Effect of apelin hormone on renal ischemia/reperfusion induced oxidative damage in rats

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ABSTRACT
Apelin is a peptide hormone defined as a ligand for G-protein clamped receptor (APJ) receptor. It is indicated in the literature both apelin and APJ are synthesized on the peripheral tissues including the renal tissues. Which roles does the apelin play on the renal tissue has not been completely illuminated yet. This study is designed to determine the possible protective effect of apelin-13 on the kidney I/R injury. Adult male Sprague-Dawley rats were used in this study. In the sham group, right kidneys of the animals were dissected. In the I/R group, right kidney was dissected and ischemia of 45 min was performed, and then reperfusion was applied for 3 h. In the treatment groups, three different doses of apelin were injected at the beginning of the ischemia unlike the I/R group. BUN, Cre, Na, K, Cl, total protein and albumin from serum samples were determined and TNF-α, IL-1β, IL-6, TAS and TOS parameters were read with ELISA reader. MDA, SOD, CAT and GSH-Px enzyme activations from renal tissues were measured. In comparison with the sham and I/R groups, while the serum BUN, CRE, CI and TNF-α levels showed an increase in the groups on which the apelin-13 was applied, Na, total protein, albumin, TAS levels decreased. Serum TOS level of other groups showed an increase by comparison with the sham group. Our results showed that apelin-13 applied after I/R increased the antioxidant enzyme activity in a dose dependent manner, prevented the lipid oxidation and improved the renal functions.

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Introduction
Acute renal failure (ARF) characterized by the waste product accumulation, not being able to have the urine concentrated and not being able to protect the electrolytes and the disturbance of the fluid balance could develop on the renal tissue exposed to the ischemia.1,2 ARF characterized by the waste product accumulation, not being able to have the urine concentrated and protect the electrolytes and the disturbance of the fluid balance could develop on the renal tissue exposed to the ischemia. It is a frequent clinical syndrome with its negative results3,4 and it is characterized by the change of blood levels of nitrogenous and biochemical wastes observed as a result of the sudden decline in the glomerular filtration rate.5–7 Renal blood flow may decrease or wholly stop in the events of kidney transplant, renal angioplasty, urological and vascular events where aorta is clamped from above the renal artery or where the renal pedicle is clamped.8 Reversion of blood flow to the ischemic tissue may improve the cellular functions; but reperfusion leads to more damage on the tissue paradoxically when compared to the damage formed by the ischemia.9,10

Many mechanisms including the reactive oxygen species (ROS) formed upon the molecular oxygen influx to the cell and its derivations at first are involved on such damage that is observed in the reperfusion process.11,12 Local and systematic inflammatory response arises after the ischemia/reperfusion (I/R) damage and it is known that pro-inflammatory agents increases on the plasma correspondingly.13,14 Under normal circumstances, tissues have enzymes like superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT) that remove the reactive oxygen types rapidly. Endogenous antioxidant defense systems of the tissues remain insufficient during the ischemia and especially in the reperfusion process in which the reoxygenation takes place.15

Previous studies showed that some hormones could prevent the free radical related damage.16,17 Apelin isolated from the digestive juice of cattle by Tatemoto et al. in 1998 is a protein hormone defined for adipose tissue family.18 As well as being an endogenous ligand of G-protein clamped receptor (APJ), this hormone shows its effects over APJ. It is known that the form of apelin whose effects change according to the forms and which
Apelin and APJ are present in the tissues of many mammals like kidney, brain, lung and spleen. Reaux et al. putting forward a hypothesis which states that apelin-13 form has a fluid homeostasis regulatory impact indicates that central apelin-13 application has an impact on water consumption. Taheri et al. reports that apelin-13 that they applied to the rats in different concentrations leads to a high level of water consumption. It is indicated that while the central application of the apelin affects the fluid homeostasis, its peripheral application decreases the blood pressure. Studies carried out reports that apelin decreases the oxidative stress in the cardiomyocytes and veins of smooth muscle cells. In their studies, Sagiroglu et al. report that apelin-13 applied before operation has a protective effect similar to leptin against renal I/R damage. Besides, they report that apelin is also effective in peripheral tissues especially the renal functions by means of antidiuretic hormone and other mediators.

This study was carried out to examine the possible protective role of apelin-13, which is the most active form of apelin and has the highest biological activity, against the oxidative damage to be formed due to renal I/R.

Materials and methods

All applications in the study approved by the Board of Ethics Committee on Animal Experiments of Firat University (Meeting no.: 2013/2; Decision no.: 32, Annex 1) were performed according to the protocol of the ethics committee. Apelin-13 hormone used in the experiment was bought from Sigma- Aldrich Co. (St Louis, MO; Catalog no.: A6469). Just before the initiation of experiment, three different concentrations (1, 10 and 100 μg/kg) of apelin-13 were prepared in physiological saline solution (SF). Thirty-five male rats of Sprague-Dawley type between the weights of 320–370 g were used in the experiment. During the experimental period, rats were kept at temperature range between 21–22 °C and in the 12 h of brightness–darkness period. Rats ate standard rat food as ad libitum and drank tap water. Rats were divided into five groups randomly in a way to have close weights (n = 7). The rats were subjected to 12 h light/dark cycle in temperature- and humidity-controlled environment, and they were put in cages and provided continuous access to water and standard food pellets there. Rats were deprived of food for 24 h before the procedure but the rats had free access to water except for the last 1 h prior to the experiment.

Experimental groups

Animals were anesthetized by 8mg/kg of xylazine (Rompun, Bater, Istanbul/Turkey) and 70 mg/kg of ketamine (Ketalar, Eczacibaş, Istanbul/Turkey) intramuscular. Groups and the applications performed are stated below:

- Sham group (Sham): Right kidneys of animals were dissected.
- I/R group: Right kidney was dissected and ischemia of 45 min was performed, and then reperfusion was applied for 3 h.
- Apelin groups (injected 1, 10 and 100 μg/kg apelin = APLN-1, 10 and 100 μg): Right kidney was dissected and ischemia of 45 min was performed on the left kidney and then reperfusion was applied for 3 h. At the beginning of the ischemia, apelin-13 intraperitoneal was given.

Animals were sacrificed by being decapitated after the experiment and their blood and left renal tissues were taken. Blood samples were centrifuged at 3500 rpm for 15min and their serums were taken. Tissues collected were preserved at −80 °C. Serum blood urea nitrogen (BUN), creatinine (Cre), Cl, Na and K levels were determined by using Olympus AU 600 (Optical Co., Ogaki, Japan) auto analyzer at the central laboratory of Firat University.

Serum tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β) and interleukin-6 (IL-6) levels of the groups were determined by using the rat specific ELISA kits and by studying in compliance with the kit protocols (TNF-α, EEL-R0019; IL-1β, E-EL-R0012; IL-6, E-EL-R0015; Elabscience, Beijing, PR China). Total antioxidant levels of samples were determined by using total antioxidant status (TAS) kit (Catalog no: RL0017, Rel Assay, Gaziantep, Turkey) and their total oxidant levels were determined by using total oxidant status (TOS) kit of Rel Assay Brand (Catalog no: RL0024, Turkey).

Analyses of renal tissues

Renal tissue was weighed after being washed with SF. Tissue sample of 1 g was put into the glass tube and homogenization process was realized by adding the 10 mL, 0.2 M Tris–HCl tampon to it. Glass tubes were taken to a pot full of ice in order for not to have a lesion during the homogenization process. Tissues were
Statistical evaluation of the data obtained in this thesis study carried out by using a statistical software package program. Compatibility of the data with the normal distribution was evaluated according to the Kolmogorov–Smirnov test and it is observed that they are not compatible with the normal distribution. Differences between groups were determined by using the Wallis H test, and Dunn test for the multiple comparisons. Results were expressed in median (min–max.). Value of \( p < 0.05 \) was accepted as significantly value in the statistical evaluation.

**Results**

**Serum analyses**

When compared with the sham group, an increase in the BUN, Cre, K, TOS, TNF-\( \alpha \), IL-1\( \beta \) and IL-6 values and a meaningful decline in Na and TAS parameters are observed after the ischemia in the serum samples used in the study (Tables 1 & 2). This finding seen in BUN and Cre values makes us think that acute tubular renal damage occurs on the kidney after I/R. When the groups to which the Apelin-13 is applied are examined, declines in the levels of BUN and Cre are statistically important in the APLN-10 \( \mu \)g and APLN-100 \( \mu \)g group (Table 1). Basic physiological effect of ARF is the accumulation of water, metabolic products and electrolytes in the blood and extracellular fluid.

When compared with the sham-control group, levels of TAS showed a significantly decline in APLN-1 \( \mu \)g groups (\( p < 0.05 \)). When compared with I/R group, a significantly increase is observed on the TAS level of APLN-10 \( \mu \)g and APLN-100 \( \mu \)g groups (\( p < 0.05 \)). When compared with sham-control group, a significantly increase is observed on the TOS level of APLN-1 \( \mu \)g, APLN-10 \( \mu \)g and APLN-100 \( \mu \)g groups (\( p < 0.05 \)). When compared with I/R group, a meaningful decline is observed on the TOS level of APLN-10 \( \mu \)g and APLN-100 \( \mu \)g groups (\( p < 0.05 \), Table 2).

Serum TNF-\( \alpha \) level increased meaningfully in I/R, APLN-1 \( \mu \)g and APLN-10 \( \mu \)g groups when compared with the sham-control groups (\( p < 0.05 \)). On the other hand, when compared with the I/R group, a significantly decline is observed on TNF-\( \alpha \) level of APLN-100 \( \mu \)g group (\( p < 0.05 \)). When compared with the I/R group, a decline is observed on the IL-1\( \beta \) level of APLN-100 \( \mu \)g group (\( p < 0.05 \)). Meaningful declines are observed on the IL-6 level of APLN-10 \( \mu \)g and APLN-100 \( \mu \)g groups when compared with the I/R group (\( p < 0.05 \), Table 2).

**Analyses of renal tissues**

**SOD, CAT, GSH-Px enzyme activities**

Statistically meaningful decline is observed in the SOD, CAT enzyme activity of I/R and APLN-1 \( \mu \)g groups when compared with sham-control group (\( p < 0.05 \), Table 3). A decline is observed in the GSH-Px enzyme activity of I/R,
APLN-1μg and APLN-10μg groups when compared with the sham-control group ($p<0.05$, Table 3). Statistically significant increases are observed in SOD, CAT, GSH-Px enzyme activity of APLN-10μg and APLN-100μg groups when compared with the I/R group ($p<0.05$, Table 3).

**Determination of malondialdehyde**

When compared with the sham-control group, a significantly increase is seen on MDA level of I/R, APLN-1μg and APLN-10μg groups ($p<0.05$). When compared with I/R group, a meaningful decline is observed on MDA level of APLN-10μg and APLN-100μg groups ($p<0.05$, Table 3).

**Discussion**

ROS generated in relation with the reperfusion of ischemic tissue plays a significant role in the pathogenicity of I/R damage. Excessive formation of ROS and deterioration of antioxidant defense system during I/R lead to an oxidative damage. Effects of peptides released

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**Table 1.** Comparison of serum parameters after apelin-13 injection.

| Groups       | Sham | I/R     | APLN-1μg | APLN-10μg | APLN-100μg | Sig. ($p$) |
|--------------|------|---------|----------|-----------|------------|------------|
| BUN (mg/dL)  | Median 21.56 | 32.61$^a$ | 29.62$^a$ | 28.75$^{ab}$ | 25.14$^b$ | <.0001     |
|              | Max   24.51  | 35.4    | 32.0     | 30.85     | 29.17      |            |
|              | Min   18.12  | 29.57   | 27.13    | 26.53     | 24.86      |            |
| Cre (mg/dL)  | Median 0.41  | 0.72$^a$ | 0.66$^a$ | 0.62$^{ab}$ | 0.59$^b$  | <.0001     |
|              | Max   0.46   | 0.8     | 0.7      | 0.67      | 0.66       |            |
|              | Min   0.34   | 0.65    | 0.59     | 0.58      | 0.47       |            |
| Na (nmol/L)  | Median 140   | 132$^a$ | 133$^a$  | 134$^a$   | 136        |            |
|              | Max   143    | 139     | 135      | 138       | 138        |            |
|              | Min   136    | 129     | 130      | 128       | 132        |            |
| K (mEq/L)    | Median 6.5    | 8.2$^a$ | 7.8$^a$  | 7.6$^a$   | 7.4$^b$   | <.0001     |
|              | Max   7      | 8.4     | 8.7      | 8.2       | 7.8        |            |
|              | Min   6.1    | 8       | 6.8      | 7.2       | 7          |            |
| Cl (nmol/L)  | Median 121   | 125     | 124      | 124       | 124        |            |
|              | Max   123    | 132     | 130      | 127       | 127        | 0.104      |
|              | Min   116    | 120     | 118      | 118       | 118        |            |
| T.protein (g/dL) | Median 5.7    | 5.2$^a$ | 5.2$^a$  | 5.4$^a$   | 5.5$^b$   | <.01       |
|              | Max   5.8    | 5.4     | 5.7      | 5.6       | 5.8        |            |
|              | Min   5.4    | 4.5     | 5.0      | 5.0       | 5.2        |            |
| Albumin (g/dL) | Median 3.4    | 3.1$^a$ | 3.2$^a$  | 3.3       | 3.3        | <.01       |
|              | Max   3.7    | 3.4     | 3.3      | 3.4       | 3.5        | <.05       |
|              | Min   3.3    | 2.8     | 3.0      | 3.1       | 3.1        |            |

Data analysis was performed using the Kruskal–Wallis H test and the Dunn test in the multiple comparisons. Values were expressed as median (min–max).

$^a p<0.05$: compared with sham group and $^b p<0.05$: compared with I/R group was considered statistically significant ($n=7$).

**Table 2.** Serum parameters measuring by ELISA reader.

| Groups       | Sham | I/R     | APLN-1μg | APLN-10μg | APLN-100μg | Sig. ($p$) |
|--------------|------|---------|----------|-----------|------------|------------|
| BUN (mg/dL)  | Median 21.56 | 32.61$^a$ | 29.62$^a$ | 28.75$^{ab}$ | 25.14$^b$ | <.0001     |
|              | Max   24.51  | 35.4    | 32.0     | 30.85     | 29.17      |            |
|              | Min   18.12  | 29.57   | 27.13    | 26.53     | 24.86      |            |
| Cre (mg/dL)  | Median 0.41  | 0.72$^a$ | 0.66$^a$ | 0.62$^{ab}$ | 0.59$^b$  | <.0001     |
|              | Max   0.46   | 0.8     | 0.7      | 0.67      | 0.66       |            |
|              | Min   0.34   | 0.65    | 0.59     | 0.58      | 0.47       |            |
| Na (nmol/L)  | Median 140   | 132$^a$ | 133$^a$  | 134$^a$   | 136        |            |
|              | Max   143    | 139     | 135      | 138       | 138        |            |
|              | Min   136    | 129     | 130      | 128       | 132        |            |
| K (mEq/L)    | Median 6.5    | 8.2$^a$ | 7.8$^a$  | 7.6$^a$   | 7.4$^b$   | <.0001     |
|              | Max   7      | 8.4     | 8.7      | 8.2       | 7.8        |            |
|              | Min   6.1    | 8       | 6.8      | 7.2       | 7          |            |
| Cl (nmol/L)  | Median 121   | 125     | 124      | 124       | 124        |            |
|              | Max   123    | 132     | 130      | 127       | 127        | 0.104      |
|              | Min   116    | 120     | 118      | 118       | 118        |            |
| T.protein (g/dL) | Median 5.7    | 5.2$^a$ | 5.2$^a$  | 5.4$^a$   | 5.5$^b$   | <.01       |
|              | Max   5.8    | 5.4     | 5.7      | 5.6       | 5.8        |            |
|              | Min   5.4    | 4.5     | 5.0      | 5.0       | 5.2        |            |
| Albumin (g/dL) | Median 3.4    | 3.1$^a$ | 3.2$^a$  | 3.3       | 3.3        | <.01       |
|              | Max   3.7    | 3.4     | 3.3      | 3.4       | 3.5        | <.05       |
|              | Min   3.3    | 2.8     | 3.0      | 3.1       | 3.1        |            |

Data analysis was performed using the Kruskal–Wallis H test and the Dunn test in the multiple comparisons. Values were expressed as median (min–max).

$^a p<0.05$: compared with sham group and $^b p<0.05$: compared with I/R group was considered statistically significant ($n=7$).
from the adipose tissue are frequently examined in the I/R studies performed on different tissues in recent years and it is indicated that they have significant protective roles. Apelin is also a peptide released from adipose tissue and plays significant roles in many physiological processes such as energy metabolism especially, nutritional behavior and reproduction function. Besides, studies carried out recently show that apelin may have a good antioxidant effect.

Yang et al. reported in their study that apelin could activate multiple protective mechanisms to prevent heart, brain, liver and kidney injury. Apelin/APJ system may be a promising therapeutic target for ischemic and other related diseases.

Foussal et al. reports in their study on the cardiac hypertrophy that apelin increases the CAT activity and decreases the oxidative stress in order to protect the heart functions. In another study, MDA level and liver functions in the I/R damage formed were examined for three days. Researchers determined that peptides showed protective effects in leptin and apelin-13 applications where they examine the MDA, GSH, AST, ALT and GGT levels.

It is stated in another study that APJ mRNA is present in all nephron segments and effective on the tubular function. Researchers determined in the in vitro studies they carried out that apelin decreases the inflammation in adipocytes and ROS production, and increases the CAT, SOD, GSH-Px expression of antioxidant enzyme and the apelin application onto the cardiomyocytes increases the CAT mRNA level and enzyme activity.

Increasing concentrations of apelin-13 was used in our study in contrast to other studies, thus the protective effect of apelin that it shows in increasing doses was attempted to be determined. It is seen that apelin-13 increases the SOD, CAT, GSH-Px enzyme activity decreased after I/R and decreases the increasing MDA and TOS levels, and therefore it shows antioxidant property.

It is known that ischemic tissue reperfusion leads to an increase in the inflammatory reactions. Inflammatory process leads to a ROS release, aggregation of inflammatory mediators and at the same time damage on the ischemic tissue caused by the immune cells. Inflammatory mediators like TNF-α, IL-1β, IL-6 are cytokines released after renal I/R. When we examine the cytokine levels in our study, TNF-α, IL-1β, IL-6 levels increased in I/R group when compared with the sham-control group. It is seen that while the IL-6 level decreases in APLN-10 group, TNF-α, IL-1β, IL-6 levels in APLN-100 group decreased.

It is reported in the study carried out by Day et al. that apelin application prevents the progression of the diabetic nephropathy. It is reported in the study carried out by Pan et al. that apelin application onto the sepsis model decreases the cardiac deterioration and inflammatory response similarly.

When we generally evaluate our results, it is seen that I/R applied onto the kidney leads to an oxidative stress and an inflammatory cytokine increase at the same time. As a result of the I/R application, it is seen that BUN and Cre levels that have a significant place in following up the renal functions increase. It is determined that BUN and Cre levels decrease by the protective effect of apelin-13 of peptide structure and this situation reminds us of the regulatory effect of renal functions.

This study, the molecular steps of which needed to be illuminated by more detailed studies, suggests that apelin-13 may be a new agent in preventing the I/R damage.

### Table 3. Biochemical analysis of renal tissue.

| Groups         | Sham  | I/R  | APLN-1 μg | APLN-10 μg | APLN-100 μg | Sig. (p) |
|---------------|-------|------|----------|------------|-------------|---------|
| SOD (U/mg Protein) | Median | 1.25 | 0.66a | 0.73a | 0.83ab | 1.07b | <0.0001 |
| Max | 1.51 | 0.76 | 0.77 | 0.99 | 1.20 | <0.0001 |
| Min | 1.06 | 0.41 | 0.67 | 0.72 | 0.75 | <0.0001 |
| CAT (K/mg Protein) | Median | 0.06 | 0.05a | 0.06a | 0.06b | 0.06b | <0.0001 |
| Max | 0.07 | 0.05 | 0.06 | 0.06 | 0.07 | <0.0001 |
| Min | 0.06 | 0.04 | 0.05 | 0.05 | 0.05 | <0.0001 |
| GSH-Px (U/g Protein) | Median | 506.28 | 352.15a | 456.15a | 459.20a | 474.45ab | <0.0001 |
| Max | 513.46 | 365.03 | 468.98 | 469.56 | 486.38 | <0.0001 |
| Min | 499.81 | 344.13 | 450.82 | 452.19 | 468.98 | <0.0001 |
| MDA (nmol/mg Tissue) | Median | 0.10 | 0.13a | 0.13b | 0.12ab | 0.12b | <0.0001 |
| Max | 0.11 | 0.15 | 0.14 | 0.13 | 0.12 | <0.0001 |
| Min | 0.10 | 0.13 | 0.12 | 0.12 | 0.12 | <0.0001 |

Data analysis was performed using the Kruskal–Wallis H test and the Dunn test in the multiple comparisons. Values were expressed as median (min–max). a p < 0.05: compared with sham group and b p < 0.05: compared with I/R group was considered statistically significant (n = 7).
Disclosure statement
Authors declare no conflict of interest.

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