV). Furthermore, the tissue GSH contents in the group IV remained unaltered compared to the controls (Table IV).

**Discussion**

Diclofenac sodium, a phenyl acetic derivate, is used as anti-pyretic, analgesic, and anti-rheumatoid drug [7, 33]. Diclofenac, like other NSAIDs, exhibits its effect through inhibition of prostaglandin synthesis at low doses but it can also induce independent events such as increased BUN and creatinine concentrations and activity of some enzymes (AST, ALT, ALP) [18, 34], increase of the proteoglycan synthesis from chondrocytes and inhibition of the membrane ion exchange [9, 29].

Significant decreases of tissue GSH contents associated to MDA accumulation reflected cellular damage in kidney and in liver induced by diclofenac. These findings were significantly correlated with increases of biochemical markers (BUN, creatinine, AST, ALT and ALP activities) in serum, suggesting liver and kidney failures. Moreover, the changes in tissue GSH and MDA contents, in serum BUN and creatinine concentrations and in enzyme activities were proportional to the diclofenac doses.

NSAIDs have been shown to target renal tissue [15, 28] and are well known to be nephrotoxic. Their nephrotoxic effects are due to the induction of the Mitochondrial Membrane Permeability Transition (MMPT), a phenomenon where mitochondrial degeneration is initiated by calcium ion flow into mitochondria due to peroxidants, inorganic phosphate inducer or reactive oxygen species [19, 26]. Serum creatinine and BUN are important indicators of kidney damage. In the present study, diclofenac treatment at moderate and high doses (5 and 10 mg/kg/day) has induced significant increases of these markers, suggesting the occurrence of renal injury as previously reported by AYDIN et al. [6]. In parallel, the accumulation of MDA, an indicator of lipid peroxidation, was observed in kidneys from diclofenac treated mice whereas the tissue GSH pool was significantly lowered. These findings demonstrated the occurrence of an oxidative stress and of lipid peroxidation in kidneys, probably due to ROS produced during diclofenac sodium metabolism. However, ERTEKIN et al. [17] previously failed to evidence such a renal oxidation induced by dipyrone (metamizole sodium) in dogs. This discrepancy may be related to the drug and the doses used and to the sensitivity of the target animal specie. By contrast, co-treatment with L carnitine significantly reduced the variations of the serum concentrations of the renal markers and of kidney GSH and MDA contents induced by a high dose (10 mg/kg/day) of diclofenac in mice. These results demonstrated a protective effect of L carnitine, probably due to inhibition of lipid peroxidation as previously reported [3, 21-23, 30].

Hepatotoxicity due to diclofenac sodium has been related to the consumption of cytosolic glutathione and protein thiols as well as to the pyridine nucleotide oxidation [29]. In the present study, mice treated with diclofenac at moderate and high doses exhibited lower liver GSH content and a higher MDA content than the control mice, suggesting a strong production of free radicals and the induction of lipid peroxidation phenomena. As previously reported [10, 13, 27], increases of serum enzyme activities (ALT, AST and ALP) observed in parallel in diclofenac treated mice also revealed liver damage. When mice were simultaneously treated with L carnitine, firstly increases of hepatic enzyme activities were significantly reduced, secondly MDA accumulation in liver was notably depressed and thirdly GSH was not intensively consumed. Moreover, treatment with L carnitine alone has significantly improved the liver anti-oxidant capacity by increasing the GSH pool. These observations revealed the blockade of free radical production leading to lipid peroxidation and that L carnitine has promoted the anti-oxidant capacities of the organism as previously reported [2, 3, 5, 16, 21-24, 30].

In conclusion, results obtained in this study demonstrated that use of high doses of diclofenac sodium has induced lipid peroxidation and impaired antioxidant defense in mice and that the use of L-carnitine has reduced the diclofenac induced oxidative stress. This may suggest that L-carnitine enhanced antioxidant defense and may be used as a cell protector.
**Table 1:** Pilot study: variations of serum biochemical parameters (BUN, creatinine, AST, ALT and ALP) according to the doses of diclofenac sodium subcutaneously injected to mice (n = 8 in each group) for 5 days. Results are expressed as mean ± standard errors.

<table>
<thead>
<tr>
<th>Serum parameter</th>
<th>Control (0 mg/kg)</th>
<th>Low dose (2.5 mg/kg)</th>
<th>Moderate dose (5 mg/kg)</th>
<th>High dose (10 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUN (mg/L)</td>
<td>188.1 ± 9.0c</td>
<td>227.0 ± 11.5bc</td>
<td>311.3 ± 14.3b</td>
<td>634.2 ± 38.8a</td>
</tr>
<tr>
<td>Creatinine (mg/L)</td>
<td>4.8 ± 0.2c</td>
<td>5.4 ± 0.3bc</td>
<td>6.1 ± 0.3b</td>
<td>14.3 ± 0.4a</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>42.38 ± 2.40c</td>
<td>51.75 ± 7.40bc</td>
<td>60.63 ± 3.91b</td>
<td>95.88 ± 7.22a</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>115.00 ± 7.08c</td>
<td>126.38 ± 4.14bc</td>
<td>147.50 ± 5.88b</td>
<td>246.75 ± 9.97a</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>38.13 ± 1.82c</td>
<td>44.38 ± 1.86bc</td>
<td>49.25 ± 2.69b</td>
<td>73.50 ± 3.14a</td>
</tr>
</tbody>
</table>

Different superscripts in the same line indicate significant differences (p < 0.05).

**Table 2:** Pilot study: variations of MDA and GSH contents in kidneys and in liver of mice treated subcutaneously with different doses of diclofenac sodium (0 to 10 mg/kg) for 5 days (n = 8 in each group). Results are expressed as mean ± standard errors.

<table>
<thead>
<tr>
<th>Tissue parameter</th>
<th>Control (0 mg/kg)</th>
<th>Low dose (2.5 mg/kg)</th>
<th>Moderate dose (5 mg/kg)</th>
<th>High dose (10 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA\textsubscript{kidney} (µmol/g)</td>
<td>0.192 ± 0.01c</td>
<td>0.213 ± 0.01bc</td>
<td>0.228 ± 0.01b</td>
<td>0.272 ± 0.01a</td>
</tr>
<tr>
<td>MDA\textsubscript{liver} (µmol/g)</td>
<td>0.242 ± 0.01c</td>
<td>0.263 ± 0.01bc</td>
<td>0.286 ± 0.01b</td>
<td>0.347 ± 0.01a</td>
</tr>
<tr>
<td>GSH\textsubscript{kidney} (mg/g)</td>
<td>0.261 ± 0.01a</td>
<td>0.238 ± 0.01bc</td>
<td>0.223 ± 0.01b</td>
<td>0.181 ± 0.01c</td>
</tr>
<tr>
<td>GSH\textsubscript{liver} (mg/g)</td>
<td>0.298 ± 0.01c</td>
<td>0.273 ± 0.01ab</td>
<td>0.242 ± 0.01b</td>
<td>0.198 ± 0.01c</td>
</tr>
</tbody>
</table>

Different superscripts in the same line indicate significant differences (p < 0.05).

**Table 3:** Variations of serum biochemical parameters (BUN, creatinine, AST, ALT and ALP) according to the mice treatment: group I (control, 0.9% NaCl, 20 mL/g/day for 5 days, SC), group II (L carnitine, 500 mg/kg/day for 5 days, SC), group III (Diclofenac sodium, 10 mg/kg/day for 5 days, SC) and group IV (Diclofenac sodium, 10 mg/kg/day for 5 days, SC + L carnitine, 500 mg/kg/day for 5 days, SC) (n = 8 in each group). Results are expressed as mean ± standard errors.

<table>
<thead>
<tr>
<th>Serum parameter</th>
<th>Group I (NaCl)</th>
<th>Group II (L carnitine)</th>
<th>Group III (Diclofenac)</th>
<th>Group IV (L carnitine + diclofenac)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUN (mg/L)</td>
<td>152.1 ± 4.3c</td>
<td>141.3 ± 4.6c</td>
<td>592.7 ± 26.0a</td>
<td>427.7 ± 21.3b</td>
</tr>
<tr>
<td>Creatinine (mg/L)</td>
<td>5.2 ± 0.2c</td>
<td>4.9 ± 0.2c</td>
<td>12.4 ± 0.3a</td>
<td>9.4 ± 0.4b</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>49.63 ± 1.86c</td>
<td>46.75 ± 2.06c</td>
<td>96.43 ± 4.67a</td>
<td>79.97 ± 2.68b</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>93.38 ± 2.12c</td>
<td>89.13 ± 1.80c</td>
<td>210.78 ± 9.03a</td>
<td>156.75 ± 4.84b</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>44.50 ± 2.47c</td>
<td>41.13 ± 1.57c</td>
<td>210.78 ± 9.03a</td>
<td>66.63 ± 1.72b</td>
</tr>
</tbody>
</table>

Different superscripts in the same line indicate significant differences (p < 0.05).

**Table 4:** Variations of MDA and GSH contents in kidneys and in liver according to the mice treatment: group I (control, 0.9% NaCl, 20 mL/g/day for 5 days, SC), group II (L carnitine, 500 mg/kg/day for 5 days, SC), group III (Diclofenac sodium, 10 mg/kg/day for 5 days, SC) and group IV (Diclofenac sodium, 10 mg/kg/day for 5 days, SC + L carnitine, 500 mg/kg/day for 5 days, SC) (n = 8 in each group). Results are expressed as mean ± standard errors.

<table>
<thead>
<tr>
<th>Tissue parameter</th>
<th>Group I (NaCl)</th>
<th>Group II (L carnitine)</th>
<th>Group III (Diclofenac)</th>
<th>Group IV (L carnitine + diclofenac)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA\textsubscript{kidney} (µmol/g)</td>
<td>0.226 ± 0.01c</td>
<td>0.210 ± 0.01c</td>
<td>0.281 ± 0.01a</td>
<td>0.252 ± 0.01b</td>
</tr>
<tr>
<td>MDA\textsubscript{liver} (µmol/g)</td>
<td>0.247 ± 0.01c</td>
<td>0.222 ± 0.01c</td>
<td>0.323 ± 0.01a</td>
<td>0.283 ± 0.01b</td>
</tr>
<tr>
<td>GSH\textsubscript{kidney} (mg/g)</td>
<td>0.275 ± 0.01b</td>
<td>0.307 ± 0.01a</td>
<td>0.216 ± 0.01c</td>
<td>0.248 ± 0.01b</td>
</tr>
<tr>
<td>GSH\textsubscript{liver} (mg/g)</td>
<td>0.311 ± 0.01b</td>
<td>0.367 ± 0.01a</td>
<td>0.243 ± 0.01c</td>
<td>0.292 ± 0.01b</td>
</tr>
</tbody>
</table>

Different superscripts in the same line indicate significant differences (p < 0.05).
References


