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The effects of TRPM2, TRPM6, TRPM7 and TRPM8 gene expression in hepatic ischemia reperfusion injury

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Abstract. – OBJECTIVE: Mammalian transient receptor potential melastatin (TRPM) channels are a form of calcium channels and they transport calcium and magnesium ions. TRPM has eight subclasses including TRPM1-8. TRPM2, TRPM6, TRPM7, TRPM8 are expressed especially in the liver cell. Therefore, we aim to investigate the effects of TRPM2, TRPM6, TRPM7, and TRPM8 gene expression and histopathologic changes after treatment of verapamil in the hepatic ischemia-reperfusion rat model.

MATERIALS AND METHODS: Animals were randomly assigned to one or the other of the following groups including sham (n=8) group, verapamil (calcium entry blocker) (n=8) group, I/R group (n=8) and I/R- verapamil (n=8) group. TRPM 2, 6, 7, 8 gene expression level was assessed by Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) and histopathologic changes were determined by the hematoxylin and eosin (HE) examination.

RESULTS: The expression level of TRPM 2, 6, 7, and 8 genes was significantly higher in ischemia-reperfusion (I/R), verapamil, IR-verapamil groups compared to sham group. The p-values were 0.0024, < 0.0001, 0.0002, 0.006 for TRPM2, TRPM6, TRPM7, and TRPM8, respectively. Severe necrotic, degenerative differentiations and severe hemorrhagic areas were observed in hepatocytes from IR group. Also, moderate necrotic and degenerative differentiations and moderate hemorrhagic areas were observed in hepatocytes from IR-verapamil group.

CONCLUSIONS: This is the first study reporting an association between the expression level of TRPM 2, 6, 7, 8 in a hepatic ischemia-reperfusion rat model. Moreover, TRPM 2, 6, 7, 8 affect hepatic ischemia-reperfusion.

Key Words
Ischemia, Reperfusion, TRP, TRPM, Verapamil, Calcium entry blocker.

Introduction

Ischemia is the lack of oxygen and nutrients and the cause of mechanical obstruction in several tissues[1]. Hepatic ischemia and reperfusion is a serious complication and cause of cell death in liver tissue[2]. The resulting ischemic liver tissue injury increases free intracellular calcium. Intracellular calcium has been defined as an important secondary molecular messenger ion, suggesting calcium's effective role in cell injury during ischemia-reperfusion, when elevated from normal concentrations. The high calcium concentration leads to depressed compensative vasodilation following a period of vasoconstriction. All these reasons, including the lack of oxygen and nutrients start the pathway of apoptosis and necrosis[3]. Verapamil, which is a member of the dihydropyridine family, is a calcium entry blocker[4]. Two studies have reported that the addition of verapamil in perfusate and the iv/ip pretreatment showed beneficial effects in ischemia-reperfusion injury prevention on animal models. Moreover, verapamil has a vital effect on calcium homeostasis and could reduce ischemia and reperfusion in the injured liver[5,6]. The calcium channel blockers are also used to improve the post ischemic myocardial function[7] and the survival of ischemic skin flaps[8].

The mammalian transient receptor potential (TRP) channels are a form of calcium channels and they transport calcium, magnesium and trace metal ions[9]. Therefore, TRPs cause changes in calcium, magnesium and trace metal ions concentrations. These contributions are pivotal for several physiological processes including pheromone signaling, temperature sensation, cell proliferation,

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and muscle contraction[10]. Almost every cell type expresses TRPs which are localized in the plasma membrane[9]. TRP superfamily includes TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPML (mucolipin), TRPP (polycystin), TRPA (ankyrin transmembrane protein) and TRPN (nom PC-like)[11]. The family of TRPMs has eight subclasses including TRPM1-8 and they are expressed in different cells. TRPM2, TRPM6, TRPM7, TRPM8 are expressed especially in the liver cell[12]. Moreover, TRPM7 has been found to be involved in delayed neuronal death after ischemia[13].

There is no study about the association between the application of calcium entry blocker aiming at preventing ischemia-reperfusion liver injury and TRPM calcium channels. To understand the roles of TRPM calcium channels in liver tissue, it is important to explain the pathogenesis of ischemia-reperfusion. Therefore, we aim to investigate the effects of TRPM2, TRPM6, TRPM7, and TRPM8 gene expression and histopathologic changes after treatment of the calcium entry blocker in the hepatic ischemia-reperfusion rat model.

Materials and Methods

In vivo Experiment

The experiments were carried out on 32 male Wistar rats (average body weight 225±25 g) housed in an environmentally controlled room (24°C to 26°C temperature, 50% to 60% moisture rate) with a 12:12 h light: dark cycle, and kept on commercial rat chow and tap water *ad libitum*. The experimental protocol was in accordance with EU directive 2010/63 for the protection of animals used for scientific purposes. This investigation was also approved by the local Committee on the Ethics of Animal Experiments of the Mustafa Kemal University, Hatay, Turkey (2014/5-9).

Experimental Design

The animals were randomly assigned to one or other of the following groups including sham, verapamil (calcium entry blocker), I/R group and I/R- verapamil groups.

Sham Group: rats were subjected to glucose pretreatment and surgical procedures, except for the induction of liver ischemia, but including liver resection (n=8).

Verapamil (Calcium entry blocker) group: rats were treated with verapamil as a pretreatment (1.25 mg/kg) and to none of them ischemia-reperfusion (n=8) was applied.

Ischemia-reperfusion group (I/R): none of the rats were treated with any substance. Ischemia was applied to the left hepatic artery and the portal vein by a clamp for 60 min after the laparotomy. Within 60 min after reperfusion, relaparotomy was performed on all group members, and liver and blood of the subjects were isolated (n=8).

Ischemia-reperfusion/calcium entry blocker (I/R-verapamil): as pretreatment, verapamil (1.25 mg/kg) was administered orally to the rats 30 min before anesthesia. Ischemia was applied to the left hepatic artery and the portal vein by a clamp for 60 min after the laparotomy. Within 60 min after reperfusion, relaparotomy was performed on all group members, and liver and blood were isolated (n=8).

Surgical Procedures

Rats were anesthetized with intraperitoneal injections of xylazine (12 mg/kg) and ketamine (80 mg/kg), and placed in a supine position on a temperature-controlled heating table, maintaining the body temperature in the range of 36.5-37.5°C. Rats were allowed to breathe spontaneously during surgery. For the preparation of the liver, abdominal skin was shaved and sterilized with 70% of ethyl alcohol. After midline laparotomy and subcostal incisions, the liver was carefully mobilized from all the ligamentous attachments. The left hepatic artery and the portal vein were clamped for 60 min using an atraumatic vascular clamp for complete ischemia of the median and left hepatic lobes. Then, the edges of the abdominal incision were approximated to each other and covered by a piece of gauze soaked with warm isotonic saline to prevent the undue loss of body fluids. After the removal of the clamp, the median and left hepatic lobes were removed and the abdomen was properly irrigated with isotonic saline. During IR periods, the abdomen was covered with a plastic wrap to minimize fluid loss via evaporation. At the end of ischemia, the abdomen was closed with continuous stitches using Vicryl (Ethicon Endo-Surgery, Inc., Cincinnati, Ohio, USA). 3/0 sutures and the animals were returned to their cages. After 60 min of reperfusion, animals were anesthetized with intraperitoneal injection of ketamine and xylazine (80 and 12 mg/kg, respectively) and were sacrificed by exsanguination via right ventricular puncture, then blood and histological samples were taken.

Hepatic Histopathologic Evaluation

After ischemia-reperfusion, the liver specimens were removed and placed in 10 % of formalin. Then, tissues were embedded in paraffin after alcohol and xylene according to the standard protocol. Five mi-

Table I. Primers used for TRPM gene expressions.

Primers		
β-Actin	Left	5'-CCC GCG AGT ACA ACC TTC T-3'
β-Actin	Right	5'-CGT CAT CCA TGG CGA ACT-3'
TRPM2	Left	5'-AAT TTG CTC ATC GCC ATG TT-3'
TRPM2	Right	5'-GAT CTG GTC TGT GTG CTC CTG-3'
TRPM6	Left	5'-GCA AGA ACT GGC TTT CCG TG-3'
TRPM6	Right	5'-ATC CGG GTC CTC TTG CAT CT-3'
TRPM7	Left	5'-AGA CGC TTT CCG ATA GAT GG-3'
TRPM7	Right	5'-CTA TCC AGG ATT TCT GGG ACA T-3'
TRPM8	Left	5'-GCC CAG TGA TGT GGA CAG TA-3'
TRPM8	Right	5'-GGA CTC ATT TCC CGA GAA GG-3'

crometer thick sections were stained with hematoxylin and eosin (HE) for light microscopy studies in a double-blind manner. The hepatic damage was evaluated with a histopathologic scoring system. The assessment was expressed as the sum of the individual score grades from 0 (no findings), 1 (mild), 2 (moderate), to 3 (severe) for each of the following six parameters: cytoplasmic color fading, vacuolization, nuclear condensation, nuclear fragmentation, nuclear fading and erythrocyte stasis.

RNA Isolation and Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) and Gene Expression Analysis

Total RNA was isolated by using commercially available kits (RNeasy Mini Kit, Qiagen, Hilden, Germany). cDNA was obtained through the use of the reverse transcription assay kit (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Foster City, CA, USA). Briefly, 10X reverse transcriptase buffer (2 µl), dNTP mix (0,8 µl), 10X RT random primers (2 µl) (Table I), AMV reverse transcriptase (1 µl), mRNA and RNase free water (4,2 µl) were mixed to obtain cDNA. The reaction mixture was incubated at 25°C for 10 min and 37°C for 120 min for reverse transcription and heated at 85°C for 5 min to inactivate AMV reverse transcriptase. The cDNA obtained was stored at −20°C until tested. Then, cDNA was denatured at 95°C for 10 min, at 95°C for 15 s annealed at 60°C for 1 min (TRPM2, TRPM6, TRPM7, TRPM8), and extended at 95°C for 3 min and at 72°C for 30 s. The reaction mixture was subjected to 40 cycles of PCR following an initial 15 s denaturation step at 95°C.

The mRNA expressions of each of TRPM and β-actin as housekeeping gene were analyzed by quantitative reverse transcriptase PCR using the Rotor-Gene Q (Qiagen, Hilden, Germany).

Statistical Analysis

Data were analyzed by using GraphPad Prism 5 program (GraphPad Software Inc., La Jolla, CA, USA). Data were expressed as mean values ± standard error of the mean (SEM). For normally distributed data, the one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison post-hoc test was used to test significant differences. p-values < 0.05 were considered as statistically significant.

Results

Data of TRPM 2, 6, 7, 8 Gene Expressions

Data were analyzed by using Rotor Gene Q Series Software and the numbers of positive chambers were corrected to estimate the true number of copies. Detected numbers were used to determine the number of copies in the original sample. Beta Actin was used as the housekeeping gene for normalization of the expressions. The expression levels of TRPM genes were compared in two manners; the expression levels of TRPM genes without treatment were named as "basal expression level." The basal expression levels in sham group, verapamil group, I/R group and I/R-verapamil group were compared. Verapamil and sham groups were considered as control group. The expression levels of TRPM genes, with and without treatment, in each liver tissue were compared within it.

Data were shown to represent the Average ΔCT± SEM. TRPM2 expression was highest in the I/R group (Average ΔCT ±SEM: 1.190±0.04) and the values of Average ΔCT were 1.036±0.02, 0.983±0.05 and 1.048±0.02 for sham, verapamil and I/R-verapamil groups respectively (Figure 1). The level of TRPM6 gene expressions was

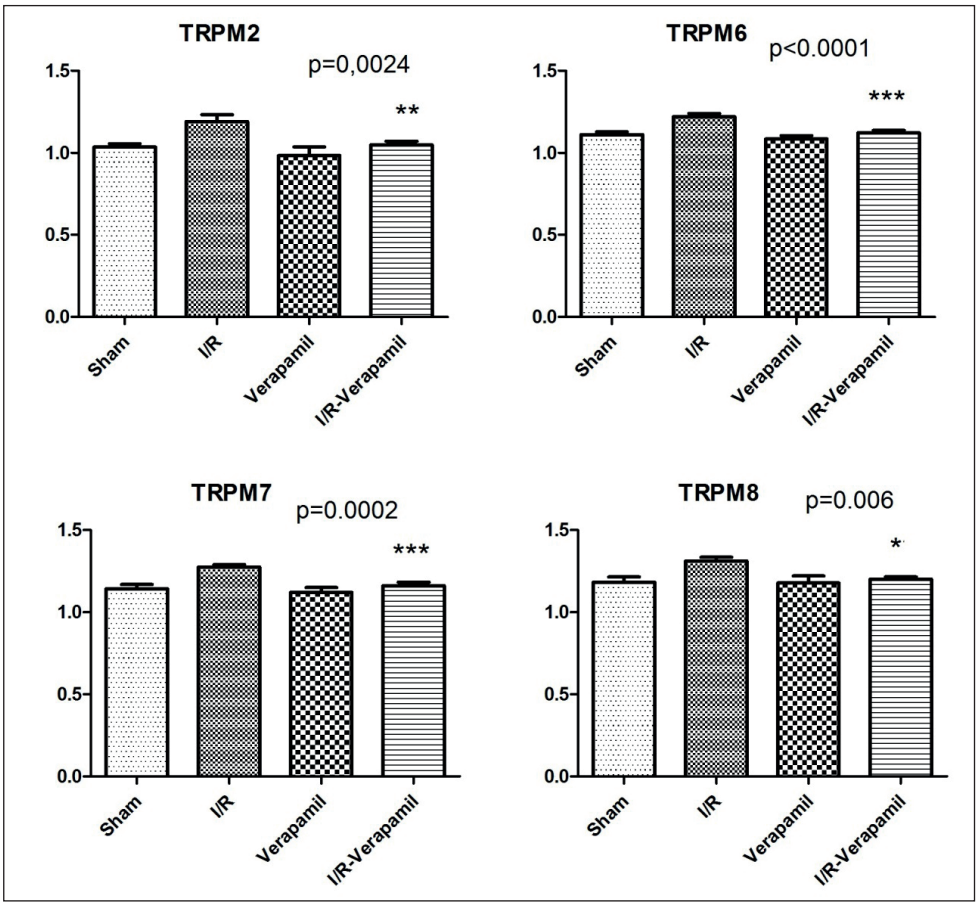

Figure 1. Level of TRPM2, TRPM6, TRPM7, TRPM8 gene expression in sham, ischemia-reperfusion (I/R), verapamil, ischemia-reperfusion with the treatment of verapamil (I/R-verapamil) from liver tissue in Wistar rats.

1.111±0.02, 1.219±0.02, 1.085±0.02, 1.121±0.02 for sham, I/R, verapamil and I/R-verapamil groups, respectively (Figure 1). For the TRPM7 gene, the value of expressions was 1.143±0.03, 1.274±0.02, 1.121±0.03, 1.162±0.02 in hepatocytes from sham, I/R, verapamil, I/R-verapamil groups, respectively (Figure 1). TRPM8 expression was modest in verapamil group (Average ΔCT ±SEM: 1.178±0.04) and other expression levels were 1.181±0.04, 1.312±0.02, 1.201±0.01 for sham, I/R and I/R-verapamil groups (Figure 1). The *p*-values were 0.0024, < 0.0001, 0.0002, 0.0060 for TRPM2, TRPM6, TRPM7 and TRPM8, respectively. Statistically significant differences in gene expression were determined relative to control rats by an ANOVA test. According to Bonferroni's multiple comparison post-test, the values of mean differences, *t*-value, *p*-value, confidence interval differences (95%) were assessed in Table II.

Table II. Comparison of the gene expressions in groups (ΔCT ±SEM).

	Sham	I/R	Verapamil	I/R-Verapamil	*p*
TRPM2	1.036±0.02	1.190±0.04[a,**]	0.983±0.05	1.048±0.02[b,**]	0.0024[a,b]
TRPM6	1.111±0.02	1.219±0.02[a,***]	1.085±0.02	1.121±0.02[b,***]	<0.0001[a,b]
TRPM7	1.143±0.03	1.274±0.02[a,***]	1.121±0.03	1.162±0.02[b,***]	0.0002[a,b]
TRPM8	1.181±0.04	1.312±0.02[a,*]	1.178±0.04	1.201±0.01[b,*]	0.006[a,b]

ΔCT±SEM; *: $p<0.05$; **: $p<0.01$; ***: $p< 0.001$; [a]: I/R vs. Sham, [b]: I/R-Verapamil *vs*. I/R.

Histopathologic Evaluation

Histopathologic evaluation was shown in Table III. Furthermore, the findings of the histopathologic analysis showed necrosis, hemorrhage and necrosis in liver tissues. The changes became more severe among shame, I/R, verapamil and I/R-verapamil groups. Liver cells in sham and verapamil groups were normal for the HE examination (0: no findings). Severe necrotic, degenerative differentiations and severe hemorrhagic area were observed in liver tissues from the IR group. Moderate necrotic and degenerative differentiations and moderate hemorrhagic area were also observed in hepatocytes from IR-verapamil group (Figure 2).

Severe necrotic, degenerative differentiations and severe hemorrhagic areas were observed in hepatocytes from IR group. Also moderate necrotic and degenerative differentiations and moderate hemorrhagic areas were observed in hepatocytes from IR-verapamil group.

Discussion

In ischemia, the restriction of nutrient and oxygen leads to changes in cell metabolism, including especially the efflux of calcium, which causes necrotic or apoptotic cell death. In reperfusion, the reentering of oxygen and nutrients renovates the mitochondrial membrane potential and the so rapid mitochondrial calcium uptake[14]. Therefore, calcium plays an important role in ischemia-reperfusion, homeostasis of cells and in calcium ion channels such as TRPM.

However, the mechanisms of ischemia-reperfusion are totally unclear. The present study revealed that the level of TRPM2, TRPM6, TRPM7, TRPM8 gene expressions was affected by ischemia-reperfusion and this effect can be related to the concentration of calcium ion changes during ischemia and reperfusion. The second member of the TRPM family of cation channels is TRPM2. The activation of TRPM2 causes increases in intracellular calcium levels. The level of calcium concentration may serve signaling roles in the secretory cells (e.g., hepatocyte) through the release of cytokines, neurotransmitters, and insulin. Furthermore, this can induce apoptotic and necrotic cell death under oxidative stress in extreme situations including ischemia-reperfusion[16]. Previous studies have shown that verapamil has a protective effect for hepatic, renal and cardiac ischemia-reperfusion injuries[17]. Erdogan et al[6] reported that verapamil treats ischemia-reperfusion injury. Unfortunately, the interaction among calcium channel blockers, ischemia injury and reactive oxygen species are not clear.

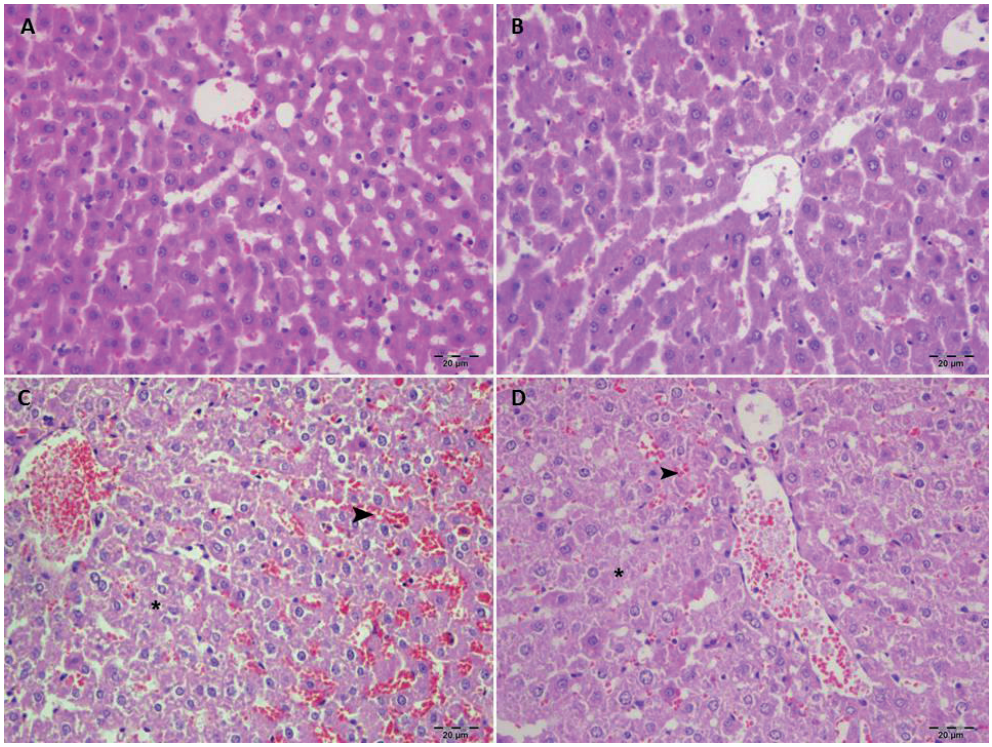

Figure 2. Photomicrographs of liver tissue HE stain (magnification X100). Panel A represents Sham group; Panel B represents verapamil group; Panel C represents severe necrotic, degenerative differentiations and severe hemorrhagic area in I/R group; Panel D represents moderate necrotic and degenerative differentiations and moderate hemorrhagic area in verapamil-treated I/R group.

Table III. Histopathologic scores in groups.

	Sham	I/R	Verapamil	I/R-verapamil
1. sample	0	2	0	2
2. sample	0	3	0	2
3. sample	0	3	0	2
4. sample	0	3	0	1
5. sample	0	3	0	2
6. sample	0	3	0	2
7. sample	0	3	0	1
8. sample	0	3	0	2

0: no findings (normal), 1: mild, 2: moderate, 3: severe.

Numerous studies have demonstrated that brain, heart and kidney ischemia-reperfusions are associated with the level of calcium concentration and TRPM ion channels, especially TRPM2[18].

In *in vitro* and *in vivo* studies, TRPM2 is the cause of detrimental in brain ischemia[19-21]. There are three studies on heart ischemia-reperfusion and TRPM2. The first study[22] reported that TRPM2 and the activation of Poly (ADP ribose) polymerase 1 (PARP-1) were involved in oxidative stress-induced cardiomyocytes death. The second study[23] reported that TRPM2 is involved in Tumor Necrosis Factor-alpha (TNF-α) mediated cardiomyocytes death. The third study[24] reported that TRPM2 protects cardiomyocytes from ischemia-reperfusion injury. Scholars[25,26] showed that TRPM2 cation channels have a harmful function in ischemia-reperfusion and thus the level of TRPM2 expression was increased after 48 h of renal reperfusion in calcium channel blocker (verapamil). Our data is in agreement with the mentioned literature findings and we suggest that TRPM2 may be used as a therapeutic target for the ischemia-reperfusion treatment (Figure 1). TRPM6 and TRPM7 are closely related, and have an intrinsic kinase domain. Both of them have the same electrophysiological properties, regulate magnesium and calcium homeostasis and are expressed by each mammalian cell[27]. Touyz[28] reported that vascular TRPM6 or TRPM7 may contribute to the delivery of cellular magnesium which plays a role in hypertension. TRPM7 has an effective role in brain ischemic injury, as the seventh TRPM in cell and mitochondrial membranes[29]. Wey et al[30] have shown that the concentration of extracellular calcium and magnesium decreases during transient brain ischemia which is related with TRPM7 gene expression.

Furthermore, TRPM7 has been found to be involved in delayed neuronal death after ischemia[13]. In the *in vivo* experiment[31], the kidney was dysfunctional in the reflow injury. Meng et al[32] said that suppression of TRPM7 in renal tubules may reduce dysfunction in early renal ischemia-reperfusion injury. Dusmez et al[26] has demonstrated that TRPM6 and TRPM7 genes were expressed in the renal system. We show that TRPM 6 and TRPM7 are associated with liver ischemia and the mentioned gene expression level may protect against liver ischemia (Figure 1).

TRPM8 was first cloned from prostate tissue and it is a calcium-permeable channel protein[33]. Nocchi et al[34] demonstrated that TRPM8 shows the greatest expression level following hydrogen peroxide (H^2O^2) treatment, and the oxidative stress can modulate TRPM8 expression. Our work also demonstrates that the level of TRPM8 expression is decreased by the treatment of verapamil before ischemia-reperfusion.

Two studies[35,36] have showed that the treatment of carvacrol had no effects on hepatic histology in the hepatic tissue of ischemia-reperfusion. But, we found that the treatment of verapamil had positive effects, and the data of sham and verapamil groups were similar on hepatocytes. According to our histopathological evaluation, liver tissues were severe necrotic, apoptotic, degenerative differentiations and severe hemorrhagic in IR group, but ischemia-reperfusion with the treatment of verapamil liver tissues were moderate necrotic, apoptotic and degenerative differentiations and moderate hemorrhagic.

Conclusions

We first showed that the levels of TRPM2, TRPM6, TRPM7, and TRPM8 expressions increase in ischemia-reperfusion from hepatocytes of rats. Moreover, the mentioned genes expression levels close to normal levels in the treat-

ment of calcium entry blocker (verapamil) before ischemia-reperfusion from rat hepatocytes. So, we propose that verapamil can block TRPM2, TRPM6, TRPM7, and TRPM8 gene expression. However, we need more information on which mechanism affects TRPM2, TRPM6, TRPM7 and TRPM8 gene expression.

Author Contributions

T.B., F.K., HE and H.G. contributed to conception and design, as well as to for critical revision of the article; T.B and F.K wrote the paper. H.G and H.E contributed to data collection, analysis, and interpretation.

Confict of interest

The authors do not report any proprietary or commercial interest in any mentioned product or concept discussed in this article.

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