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Original Research Article

Protective effect of calpain inhibition on contrast induced nephropathy in rats

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ABSTRACT

Background: Although the underlying mechanisms of contrast induced nephropathy (CIN) is unclear, some mechanisms are suspected such as direct toxic effects of contrast media (CM) and oxidative stress. In this study, antioxidant, anti-apoptotic and protective effects of calpain inhibitor-1 (CAL-1) were investigated in experimental model of CIN.
Methods: Twenty-eight Sprague Dawley female rats, aged 8-10 weeks were used. CIN was induced by administration of high-osmolal CM diatrizoate (6 ml/kg) after 48 h of dehydration into the tail vein. Rats of CAL-1 and CM + CAL-1 groups were administered Cal I-1 (10 mg/kg, IP) 30 minutes before administration of CM. Kidney function parameters, renal tissue oxidative stress markers were measured. At the end of the study Renal tissues were dyed with Hematoxylin-Eosin, periodic acid shiff (PAS) and TUNEL paints.
Results: Increases of serum creatinine (Cr) and blood urea nitrogen (BUN) levels in CM group were significantly high than control group ($p<0.002$, $p<0.002$, respectively). BUN and Cr levels of CM + CAL-1 group were significantly lower than those in CM group ($p<0.002$, $p<0.007$, respectively). Tissue oxidative stress markers in CM + CAL-1 group were lower than in CM group. CM group had a significant increase in apoptotic cells with TUNEL staining. There was a significant reduction in apoptotic cells in CM + CAL-1 group compared to CM group.
Conclusions: It has been determined that CAL-1 provides protection of renal functions by preventing the increase of oxidative stress and apoptosis in the experimentally created CIN model in rats.

Keywords: Contrast-induced nephropathy, Apoptosis, Calpain inhibitor-1

INTRODUCTION

Contrast media (CM) is increasingly used in clinical practice in recent years and are very useful agents for diagnosis. With the diligent work of scientists, they have reached their current position and are used safely in many patient groups. After the application of CM, unwanted effects can be observed in the body. While some of these effects are minor reactions that do not require partial drug therapy, some of them are serious complications that can cause permanent damage that cannot be prevented without medical support.[1] In the studies conducted, it is determined as the third most common cause of acute kidney failure developing in hospital and its relation with increased morbidity and mortality rate increases the importance of CIN.[2] In general, conditions such as fluid deficit, diabetes mellitus, chronic kidney disease, age, congestive heart failure are risk factors for the development of CIN. The frequency of developing nephropathy after contrast use is directly proportional to risk factors.[3]

In studies, conducted with experimental animal models, it seems difficult to create nephropathy by giving CM alone. In the literature, researchers use different methods such as

dehydration, diuretics, nephrectomy, glycerol, and NSAIDs to induce experimental contrast nephropathy.[4] Researchers use various methods for experimentally induction of CIN in rats. The common point in all methods is to create a predisposed situation for CIN by causing renal damage before CM injection.[5]

In the literature, in studies investigating the effects of CAL-1 on renal ischemia / reperfusion injury, it has been shown that apoptosis and oxidative stress in renal tubular epithelial cells are reduced and renal functions are preserved with calpain inhibition.[6]

In this study, a contrast nephropathy model was created in rats and the effects of calpain inhibition with CAL-1 on renal changes in CIN were investigated.

METHODS

This experimental animal study was conducted at Fırat University Experimental Research Center (FUDAM) from 11 April 2011 to 22 April 2011, on a total of 28 Sprague Dawley rats, which purchased from FUDAM, weighing 150-200 gram at the age of 8-10 weeks. Body weights of all rats were recorded at the beginning and end of the study. Rats were fed in specially prepared cages with 55±5% relative humidity, in an environment with a ventilation system, and their bottoms were cleaned every day. For feeding of rats, standard rat feeds in the form of pellets were used. Water was supplied with special bottles placed in special sections in the cages and with droppers at the ends. The ambient temperature of the experimental animals was kept constant between 22-240C and the animals were left in 12 hours light and 12 hours dark.

Diatrizoate (UrografinR 76%, Schering AG, Istanbul, Turkey) was used as high osmolar CM. CAL-1 ((N-Acetyl-Leu-Leu-norleucinal) R, Roche Diagnostic Systems, Manheim, Germany) was used to investigate its effects on CIN. In addition, ketamine (KetalarR, Phizer, Istanbul, Turkey) and xylazine (RompunR, Bayer, Istanbul, Turkey) were used to provide anesthesia in rats.

The most commonly used experimental CIN method, CM injection after a certain period of dehydration, was used in the study. For this purpose, after a dehydration period of 72 hours a dose of 6 ml / kg of diatrizoate injected through the tail veins with a 26-gauge intravenous cannula with to create CIN. Rats were divided into 4 groups: Control (Group I), CM (Group II), CAL-1 (Group III) and CM + CAL-1 (Group IV).

Group (N=7) Control group: no operations were performed during the 7-day experiment and they were kept alive under normal laboratory conditions. At the end of the study, blood samples were taken from rats under anesthesia and nephrectomy was performed.

Group (n=7) CM Group: The rats in this group were dehydrated for 3 days before giving CM. The next day, 6 ml / kg diatrizoate was administered through the tail veins with a 26 gauge cannula. 24 hours after CM application, blood samples were taken as in the control group and nephrectomy was performed.

Group (n=7) CAL-1 Group: After the rats were left without water for 3 days, CAL-1 at a dose of 10 mg / kg was administered intraperitoneally. One hour after the application of CAL-1, 6 ml / kg 0.9% sodium chloride was administered from the tail veins. 24 hours after the application of CAL-1, blood samples were taken as in the control group and nephrectomy was performed.

Group (n=7) CM + CAL-1 Group: The rats in the fourth group were dehydrated for 3 days and then diatrizoate was given as applied in the second group the next day. One hour before the administration of diatrizoate, CAL-1 was administered intraperitoneally at 10 mg/kg, and 24 hours later, blood samples were taken as in the other groups and nephrectomy was performed.

Baseline blood samples were drawn from the tail vein on day one under ether anesthesia while the final blood samples were withdrawn from the abdominal aorta at the end of the study (day 7). At the end of the study, general anesthesia with intraperitoneal xylazine and ketamine was applied, laparotomy was performed, and nephrectomy was performed. The right kidney was placed in boin solution and separated for histopathological evaluations. The left kidney was wrapped in aluminum foil without any processing, and stored at -700C until the day of analysis for biochemical studies. 2 ml of blood samples taken from all groups were placed in a plain biochemistry tube and allocated to measure serum blood urea nitrogen (BUN) and Cr on the same day. Other blood samples were placed in an anticoagulant tube to measure oxidative stress markers and stored at -700C until the day of analysis.

On day 1, samples were obtained for serum BUN and Cr which were used as indicators of renal functions. On day 7, blood samples were withdrawn for measurements of BUN and Cr. Chemicals and kits were from Sigma–Aldrich and Sigma Diagnostic (St. Louis, MO, USA). Measurement of malondialdehyde (MDA) in kidney tissue was made by spectrophotometer according to the method modified by Markovics et al.[7] Total superoxide dismutase (SOD) activity was measured by the method described by Sun et al.[8] Catalase (CAT) level was determined by minute spectrophotometric absorbance measurement until the change (decrease) in hydrogen peroxide level reached a constant level (s-1, k) as defined by Aebi.[9] For the evaluation of these three parameters, protein measurement from the kidney was made by the Lowry method.[10]

Kidney tissues taken from all groups were fixed in boin solution for 24 hours and then washed under tap water. Tissues washed in tap water were then passed through routine histological follow-up series. The kidney tissues were then embedded in paraffin blocks. 5-6 μm thick sections were cut through these paraffin blocks. The

sections were stained with Hematoxylin - Eosin (H and E) and periodic acid schiff (PAS) methods. The preparations obtained were examined and photographed under a research microscope (Olympus BH-2). In the evaluation of the damage to the kidney tissue; Vacuolization, degeneration, necrotic changes in the proximal and distal tubules of the kidney and peritubular fibrosis and infilitrative cell increase in the medulla were evaluated in randomized microscope fields and scored semi-quantitatively with a number from 0 to +4.

TUNEL method is widely used for detecting DNA breaks in the apoptotic signal cascade. In apoptosis, after the terminating proteins are activated, they break down target proteins in the cytoplasm and nucleus. One of these proteins is a protein that binds with DNA endonuclease. Caspases break down this protein and release the endonuclease. Ca-Mg dependent endonuclease entering the nucleus creates DNA breaks. The TUNEL method allows the detection of these DNA breaks.[11] In our study, sections obtained from paraffin blocks with a thickness of 5 µm were taken on polylyzed slides. Cells undergoing apoptosis were determined using the ApopTag plus Peroxidase in Situ Apoptosis Detection Kit (ChemiconR, cat no: S7101, USA) in line with the manufacturer's recommendations. The preparations obtained were evaluated under a research microscope (Olympus BH-2) and photographed. In the evaluation of the TUNEL staining procedure, nuclei stained blue with hematoxylin were normal, and cells stained as nuclear brown were evaluated as apoptotic. In the evaluation of TUNEL staining, the prevalence of staining was based. The prevalence of TUNEL staining was scored semi-quantitatively with a number from 0 to +4.

The data obtained were determined as mean±standard error. Statistical package for social sciences (SPSS) 16.00 computer package statistics program was used for the preparation of statistics. Analysis of variance (ANOVA) was used for comparison of data between groups, and Bonferroni test was used for post-hoc study. Mann-Whitney U test was used for comparison between two independent groups. P<0.05 values were considered significant. The study was conducted at the Experimental Research Center of Euphrates University in accordance with the standard code of ethics for experimental animal studies. The study was approved by the Fırat University Animal Experiments Ethics Committee.

RESULTS

There was no rat loss during the study. Body weight decreased significantly after dehydration in all groups in which the dehydration protocol was applied. Table 1 shows the clinical and biochemical data obtained at the end of the experiment. BUN and Cr levels were higher in group II than in other groups. When Group II (CM group) and Group I (Control group) were compared, there was a statistically significant increase in BUN and Cr levels in Group II (p <0.02 for both). Although there was an increase in BUN and Cr levels in Group III and Group IV compared to Group I, it was not found statistically significant. When group II and group IV were compared, a significant difference was found in BUN and Cr levels (p <0.07, p <0.02, respectively). When Group III (CAL-1 group) and Group IV (CM + CAL-1 group) were evaluated among themselves, no significant difference was found between the groups (Table 1).

For all groups, MDA levels and SOD, CAT enzyme activities obtained as a result of biochemical analysis in kidney tissue are shown in Table 1. A significant difference was observed between the control group and CM group in terms of the levels of CAT measured in the kidney tissue, the enzyme activity was found to be increased in the CM group (p<0.002). There was a significant decrease in CAT levels in the CM+CAL-1 group compared to the CM group (p<0.002).

Table 1: Clinical and laboratory data in study groups.

	Group I (Control) (n = 7)	Group II (CM) (n = 7)	Group III (CAL-1) (n = 7)	Group IV (CM +CAL-1) (n = 7)
Starting weight	186.8±36.9	180.5±24.4	179.8±23.4	176.9±12.3
7th day weight	191.4±33.8	154.4±21.1	155.9±19.1	156.8±16.7
BUN (mg/dl)	39.85±1.95	46±2.44[a]	41.28±1.79[b]	41.14±2.19[b]
Cr (mg/dl)	0.50±0.04	0.67±0.03[a]	0.52±0.03[b]	0.48±0.03[b,c]
SOD (U/mg)	1.36±0.27	1.88±0.64	1.84±0.54	1.72±1.05
CAT (k/g)	0.69±0.05	0.98±0.11[a]	0.83±0.13[a,b]	0.84±0.12[a]
MDA (nmol /g)	18.87±2.64	28.84±4.75[a]	20.22±2.31[b]	25.01±2.85[a,c]

SOD: Superoxide dismutase. CAT: Catalase. MDA: Malondialdehyde. BUN: Blood urea nitrogen. Cr: Creatinine

Values are given as mean ± standard deviation.
a Compared to group I: p<0.05.
b Compared to group II: p<0.05.
c Compared to group III: p<0.05

CAT levels were significantly higher in the CAL-1 group and the CM + CAL-1 group compared to the control group ($p<0.02$ for both). The MDA level was found to be significantly higher in the CM and CM + CAL-1 groups compared to the control group ($p<0.002$, $p<0.006$, respectively). Although the MDA levels were lower in the CM + CAL-1 and CAL-1 groups compared to the CM group, only the difference between the CAL-1 group and the CM group was considered statistically significant ($p<0.002$). Although the SOD level in the kidney tissue was the highest in the CM group and the lowest in the control group, no statistically significant difference was found between the groups.

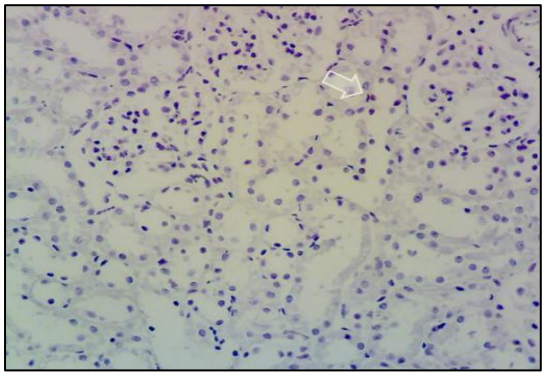

Figure 1: TUNEL positive cells (→) X 100 in the prevalence of +1 in kidney tissue of the control group.

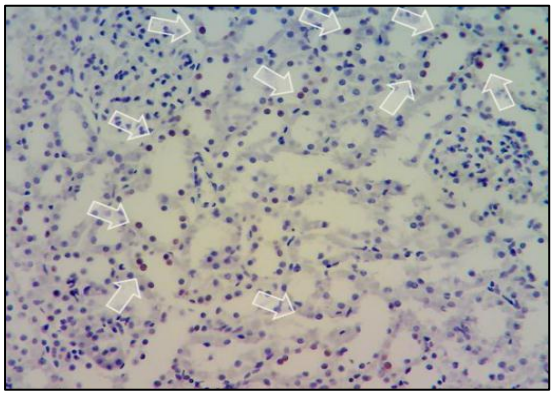

Figure 2: TUNEL positive cells (→) X 100 in the prevalence of +4 in kidney tissue of the CM.

In the cross-sections of the control group, it was noted that the glomeruli in the cortex were of normal structure and size. No pathological signs such as vacuolization, degeneration or necrotic changes were found in the tubules of the cortex and medulla. In the CM group (Group II), significant differences in the structure and size of some glomeruli in the cortex were observed. Compared to the control group, irregularities and spills in the tubule epithelium, thickening, decomposition and distortions in the basal membranes were significantly increased in the CM Group. Epithelial vacuolization, being more prominent in the tubules in the cortical region, was significantly higher than in the control group. In the CM group, flattening of the tubular epithelium, epithelial cell shedding and hyaline accumulation were observed significantly more than the control group.

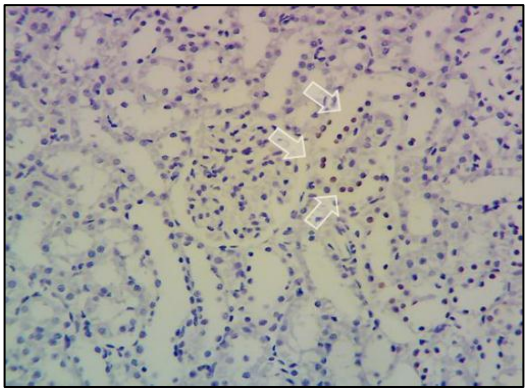

Figure 3: TUNEL positive cells (→) X 100 in the prevalence of +2 in kidney tissue of the CM+CAL-1 group.

There was an increase in inflammatory cell infiltration in the interstitial space between the glomeruli and tubules. Peritubular fibrotic areas and bridging between them in the corticomedullary region were observed more frequently in the CM group than in the other groups. Signs of tubulointerstitial damage such as interstitial inflammation, interstitial fibrosis, tubular epithelial changes and cell shedding, basement membrane thickening, dilatation, vacuolization, and hyaline accumulations were significantly less in group IV (CM + CAL-1 group). It was observed that the structures of the formations in the cortex and medulla were preserved in group III (CAL-1 group). Glomerular basement membrane had normal thickness and appearance. It was noteworthy that there was more vacuolization in the tubule epithelium compared to the control group. Other findings of tubular damage were not found. As a result, it has been observed that the calpain inhibition provided protective effects against the damage caused by CM in the cortex and medulla of the kidney.

As a result of the examination of TUNEL staining under light microscopy for the determination of apoptotic cells; TUNEL positivity was observed with a prevalence of +1 in the control group (Figure 1). Significantly increased TUNEL positivity in the CM group when compared to the control group was noted and was evaluated as +4 (Figure 2). TUNEL positivity was significantly decreased in the KM + CAL-1 group compared to the KM group, and it was evaluated as +2 (Figure 3).

DISCUSSION

Contrast nephropathy, which came to the fore with the first publications about nephrotoxicity due to the use of CM, was reported about 60 years ago, is one of the most important reasons limiting the intravascular use of CM. In our study, increase in serum Cr levels in the CM group is similar to the diagnosis and clinical course of CIN.[12] BUN

and Cr levels were found to be significantly higher in the CM group compared to the control group ($p<0.002$, $p<0.002$, respectively). BUN and Cr levels were found to be significantly lower in CAL-1 subjects compared to the CM group ($p<0.002$, $p<0.007$, respectively). There was no significant difference between the control and KAL-1 groups in terms of renal function. The fact that BUN and Cr values were significantly lower in the CM + CAL-1 group compared to the CM group supports the protective effect of CAL-1 on renal functions.

Recently, it has been shown that the disruption in the oxidative balance, which is thought to be associated with many clinical conditions, has a role in the pathophysiology of CIN.[13] Renal medullary ischemia and direct toxic damage to tubule epithelial cells, which are in the pathogenesis of CIN, may mediate the increase in the production of reactive oxygen species.[14] In both cases, free-oxygen radicals increases and renal damage occurs due to the resulting oxidative stress. The fact that there are many articles in the literature showing that some antioxidant agents have protective effects also supports this hypothesis.[15] Toprak et al showed that after 72 hours of dehydration, rats lost 30% of their weight and that 6 ml / kg of diatrizoate administered intravenously after dehydration significantly increased serum creatinine (Cr) levels compared to the control group and they have been determined that nebivolol, which is known to have antioxidant effects, has a protective role on CIN.[16]

No effective treatment for contrast-induced nephropathy has been identified so far, and therefore identification of CIN risk and effective preventive strategies are ways to reduce the occurrence of contrast-induced nephropathy. The proper use of CM and hydration can reduce the occurrence of CIN.[18] Different results have been reported regarding the benefits of antioxidants. NAC, the best known of these antioxidant agents, is the most widely studied agent and the most commonly used agent in addition to hydration in clinical practice. Koç et al showed in the experimental CIN model in rats, that the BUN, Cr and MDA levels of the rats in the CIN group were significantly higher than the control group and that the use of NAC or beta glucan decrease MDA levels and other oxidative stress markers.[18]

Apoptosis, called programmed cell death, is a form of death that plays a role in the elimination of cells during proliferation and differentiation.[19] The relationship between calpain and apoptosis in renal damage due to hypoxic conditions or nephrotoxic agents has been shown in many studies. In a study with cisplatin, well-known nephrotoxic effects of, it was observed that calpain and caspase systems caused cell death by increasing apoptosis in renal damage caused by cisplatin, and apoptosis decreased and renal damage improved with inhibition of these systems.[20] In our study, as a result of examining the preparations stained with TUNEL method under light microscopy for the determination of apoptotic cells; a significant increase in apoptotic cells was observed in the CM group compared to the control group.

In an experimental CIN study, apoptosis investigated by TUNEL method, parallel with the results of our study, an increase in TUNEL staining was found in the groups in which CM was applied, and another finding in the study was that apoptosis was found to be significantly increased in diabetic rats compared to normal rats.[21] In a study conducted with telmisartan, an angiotensin receptor blocker, it was shown that the increase in apoptosis caused by contrast nephropathy in rats was prevented by the administration of telmisartan and renal functions were preserved.[22]

In our study, a significant decrease in apoptotic cells was observed in the CM + CAL-1 group compared to the CM group. The decrease in TUNEL-positive stained cells with the application of CAL-1 may be due to the impairment of signal transmission and the prevention of the increase in membrane defects and apoptosis by calpain inhibition. In addition, CAL-1, due to its antioxidant properties, may prevent apoptosis that develops due to oxidative stress.

A few potential concerns and limitations should be mentioned in this study. The CIN model created in experimental animals cannot fully simulate clinically developing CIN. The sample size used in this study is very small. Therefore, statistical power in determining the differences between groups is decreased. Different doses of CAL-1 were not given, because of that dose-dependent effects of CAL-1 on CIN were not demonstrated.

CONCLUSION

As a result in our study; It has been determined that CAL-1, a calpain inhibitor, provides protection of renal functions by preventing the increase of oxidative stress and apoptosis in the experimentally created CIN model in rats. In-vitro or in-vivo studies with calpain inhibitors may provide different perspectives to the treatments applied in many diseases. The major obstacle to the clinical use of calpain inhibitors appears to be their inhibition of all cysteine proteases and other proteolytic enzymes due to their lack of specificity. In the future, with further and detailed studies, new treatment approaches can be created in many diseases by elucidating the pathophysiological mechanisms associated with the calpain system and completing the deficiencies of calpain inhibitors.

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