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MicroRNA-125b as a new potential biomarker on diagnosis of renal ischemia–reperfusion injury

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ABSTRACT

Background: Acute renal failure is commonly seen in the perioperative period. Ischemia–reperfusion (IR) injury plays a major role in acute renal failure and delayed graft function. MicroRNAs (miRs), which are pivotal modulators of cell activities, offer a major opportunity for affective diagnosis and treatment strategies because they are tissue specific and in the center of gene expression modulation. The effect of bardoxolone methyl (BM) on miR-21, miR-223-5p, and miR-125b in renal IR injury was evaluated in this study.

Methods: Wistar-Albino rats (12-16 wk old, weighing 300-350 g) were used in the study. Rats ($n = 6$) were randomized into three groups (control, IR, and BM + IR). Tissue levels of miRs were analyzed with reverse transcription polymerase chain reaction.

Results: Significant reduction of urea and total oxidant status, increase of total antioxidant status, and oxidative stress index were identified in the IR + BM group compared with the IR group. Significant increases of miR-21 (2842.82-fold) and miR-125b (536.8-fold) were identified in the IR group compared with the control group; however, miR-223-5p levels did not show any significant difference. Also, miR-21 and miR-125b were significantly reduced in the IR + BM group compared with the IR group. Reduced histopathologic changes were observed in the IR + BM group. A significant decrease in the number of tunel-positive cells was identified in the IR + BM group compared with the IR group.

Conclusions: miR-125b was significantly increased in IR injury; thus, miR-125b can be a potential novel marker that can be used in diagnosis and treatment of renal IR injury. BM reduces miR-21 and miR-125b in case of IR injury and makes functional and histopathologic repairs.

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Introduction

Acute renal failure (ARF) is widely seen in the perioperative period. Ischemia–reperfusion (IR) injury plays a central role in ARF, delayed graft function, and long-term graft survival.[1-3] Even with modern technological advances, ischemic ARF is one of the most important clinical problems. Novel treatment strategies are required to lower acute mortality and to prevent chronic kidney failure progression.

MicroRNAs (miRs) are single-stranded RNA molecules of 21-23 nucleotides in length. miRs regulate cellular processes such as proliferation, differentiation, and apoptosis[4,5] by playing a central role in modulation of gene expression.[6,7] Being tissue specific and playing a pivotal role in modulation of gene expressions grant miRs a major opportunity for diagnosis and treatment of diseases. Even with advances in the last decade, affective novel and therapeutic approaches are still lacking in ARF. It has been shown that various characteristics of IR injury such as fibrosis and apoptosis are regulated by miRs.[8] miRs, which are pivotal modulator of cell activities, give hope for affective diagnostic and therapeutic strategies for kidney diseases such as acute and chronic kidney failure.[9]

It has been shown that miR-21 plays an important role in renal IR injury.[10] Previous studies showed that miR-125b and miR-223-5p are related to primary renal cell carcinoma.[11,12] Because miRs are tissue specific, we hypothesized that miR-125b and miR-223-5p might be a potential biomarker in renal IR injury. To the best of our knowledge, miR-125b and miR-223-5p have not been studied in renal IR injury. Bardoxolone methyl (BM) is a synthetic oleanane triterpenoid. Late-phase studies of BM in chronic kidney failure treatment still continue.[13] It has been shown that BM repairs ischemic ARF functionally and pathologically.[14] However, its exact mechanism is yet unclear.

We examined functional and histopathologic effect of BM on miR-21, miR-223-5p, and miR-125b in experimental renal IR injury.

Methods

This study was conducted in Dumlupinar University Experimental Research Department after obtaining local ethic board approval. This study was done in compliance with the Guidelines for the Care and Use of Laboratory Animals published by the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council.

Animals

Wistar-Albino rats (12-16 wk old, weighing 300-350 g) were used in the present study. All rats were kept in polycarbonate cages at 22°C temperature under a cycle of 12 h of light and 12 h of darkness. Rats were fed at will with fresh water and standard rat food.

Chemical agent

BM (methyl 2-cyano-3,12-dioxooleana-1,9(11) dien-28-oate, CDDO-Me) was purchased from Selleck Chemicals (Houston, TX).

Experimental study design

Rats were randomized into three groups: (group 1 [c]) rats underwent laparotomy but not IR; (group 2 [IR]) rats underwent 60 min of ischemia followed by 120 min of reperfusion[15]; (group 3 [IR + BM]) rats underwent 60 min of ischemia followed by 120 min of reperfusion. In addition, 1 mL of 20 mg/kg of BM was administered orally with gavage 5 h before IR.[14]

Surgical procedures

Rats were anesthetized with 10 mg/kg xylazine hydrochloride (Rompun, Bayer, Istanbul, Turkey) and 70 mg/kg ketamine (Ketalar, Pfizer, Istanbul, Turkey) and then placed on homoeothermic tables to maintain a body temperature of 37.1°C. Abdominal wall was sterilized with povidone iodine. Abdomen was incised and both renal arteries and vein were reached.

Both renal peduncles were clamped with atraumatic vascular clamp (Vascu Stop Bulldog Clamp, Istanbul, Turkey) for 60 min. After 60 min of ischemia, clamps were released and left for 120 min of reperfusion. Abdominal wall was closed and monitored for 120 min of reperfusion period. Blood samples were taken into polypropylene tubes from abdominal artery of each rat at the end of 120 min. Later on, nephrectomy was performed on rats and rats were decapitated. Blood samples were centrifuged at 1500 × *g* for 4 min at +4°C, and serum samples were stored in polypropylene tubes at −80°C. Renal tissue samples were rinsed with cold heparinized phosphate-buffered saline to remove blood cells and clot. A part of each tissue sample was fixed with 10% buffered formalin for histopathologic and immunohistochemical measurement. In addition, a sample of each tissue was placed in polypropylene tubes and stored for biochemical and molecular analysis at −80°C.

Biochemical analysis

Preparation of renal tissue homogenates

Renal tissue was mixed with cold solution (50 mmol/L phosphate buffer, pH 7.40) and homogenized with mechanic homogenizer (Analytik Jena SpeedMill PLUS, Jena, Germany) for biochemical analysis. Mixtures were centrifuged at 10.000 × *g* for 15 min at +4°C.

Measurement of serum urea level

Serum urea levels were measured using Beckman Coulter AU680 analyzer (Beckman Coulter, Miami, FL). Serum urea levels are reported as milligrams per deciliter.

Measurement of total antioksidan status, total oksidan status, and oxidative stress index values

Tissue total antioksidan status (TAS) and total oksidan status (TOS) values were measured with commercial reagents (Rel Assay Diagnostic, Gaziantep, Turkey) using Beckman Coulter AU680 analyzer. This method automatically measures the method developed by Erel.[16] TAS values are expressed as Trolox equivalent per milligram of protein. TOS values are reported as H_2O_2 equivalent per milligram of protein. For calculating oxidative stress index (OSI), which is a percentage ratio of TOS value to TAS value, millimole value of TAS unit was changed to micromole value as in TOS value.[17]

miR analysis

Total RNA isolation and real-time PCR

The kidney was divided into small pieces in trizol solution by a lancet on ice. Total RNA was isolated from the control and experiment rat kidney tissue groups with TRIzol reagent (Invitrogen, Waltham, MA) according to the manufacturer's protocol and quantitated with a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA). miR complementary DNA was synthesized by TaqMan miR Reverse Transcription kit according to the manufacturer's procedure (ThermoFischer Scientific). Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed with StepOne RT-PCR instrument using miR-223-5p, miR-21, and miR-125b specific primers to analyze the miR expression. Relative quantification of miR-223-5p, miR-21, and miR-125b was analyzed by RT-PCR using TaqMan Universal Master Mix II, noUNG, miR qPCR profiling kits. U6 was used as rat endogenous controls.

Histopathologic examinations

Renal tissue samples were fixed in 10% formalin embedded into paraffin blocks and sectioned at 4 lm. Samples were stained with hematoxylin and eosin and then evaluated in a light microscope (BX51; Olympus, Tokyo, Japan) by a pathologist who was blind to the study groups. Study groups were scored according to the degree of renal injury.[18] This scoring method is defined in Table 1.

Table 1 – Histopathologic renal damage criteria according to Jablonski *et al.*[18]

Score	Criteria
0	Normal
1	Necrosis of individual cells
2	Necrosis of cells in adjacent proximal convoluted tubules, with survival of surrounding tubules
3	Necrosis confined to the distal third of the proximal convoluted tubule with a band of necrosis extending across the inner cortex
4	Necrosis affecting all three segments of the proximal convoluted tubule

We used *in situ* tunnel to evaluate renal apoptosis. Formalin-fixed tissues were deparaffinized in xylene and rehydrated using graded concentrations of ethanol in water. Deparaffinization was performed using three changes of xylene. Then samples were rehydrated with descending levels of alcohol series, soaked in water for 5 min, and deparaffinized. Apoptotic DNA fragmentations (ApopTag Peroxidase In Situ Apoptosis Detection Kit; Millipore, Billerica, MA) were identified according to the manufacturer's procedure. Olympus BX51 light microscope was used for examination. Tunel-positive cells were counted in areas chosen randomly. Apoptotic index was defined as a percentage of apoptotic cells.

Statistical analysis

The miR expression analysis of the findings has been made with ΔΔ*CT* method and quantitated with a computer program. The comparison of the groups has been performed with "volcano plot" analysis from "RT2 Profiles PCR Array data analysis," which is assessed statistically using the "Student *t*-test."

Repeated measures analysis of variance and *post hoc* Tukey test were used for repeated measures of spike frequency and amplitude values. One-way analysis of variance and *post hoc* Tukey test were used to compare the spike frequency and amplitude values of the different dose groups. $P < 0.05$ was considered statistically significant. The statistical analyses were performed with the statistical package program SPSS version 20.0 (SPSS Inc., Chicago, IL).

Results

In the present study, it was found that urea levels were statistically significantly higher in IR and IR + BM groups compared with the control group ($P < 0.001$). Urea levels were statistically significantly decreased in the IR + BM group compared with the IR group ($P = 0.001$). TAS was significantly decreased in the IR group compared with the control group ($P = 0.001$). TOS was found statistically significantly increased in the IR group compared with the control group ($P = 0.001$) and IR + BM group ($P = 0.001$). OSI was significantly decreased in the control and IR + BM groups compared with the IR group (Table 2).

miR-223-5p was increased by 1.26-fold in the control group, 10.36-fold in the IR group, and 6.95-fold in the IR + BM group compared with rat endogenous controls. Significant difference in mir-223-5p was not identified among groups. miR-125b was increased by 1-fold in the control group, 536.8-fold in the IR group, and 3-fold in the IR + BM group compared with rat endogenous controls. miR-125b was found significantly increased in the IR group compared with rat endogenous controls ($P < 0.05$). miR-125b was observed significantly decreased in the IR + BM group compared with the IR group. miR-21 was increased by 16-fold in the control group, 2842.82-fold in the IR group, and 8.52-fold in the IR + BM group compared with rat endogenous controls. miR-21 was significantly increased in the IR group than that in the rat endogenous controls ($P < 0.001$). miR-21 was found significantly

Table 2 – Comparison of biochemical and histologic data among experimental groups.

Parameters	Control	IR	IR + BM
Urea (mg/dL)	47.83 ± 2.49	86.83 ± 5.56*	72.66 ± 4.58*,α
TAS (Trolox Eq/mg protein)	1.85 ± 0.09	1.42 ± 0.23*	1.60 ± 0.24
TOS (H_2O_2 Eq/mg protein)	3.10 ± 0.60	8.65 ± 3.44*	3.26 ± 0.82α
OSI (arbitrary unit)	0.25 ± 0.07	0.92 ± 0.15*	0.20 ± 0.05α

Control = control group; IR = ischemia–reperfusion group; IR + BM = bardoxolone methyl + ischemia–reperfusion group; TAS = total antioksidan status; TOS = total oksidan status; OSI = oxidative stress index.
*$P < 0.001$ vs control.
α$P < 0.001$ vs IR.

decreased in the IR + BM group than that in the IR group ($P < 0.05$; Fig. 1).

Histologic findings

As can be seen in Figure 2, there were no inflammatory changes in proximal and distal tubules of Bowman Capsule and glomerular structure was normal. No significant histopathologic changes were identified in the control group.

Many histopathologic changes such as mild capillary congestion in glomerulus, injury on brush borders, significant necrosis on proximal convoluted tubule, and deterioration of structure were identified in the IR group. In addition, mild cellular necrosis and tubular dilatation were observed in the group treated with BM, and there was significant recovery in renal tissue compared with the IR group. Also histopathologic

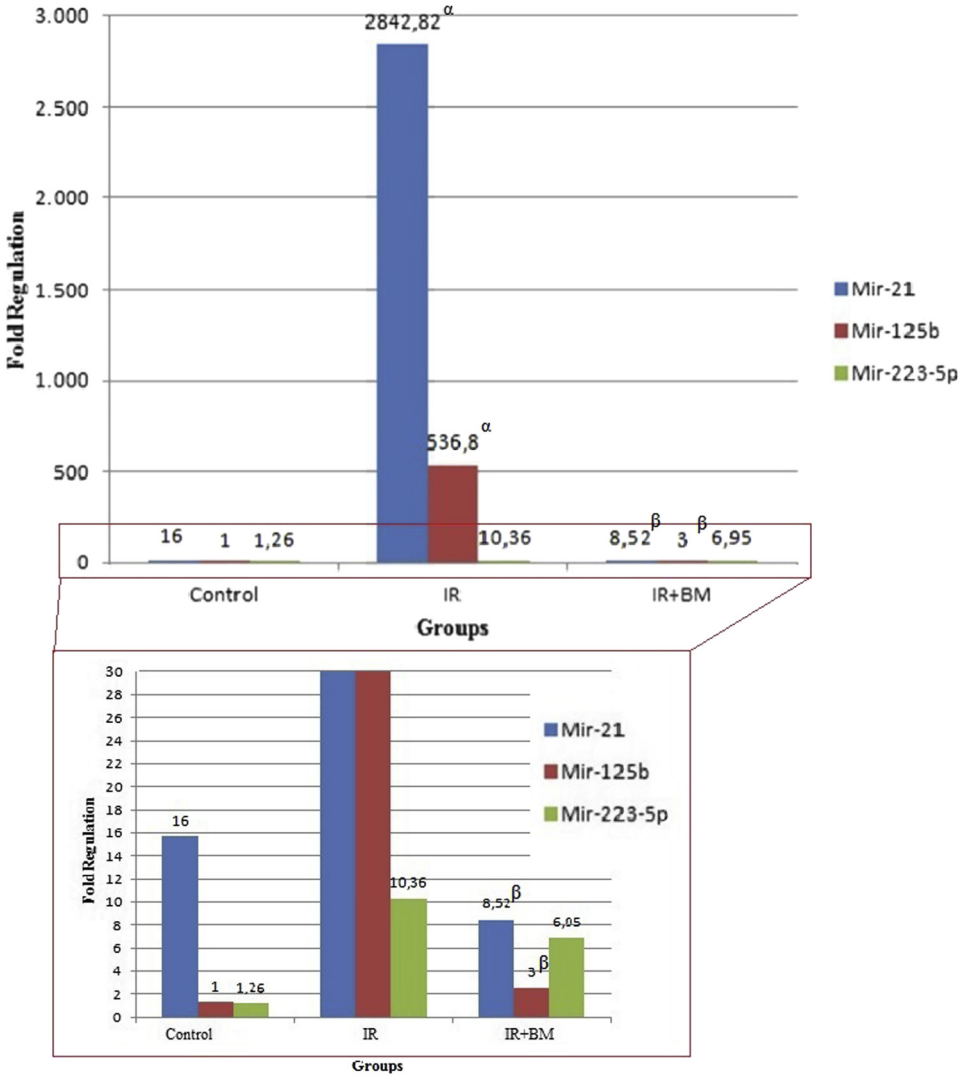

Fig. 1 – Comparison among groups of microRNA levels. Magnification is applied for better understanding of values of control and IR + BM groups. Control = control group; IR = ischemia–reperfusion group; IR + BM = bardoxolone methyl + ischemia–reperfusion group. α: It was significantly increased in IR than in rat endogenous controls ($P < 0.001$). β: It was significantly decreased in IR + BM than in IR ($P < 0.05$). The miR expression analysis of the findings has been made with the ΔΔ*CT* method. The comparison of the groups has been performed with volcano plot analysis, from RT2 Profiles PCR Array data analysis, which is assessed statistically using the Student *t*-test. (Color version of figure is available online.)

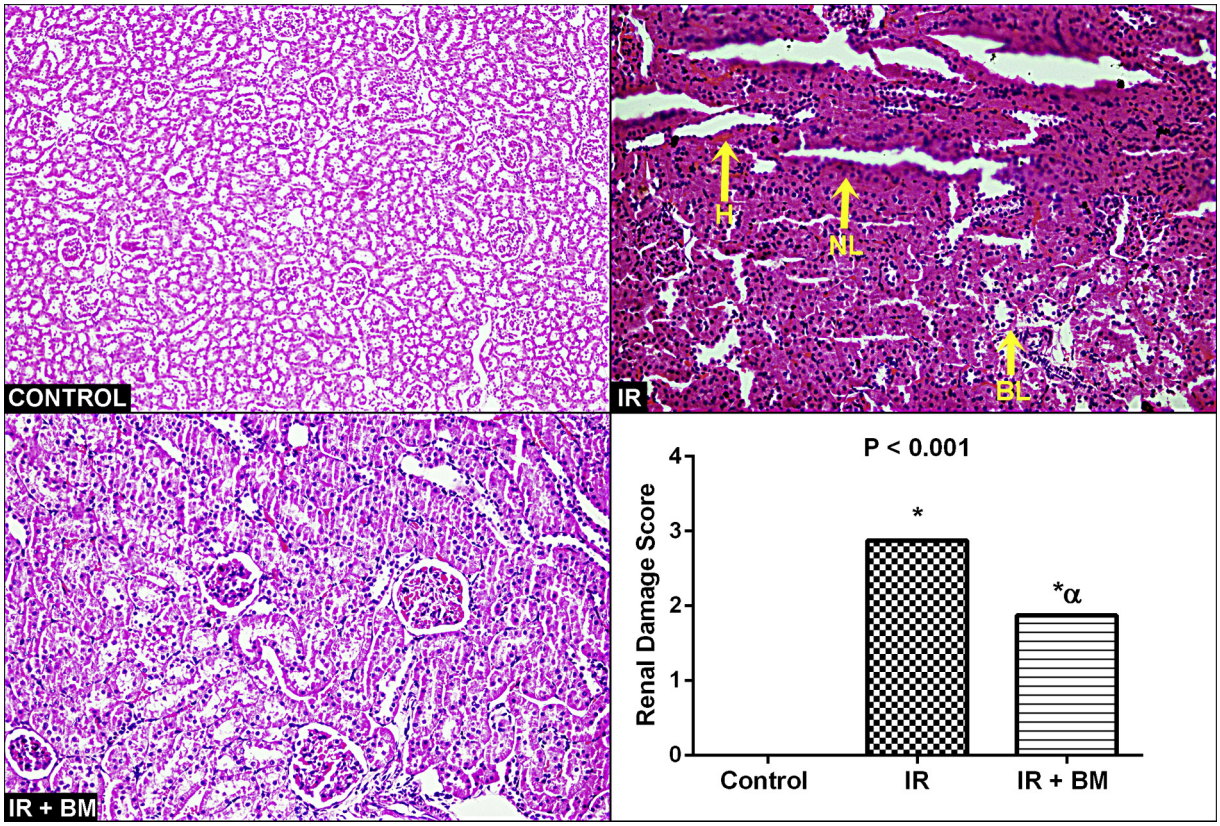

Fig. 2 – Representative photomicrographs of PAS-stained kidney tissue in the experimental groups. IR = ischemia–reperfusion group; BM + IR = bardoxolone methyl + IR group. BL: Representative histologic microphotographs of kidney tissues in the assay groups. Hematoxylin and eosin staining (×100 magnification) showed that there was no change in the control rats. Significant hemorrhage, loss of brush borders, and loss of nuclei in glomeruli were observed in the IR group (yellow arrows). The "BL" denotes brush border loss, "H" denotes hemorrhage, and "NL" denotes nuclei loss. The microscopic appearance of renal tissue samples was near normal in the BM group. P value shows the differences between all groups (one-way analysis of variance test). *P < 0.001 compared with the control group; ᵅP < 0.001 compared with the IR group (*post hoc* Tukey test). A P value <0.05 was considered statistically significant. PAS = periodic acid-Schiff stain. (Color version of figure is available online.)

score was improved significantly in the BM + IR group compared with the IR group ($P < 0.05$) (Fig. 2).

Apoptosis findings

Tunel-positive cell count was significantly higher in the IR group than that in the control group. Tunel-positive cell count was significantly increased in the IR injury group than that in the control group. Tunel-positive cell count was significantly lower in the IR + BM group ($P < 0.05$; Fig. 3).

Discussion

The main findings of our study are that miR-21 significantly increased in IR injury. We showed for the first time that miR-125b is increased in renal IR injury. miR-125b might be a potential diagnosis and treatment target in renal IR injury. Also, we demonstrated BM significantly reduced miR-21 and miR-125b in renal IR model and made functional and histologic recoveries in kidney.

Because of renovascular anatomy of kidney and high energy consumption of renal tubular epithelia, kidney is very sensitive to hypoxia and ischemic injury.[19] Hypoxia has a major role in IR injury; it creates a response that includes serial complex molecular mechanism in cells and tissues.[20] Recently, many studies confirm that miRs have an important regulatory role in hypoxic response.

Recently, the relation between IR injury and miR-21 attracted attention. In accordance with our study, Godwin *et al.*[8] and Wei *et al.*[21] showed that the expression of miR-21 increases in IR injury. It has been shown that miR-21 has a major role in myocardial fibrosis and remodeling of cardiac tissue in IR injury.[22,23]

Mechanism of IR injury is complex and most of the injury during reperfusion is related to oxygen radicals.[24] In accordance with the literature, we showed that TOS and OSI activities were significantly increased in the IR group in our study. Reactive oxygen species (ROS) was shown to increase activation of miR-21b by stimulating hypoxia-inducible factor (HIF)-1α in hypoxic tissue.[25,26] Also, HIF-1α was identified in renal tubular epithelial cells in renal ischemia and after

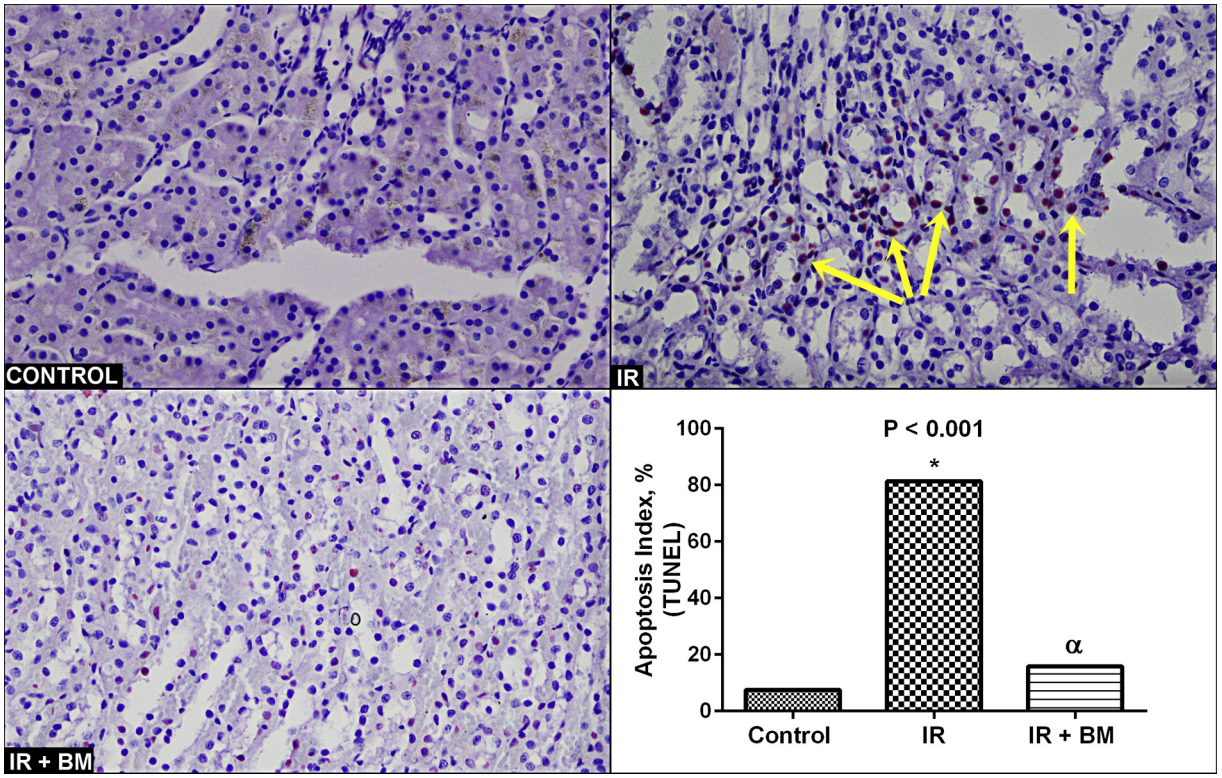

Fig. 3 – Examination of cellular apoptosis in kidney tissues in the assay groups. tunel staining (×200 magnification) showed that there was minimal apoptosis in control rats. Significant increase in the apoptosis was observed in the IR group. The positive tunel reaction is visible as dark brown spots in the cells (yellow arrows). The BM significantly reduced the apoptotic index. Apoptosis in the kidney tissue was examined using the tunel method. The number of tunel-positive cells per 100 cells was calculated from randomly selected fields. The apoptotic index was calculated as the percentage of apoptotic (positive stained) cells. *P* value shows the differences between all groups (one-way analysis of variance test). *$P < 0.001$ compared with the control group; $^{\alpha}P < 0.001$ compared with the IR group (*post hoc* Tukey test). A *P* value < 0.05 was considered statistically significant. (Color version of figure is available online.)

reperfusion.[27] It has been shown that HIF-1α might increase activation of miR-21 in hypoxic conditions.[26] It also has been shown that miR-21 is the hypoxia-related miR in humoral tissues.[26,28] In our IR group, increase of miR-21 might be related to increase of ROS because of ischemia and HIF-1α stimulating miR-21.

Previous studies identified miR-125b and miR-223 in primary renal cell carcinoma.[11,12,29] Because miRs are tissue specific, we hypothesized that miR-125b and miR-223 might be a potential biomarker in renal IR injury. In conjunction with our study, miR-125b increase was shown after ischemic stroke. Also, upregulation of miR-125b was identified as a result of acute cerebral ischemia. It has been shown that increase of miR-125b in early phase of ischemic stroke has a diagnostic value for ischemic stroke.[30,31] Also, miR-125b was shown to increase in hypoxia.[26] Likewise, oxidative stress increase was shown to augment expression of miR-125b.[32] Increase of miR-125b in the renal ischemic group of our study might be because of hypoxia or oxidative stress caused by ischemia.

We did not identify significant difference in miR-223 in renal IR injury. miR-223 was reported to be associated with brain and liver IR models; however, there is no report of association with renal ischemia in the literature. This might be related to tissue- and cell-specific nature of miRs.

In accordance with our study, previous studies have shown that ROS increases in the early phase of IR injury.[33] ROS increase in IR was shown to be related to severity of necrosis, apoptosis, and early inflammation in renal tissue.[34] Effective IRI treatment strategies must include anti-inflammatory regulatory and antioxidant defense systems.

BM is a synthetic triterpenoid derived from the natural product of oleanolic. In accordance with the literature, we showed that BM reduces an OSI and increases total antioxidant activity.[35] Previous studies reported that nuclear factor κB (NF-κB) has a major role in the regulation of miR gene transcription. BM is similar to cyclopentenone prostaglandin, which is a potent NF-κB inhibitor.[36,37] Also, it has been shown that NF-κB activity increases gene transcription of miR-125b.[38] NF-κB was shown to activate miR-21 directly.[39,40] Decrease of miR-21 expression was shown with decrease of NF-κB transcriptional activity.[41] The reducing effect of BM treatment on miR-125b and miR-21 might be because of the reducing effect of BM on NF-κB activity. Protective effects of BM were shown in ischemic ARF in accordance with our findings.[14,42] Also, a recent phase 2 randomized controlled, double-blind study reported lower serum creatinine levels with BM.[43]

Conclusions

The expression of miR-125b increases in IR injury. miR-125b might be a potential novel marker that might have a use in renal IR injury. In addition, BM reduces miR-21 and miR-125b expressions in IR injury and makes functional and histopathologic recoveries.

Limitations

One might think that miR levels might be affected from degradation or environmental impacts. It has been shown that half-life of miR-125b is 225 h and miR-21 levels are stable for 70 h.[44]

In addition, it has been shown that miRs can be used for RNA analysis for 5 y if they are stored in 20 min at −80°C after they are sampled from tissues with IR injury.[45] We stored samples at −80°C after sampling them from tissues. These samples were evaluated 3 mo later after storing at −80°C. Also to rule out further concerns, we performed miR expression assays via TaqMan Master Mix for each miR sequence. In this analysis, lower C_t values indicate high amounts of targeted nucleic acid, whereas higher C_t values mean lower (and even too little) amounts of target nucleic acid. Typically, C_t values <29 cycles show abundant nucleic acids, and C_t values >38 cycles indicate minimal amounts, and possibly an infection or environmental contamination. According to our TaqMan method results, average C_t values were low for each miR sequence (16, 21, and 24 for U6; U6 is an endogenous control for real-time quantitation of miR using TaqMan miR assays).[46] Accordingly, low C_t values in our study exclude any probability of a degradation problem or environmental impact.

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