



Effects of Hyperthermia on TRPV1 and TRPV4 Channels Expression and Oxidative Markers in Mouse Brain

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Abstract

Heat stress increases the core body temperature through the pathogenic process. The pathogenic process leads to the release of free radicals, such as superoxide production. Heat stress in the central nervous system (CNS) can cause neuronal damage and symptoms such as delirium, coma, and convulsion. TRPV1 (Transient Receptor Potential Vanilloid1) and TRPV4 genes are members of the TRPV family, including integral membrane proteins that act as calcium-permeable channels. These channels act as thermosensors and have essential roles in the cellular regulation of heat responses. The objective of this study is to examine the effect of general heat stress on the expression of TRPV1 and TRPV4 channels. Furthermore, oxidative markers were measured in the brain of the same heat-stressed mice. Our results show that heat stress leads to a significant upregulation of TRPV1 expression within 21–42 days, while TRPV4 expression decreased significantly in a time-dependent manner. Alterations in the oxidative markers were also observed in the heat-stressed mice.

Keywords Central nervous system · Heat stress · TRPV channels · Oxidative markers

Introduction

Although all living organisms endeavor to achieve homeostasis, certain physical and psychological events can alter this dynamic equilibrium (Selye 1936; Levine 1991; Chrousos and Gold 1992). Heat stress as a natural hazard and a major extracellular stimulation (Park et al. 2004), influences many physiological functions and behaviors in animals (Harikai et al. 2003). Increase in body temperature not only has been proposed as an inducer of many physiological and pathophysiological responses such as hypoglycemia and systemic metabolic disorders but is also known as the main risk factor of heatstroke, which is defined by an increase in

the core body temperature above 40 °C and results in a malfunction of the central nervous system (CNS) (Kregel et al. 1990; Moran et al. 1999; Bouchama and Knochel 2002). Besides increasing mortality rate, heat stroke can alter the mental status even after positive therapeutic interventions (Albukrek et al. 1997; Bouchama et al. 2007). Many studies suggest that thermal stress alters brain structures and functions, leading to neuronal loss and circuit modification, neurological defects and accelerated brain dysfunction (White et al. 2003; Sinha 2007; Kim et al. 2013). In addition, hyperthermia can affect attention, memory, and information processing (Yang and Lin 1999; Xiao et al. 2007; Sun et al. 2012). In this regard, it has been observed that thermal stress can cause cognitive impairment in both experimental animals and humans (Hancock and Vasmatazidis 2003; Gaoua 2010). During acute hyperthermia, connections in the temporal, frontal and occipital lobes are reduced and increased around the limbic system (Sun et al. 2013). At the cellular level, increases in temperature stimulate changes in gene expression in a variety of organs and tissues (Jian et al. 2008; Yan et al. 2009). These increases cause protein denaturation and disruption of critical cellular processes that lead to apoptosis and cell death (Matsuki et al. 2003). Given the fact that mitochondria and plasma membrane are

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thermosensitive, disturbance of cellular signaling mechanisms, mitochondrial dysfunction, and electrochemical depolarization have been observed in neuronal cells that were exposed to heat (Kiyatkin 2007; White et al. 2007). Global warming and the worldwide increase in the frequency and intensity of heat waves have an impact on the incidence of heat-related disease, leading to a rising concern among researchers (Rooney et al. 1998; Easterling et al. 2000). Nowadays, there is no clear therapeutic strategy to treat heat-induced mental abnormalities so getting in-depth knowledge of the molecular mechanisms that regulate cellular responses to heat is mandatory to find new methods to minimize such symptoms (Zeller et al. 2011; Sharma et al. 2012). Finding new candidate genes involved in adaptation to heat stress and cell surviving may determine the nature of the cellular response to heat stress (Lin et al. 1997; Moseley 1997). It is shown that heat can alter cellular Ca^{2+} homeostasis by modifying Ca^{2+} entrance from both internal stores and the extracellular environment (Harikai et al. 2003). TRPV1 and TRPV4 are members of the TRP ion channel family and are gated by certain lipophilic molecules, extracellular protons and stimuli such as heat or osmotic pressure changes. These nonselective cation channels are permeable to Na^+ and K^+ and highly permeable to Ca^{2+} . TRPV channels are expressed ubiquitously within the body and also in the brain, which are known as hot flashes. TRPV1 channel plays a pivotal role in the brain by participating in the transduction of pain, heat or osmotic stimuli (Kauer and Gibson 2009). As a heat sensor, TRPV1 is activated at a temperature range of $> 42^\circ\text{C}$. Furthermore, TRPV1 also interacts with endocannabinoids and participates in other basic physiological processes (Christie et al. 2018). On the other hand, the TRPV4 channel is also a heat sensor, with an activation temperature of around 37°C . Apart from the heat, TRPV4 is activated by hypo-osmotic shocks, heat stress, kinases and some lipophilic ligands (Vincent et al. 2009). Although constant research is done to unravel the role of these channels in the CNS, we are still far from having the whole picture (Kauer and Gibson 2009), particularly concerning their roles as thermosensors. TRPV1 channels open at temperatures of around and above 42°C (Caterina et al. 1997) and have been detected in the spinal cord and in several brain structures. Although the patterns of TRPV1 expression are well determined, its functions in these structures are not well understood or remain controversial (Menigoz and Boudes 2011). What is known though, is that sensory neurons from the TRPV1 knockout mice show thermosensitivity deficiencies (Caterina et al. 2000), suggesting that TRPV1 could act as a Ca^{2+} signaler of the heat response. TRPV4 is also expressed in the brain and activated around at temperature ranging from 24 to 37°C (Shibasaki et al. 2015). As mentioned before, TRPV4 is a known osmotic sensor that mediates osmotic pressure changes in the brain (Mizuno et al. 2003) and it is shown that in primary

cultures of rat neocortex it is strongly expressed in astrocytes (Benfenati et al. 2007). However, as for TRPV1, it remains unclear how this channel is activated under temperature changes and pathologic processes like brain edema produced by hyperthermia. (Sharma 2006; Hoshi et al. 2018). On the other hand, ROS have noxious effects on ion channels (Naziroğlu 2012). Recent studies showed that TRP channels can be targeted and modified by ROS (Hara et al. 2002; Wehage et al. 2002). It has been found that TRPV1 and also TRPV4 respond to oxidative stress by raising their activity. Also some TRP channels contain a molecular sensor for oxidative stress (Naziroğlu 2012). Plus, it is known that the balance between the cellular antioxidant capacity and the reactive oxygen species (ROS) plays a critical role in cellular physiology and brain development (Parellada et al. 2012). ROS has neurotoxic effects and induce oxidative damages, as seen in many neurodegenerative disorders like Alzheimer disease (AD), which are connected with oxidative damages (Kanamaru et al. 2015). With all this in mind, the following study aims to examine the changes in TRPV1 and TRPV4 expression under hyperthermia and the effect of heat stress on the brain oxidative markers. Consequently, changes in TRPV1 and TRPV4 gene and protein expression were assessed using a heat stress on model mice and the levels of oxidative stress were determined by measuring the total antioxidant capacity (TAC), superoxide dismutase (SOD), glutathione peroxidase (GPx) and the biomarker of lipid peroxidation process, malondialdehyde (MDA).

Materials and Methods

Ethics

All experiments were performed in accordance with the Iran National Committee of Ethics in Biomedical Research and approved by the Experiment Ethics Committee of the *Tabriz University of Medical Sciences* (Approval Number IR.TBZMED.VCR.REC.1397.212) and the *Tabriz University of Medical Science* guidelines for the care and use of laboratory animals.

Animal Heat Exposure and Heat Stress Protocol

7 to 10-week-old male mice ($n=50$) from the C57BL/6J strain were used to generate the heat model. Mice were randomly divided into five groups ($n=10$): (1) Control (animals not exposed to heat), (2) 7 days heat stress, (3) 14 days heat stress, (4) 21 days heat stress and (5) 42 days heat stress. Five animals were housed in the same cage with unlimited access to water and food. They were kept under a constant room temperature of $23 \pm 1^\circ\text{C}$ and a humidity of $60 \pm 10\%$ and a 12 h light/dark cycle. To generate the model, animals

from groups 2 to 5 were transferred one by one in a heat chamber maintained at 43 °C and 60 ± 10% humidity for 20 min once a day during 7, 14, 21 or 42 days according to the group. Body weight was measured before and after the heat shock and mice were returned to their cages.

Brain Tissue Preparation

One hour after last heat exposure, mice were anesthetized with ketamine and xylazine (50 and 10 mg/kg respectively) and decapitated. Whole brains were removed, frozen immediately in liquid nitrogen and stored at –80 °C for later use.

RNA Extraction and cDNA Synthesize

RNA was extracted from each brain sample using the TRIzol™ Reagent following the manufacturer's protocol. To prevent RNA degradation, all types of equipment were kept RNase free and homogenizers were treated with 1% DEPC water for 24 h and then autoclaved (Thermolyne). RNA quality was assessed by running the samples in a 1% agarose gel electrophoresis and concentration was measured with a UV spectrophotometer (Picodrop, UK). All RNA samples had 260/280 nm ratios ≥ 1.8. Samples were reverse-transcribed into cDNA (37 °C for 2 h) using the TAKARA kit (Japan).

qRT-PCR Analysis

Quantitative real-time PCR (qRT-PCR) measurements were performed using a Corbett Rotor-Gene (Corbett Life Sciences, Germany) 6000 Real-Time PCR system with the following temperature protocol: 95 °C for 5 min, followed by 40 cycles at 95 °C for 30 s, 63 °C for 20 s, and 72 °C for 20 s. Each reaction contained: 5 µL SYBR® Green Real-Time PCR Master Mixes (TAKARA, Japan), 3.6 µL H₂O, 1 µL target cDNA, and 0.2 µL of the specific forward and reverse primers. The following primers were used: TRPV1-forward: TGCTGGTGTCTGTGGTACTG, TRPV1-reverse: GCTGGAATCCTCGGGTGTAG desired band size (114 bp) and TRPV4-forward: CGCCTTCGTAGGGATCGTTG and TRPV4-reverse: GCATCGTCCGTCCTCCAC desired band size (161 bp). Samples were normalized to the expression of the mouse-endogenous control gene GAPDH (forward: TGCAGTGGCAAAGTGGAGAT and reverse: GTCTCGCTCCTGGAAGATGG) desired band size (160 bp). Relative expression changes were calculated using the $\Delta\Delta Cq$ method.

Western Blotting

100 mg of brain tissue was homogenized in 500 mg of RIPA lysis buffer (0.05 mmol/L Tris (pH 8) 150 mmol/L NaCl, 1% EGTA, 1% SDS) and 1% anti-protease cocktail (Roche) was used to extract protein and was incubated for 30 min at

4 °C, then centrifuged for 20 min at 1200 rpm at 4 °C (bo SW14rfoil). Protein concentration was determined using the Bio-Rad Protein Assay kit and measuring absorbance at 595 nm. Samples were stored at –80 °C until use. To detect protein expression, samples were diluted in a 1:1 ratio in loading sample buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.005 bromophenol blue) and boiled 5 min to denature proteins. Samples were separated on a 10% polyacrylamide gel at 150 V and transferred to a PVDF membrane for 2 h at 90 V. PVDF membranes were first incubated with blocking buffer (BSA 3%) for 2 h in TBSI then with the primary antibodies for TRPV4 (ab39260) (1:500), TRPV1 (VR1 (E-8): sc-398417) (1:100–1:1000) and B-actin (ab8227) (1:500) overnight which were diluted in 1% (w/v) skim milk in TBS-T [0/05 (v/v) Tween-20 in Tris-buffered saline]. Afterward, the membrane was incubated with an anti-rabbit secondary antibody after 3 times washing for 1 h at room temperature. Blots were enhanced for visualization using chemiluminescence (ECL) detection kit (Pierce, Rockford, IL), scanned and quantified using the Image J 1.62 software (National Institute of Health, Bethesda, Maryland, USA). Actin bands were used as a loading control to normalize protein amounts (Yousefi et al. 2017).

Immunohistochemistry

The animals were sacrificed, and brain tissues were obtained and fixed overnight in 10% formalin. Then formalin-fixed and paraffin-embedded tissues were cut (coronal 5 µm sections) immunohistochemically stained was applied through the streptavidin–biotin method with antibodies against TRPV1 and TRPV4. The tissues were deparaffinized using xylene and then dehydrated in ethanol. To retrieve antigen, microwave irradiation was applied. After cooling at room temperature, the tissues were incubated with primary antibodies overnight at 4 °C TRPV4 (1:200 ab39260) and TRPV1 [1:50 VR1 (E-8): sc-398417]. The sections were incubated with the biotinylated secondary antibodies and peroxidase-conjugated streptavidin for 1 h at room temperature, after three-time washes in TBS. The diaminobenzidine was used to visualize, and eventually the sections were counterstained with hematoxylin, after repeating the washing cycle. The mean optical density of protein was conducted by NIH image j (Bethesda, US).

Reactive Oxidative Stress Markers

Oxidative stress was assessed by measuring the content of glutathione peroxidase (GPx), superoxide dismutase (SOD), malondialdehyde (MDA), and total antioxidant capacity (TAC) in all samples using several biochemical assays as follows.

GPx Activity

GPx activity was measured using the RANSEL kit (Randox Labs, Crumlin, UK) as described in (Paglia and Valentine 1967). Oxidation of glutathione (at a concentration of 4 mmol/L) is catalyzed by GPx via cumene hydroperoxide. Glutathione reductase leads to the conversion of oxidized glutathione to reduced form with the oxidation of NADPH to NAD⁺. Absorbance at 340 nm at 37 °C was measured by spectrophotometry.

SOD Activity

SOD activity was determined using the RANSOD kit (Randox Labs, Crumlin, UK) as described in Breinholt et al. (1999). Using the supernatant, SOD activity was assessed by measuring absorbance at 505 nm with a spectrophotometer. For this method xanthine and xanthine oxidase were used to produce free super oxidase radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5 phenyl tetrazolium chloride (ITN) to generate a red dye (formazan). Substrates concentration for xanthine and ITN were 0.025 mmol/L and 0.05 mmol/L, respectively. SOD activity was measured as the degree of inhibition of the reaction.

Malondialdehyde (MDA)

MDA levels were determined using TBARS (thiobarbituric acid reaction substances) in the homogenized tissue, as described in Esterbauer and Cheeseman (1990). Samples were mixed with 1 mL of trichloroacetic acid 10% and thiobarbituric acid 67%. Samples were boiled for 15 min. N-butanol was added to the supernatant at a 2:1 ratio. The mix was centrifuged for 10 min at 1000 rpm. Pink stained substance as TBARS was determined with a spectrophotometer by reading absorbance at 532 nm. Results were calculated as nmol TBARS/mg protein.

TAC

TAC levels were determined using the RANDOX total antioxidant status kit (Randox Labs, Crumlin, UK). Briefly, ABTS® (2, 2'-azino-di-[3-ethylbenzthiazoline sulphonate]) was incubated with peroxidase (metmyoglobin) and H₂O₂ to produce the free radical cation ABTS®^{*+}. This has a relatively stable blue-green color, which can be measured at 600 nm. The idea of this assay is that antioxidants present in the sample decrease ABTS®^{*+} production and the measured absorbance decreases proportionally to their concentration.

Data Analysis and Statistics

Data were analyzed with the Statistical Package for Social Science, version 16.0 (SPSS, Chicago). D Agostino–Pearson test was used as a normality test. Data are shown as mean ± SEM (standard error of the mean). One-way ANOVA (Tukey), and Kruskal–Wallis tests were used to assess significant differences of parametric and non-parametric between groups respectively. *p* values smaller than 0.05 were considered significant. To assess the correlation of the relative quantifications, Spearman and Pearson tests were used to analyze non-parametric and parametric data, respectively.

Results

Effect of Heat Stress on TRPV1 and TRPV4 Gene Expression Levels

TRPV1 and TRPV4 expression levels were obtained with qRT-PCR. According to findings, each melt curves of TRPV1, TRPV4, and GPDH showed specific PCR products for each gene. Further, various diluted cDNAs were used in order to determine primers efficiency (Fig. 1). agarose results endorsed PCR findings (Fig. 2). Based on the D Agostino–Pearson test, TRPV1 passed the normality test (*p* value = 0.087) but not TRPV4 (*p* value = 0.001) so, ANOVA and Kruskal–Wallis were used to test significance respectively. As shown in Table 1 our results showed no significant alterations in the expression of the TRPV1 gene

Table 1 qRT-PCR analyses of RNA expression levels of TRPV1 and TRPV4 in the control and heat-stressed mice groups

Markers	Control	7 Days	14 Days	21 Days	42 Days
TRPV1	6.2E–6 (1.2E–6)	4.1E–6 (6.2E–7)	5.1E–6 (7.7E–7)	4.0E–6 (7.8E–7)	4.2E–6 (6.2E–7)
TRPV4	1.7E–3 (2.9E–4)	5.9E–4 (6.9E–5)	4.8E–4 (5.9E–5)	2.1E–4 (3.1E–5)***	1.2E–4 (1.7E–5)***

Data show no significant changes in the RNA expression levels of the TRPV1 gene compared to the control group (*p* value > 0.05). In TRPV4, data reveal significant time-dependent down regulation in 21 and 42 days group with compared to control (*p* value < 0/001)

****p* < 0.001 as compared to control group

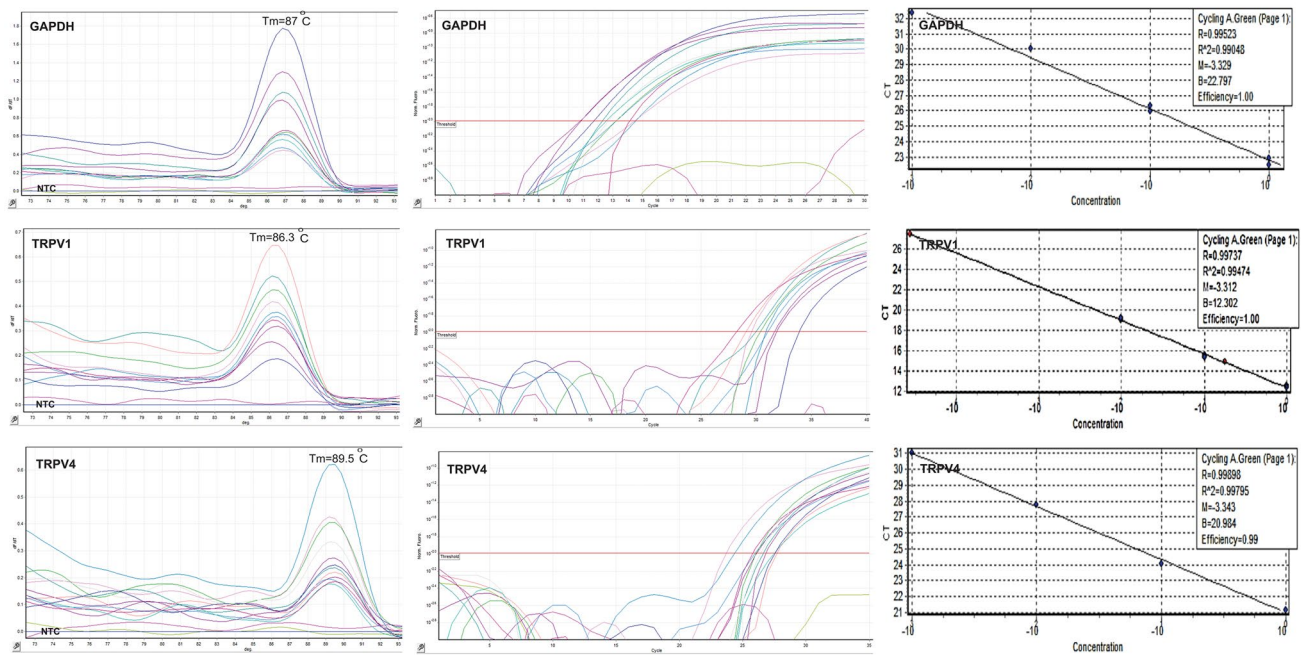


Fig. 1 qRT-PCR details for TRPV1, TRPV4 and GAPDH: melt, amplification, and standard curves which endorse PCR results, and used primer efficiency

among heat-treated groups compared to the control one (p value 0/05), but a time-dependent down-regulation of the TRPV4 gene expression occurred which was significant during 21 and 42 day groups. (p value 0/05 (Table 1, Fig. 3).

Effect of Heat Stress on TRPV1 and TRPV4 Protein Expression Levels

Western blot is used to assess the protein levels of both TRPV1 and TRPV4. According to D Agostino–Pearson test, both TRPV1 and TRPV4 were non-parametric (p value < 0.05) so, data were analyzed using Kruskal–Wallis and our findings showed that while TRPV1 protein expression did not change in the groups exposed which were exposed to heat for a short time of heat, it was significantly upregulated in mice exposed to heat during 21 and 42 days compared to the control animals (p value = 0.043 and < 0.001 respectively). TRPV4 protein expression was downregulated in a time-dependent manner in 21 and 42 heat-treated groups compared to control, with an almost 70% reduction in mice exposed to heat in 42 days (p value = 0.017 and < 0.001 respectively). These findings in TRPV4 protein expression mimicked those observed by qRT-PCR (Table 2; Fig. 4a, b).

Correlation of the Expression Between TRPV1 and TRPV4

Non-parametric Spearman test was performed to investigate the correlation between the TRPV1 and TRPV4 gene pairs. The results (Spearman test $p > 0.05$, $r = 0.276$) showed no significant correlation in the mRNA expression levels of these genes, but a clear significant inverse correlation in the protein expression levels (Spearman test $p < 0.001$, $r = -0.659$), showing that increases in TRPV1 expression levels are correlated with TRPV4 protein decrease. These results suggest that, although there is no relation between the mRNA levels, both proteins could be regulated in parallel in the cellular response to hyperthermia (Fig. 4b).

Effect of Heat Stress on TRPV1 and TRPV4 Immunohistochemistry in Mice Brain

Immunohistochemical analyses of the mice brain in control and heat intervention groups was performed in this study (Fig. 5). The results displayed that the cerebellum and entopeduncular nucleus showed the most TRPV1 and TRPV4 expression levels in the control group. Evaluating protein levels alteration for both TRPV1 and TRPV4 in seven distinct regions (entopeduncular nucleus, caudate-putamen, cortex, thalamus, hypothalamus, amygdala, and cerebellum) revealed that time-dependent heat stress manner enhances TRPV1 protein levels in cerebellum, cortex, the

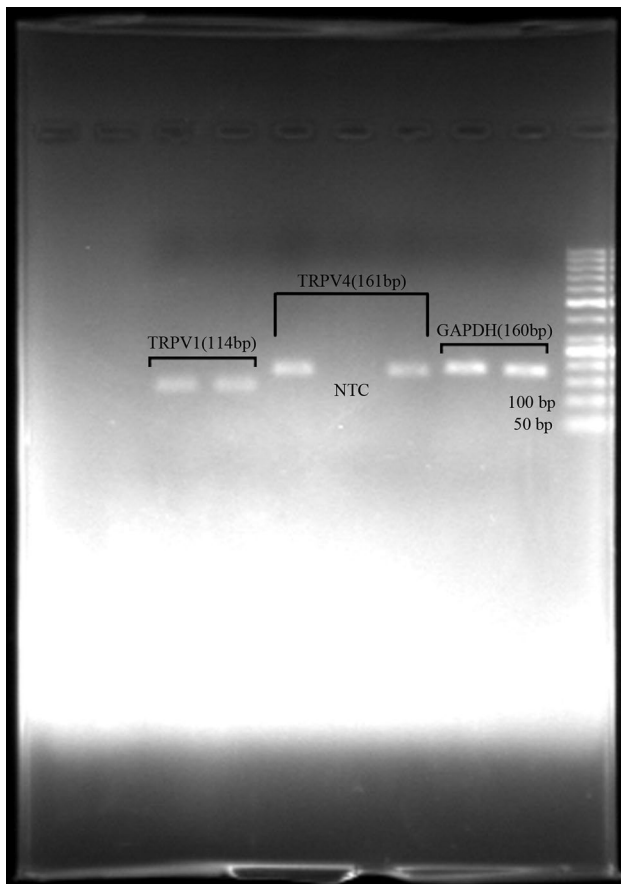


Fig. 2 Agarose gel: desired band size on agarose gel (TRPV1 114 bp, TRPV4 161 bp, GAPDH 160 bp) endorse fidelity of qRT-PCR

hypothalamus, and entopeduncular nucleus, whereas TRPV4 shows downregulation in cerebellum and cortex regions with intangible alterations in the other regions (Fig. 6).

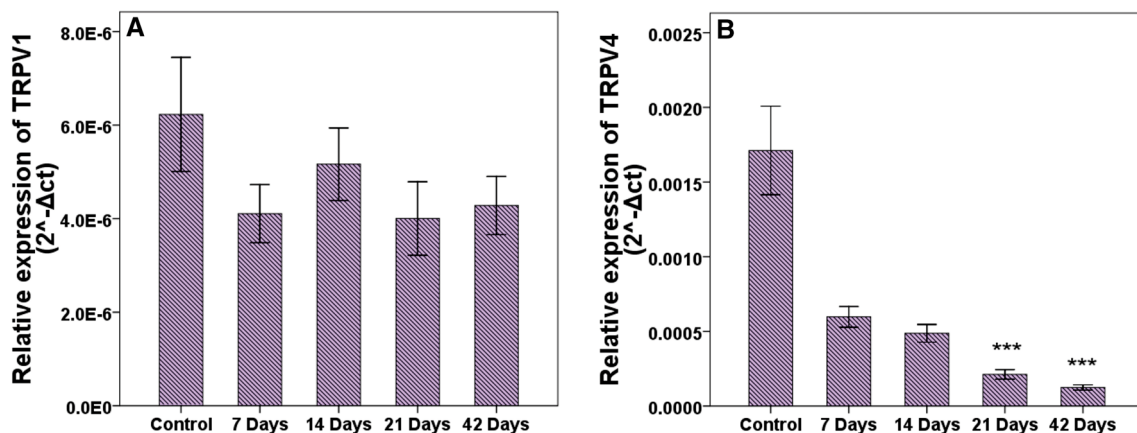


Fig. 3 Relative RNA expression of TRPV1 and TRPV4 genes: **a** qRT-PCR analyses show no significance expression of TRPV1 compared to control (p value > 0.05). **b** Time-dependent changes in TRPV4 expression levels showing the significant decrease in 21 and

Effect of Heat Stress on Oxidative Markers of Mice Brain

The obtained results show that brain levels of SOD only decreased significantly in the 21-day group compared to control ($p=0.015$), but not in the other heat-treated groups (Fig. 7a). GPx also increased significantly only in the 21-day group compared to the control animals ($p=0.009$, Fig. 7b). MDA levels appeared to be significantly increased in the 21-day group ($p=0.008$) but decreased in the following group of treatment of 42 days ($p=0.036$, Fig. 7c). Although some changes were observed in the oxidative markers, no significant alteration in the TAC level was found among groups (Fig. 7d; Table 3).

Discussion

The obtained results show that heat stress significantly increases the expression of TRPV1 protein in the brains of mice exposed to heat in 21 and 42 days. Heat exposure also affects the expression of TRPV4, decreasing its levels both at the mRNA and protein level at 21 and 42 days group. Also, for the first time as pivotal evidence present study showed involved brain regions and cell types which can contribute to TRPV1 and TRPV4 protein level changes during thermal stress. Moreover, our report also suggests an alteration of the levels of oxidative markers especially after 21 days of heat exposure.

Chronic stress is associated with pathological and psychological alterations in brain function. However, it remains unclear how heat-induced stress regulates cellular response interactions (Wang et al. 2017a, b). Ca^{2+} as an indispensable factor in signal transduction and cellular response regulator

42 days group compared to control group (p value < 0.05). (($n=9$) control group), (($n=8$) 7-day group), (($n=10$) 14-day group), (($n=8$) 21-day group), (($n=9$) 42-day group). *** $p < 0.001$ as compared to control group

Table 2 Western blot analyses of protein expression levels of TRPV1 and TRPV4 in the control and heat-stressed mice groups

Markers	Control	7 Days	14 Days	21 Days	42 Days
TRPV1	1.0 (0.00)	1.0 (0.02)	0.9 (0.01)	2.4 (0.21)*	3.4 (0.07)***
TRPV4	1.00 (0.00)	0.91 (0.01)	0.83 (0.01)	0.57 (0.05)*	0.33 (0.03)***

Protein expression levels of TRPV1 shows significant time-dependent upregulation in 21 and 42 days group with compared to control (p value < 0.05). In TRPV4 protein levels, significant time-dependent downregulation in 21 and 42 days group (p value < 0.05)

* p < 0.05 as compared to control group, *** p < 0.001 as compared to control group

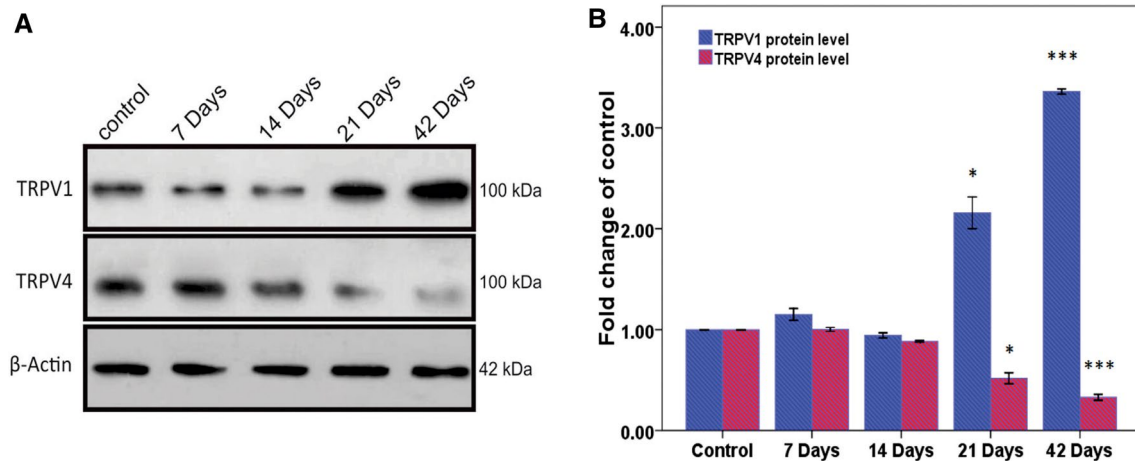


Fig. 4 a Western blot: data were obtained from three independent western blot analyses, β -actin was used as a loading control. ($n=4$ in each group). **b** Protein expression levels of TRPV1 and TRPV4 groups: TRPV1 protein levels showed significant upregulation in 21 and 42 heat intervention groups (p value < 0.05). Time-dependent

changes in TRPV4 protein levels showed a significant decrease of 21 and 42-day groups, which confirms TRPV4 gene expression data in 21 and 42-day groups. (p value > 0.05). * p < 0.05 and *** p < 0.001 as compared to control group

seems to be significant in many aspects of heat response events (Llinás 1988; Marty 1989). The TRPV1 and TRPV4 ion channels as calcium-permeable cation channels are integral membrane proteins that regulate many cell functions (Nilius and Owsianik 2011). Apart from other roles, TRPV channels are considered to be involved in neural or neuroendocrine processes. Both channels are expressed in the subfornical organ (SFO) area lacking blood–brain barriers which are considered to be the systemic osmosensing region (Ciura and Bourque 2006; Tsushima and Mori 2006; Pedersen and Nilius 2007). It seems that a combination of TRPV channels polymodal nature and stimulation sensitivity make them ideal candidates for stress response proteins that merge signaling pathways and adjust intracellular Ca^{2+} levels as a response to induced stress. Previous studies show that there is a substantial link between heat stress, both activation, and expression of TRPV1–4 channels and ROS generation. According to findings, heat stress activates these channels, and intracellular Ca^{2+} can increase subsequently. Accumulation of intracellular Ca^{2+} can affect ROS generation via distinct pathways. Initially, the entrance

of Ca^{2+} into mitochondria disrupts the respiratory chain and leads to ROS generation (Pivovarova and Andrews 2010). Second, protein kinase C (PKC) is activated by Ca^{2+} which activates nicotinic adenine dinucleotide phosphate hydrogen oxidase (NADPH oxidase) and culminates in ROS generation (Sharma et al. 1991; Nazıroğlu 2012). Third, calmodulin-kinase II (CaMKII) activation through Ca^{2+} can activate the P38MAPK pathway which results in NADPH oxidase activation (Lu et al. 2013). Furthermore, ROS products activate downstream kinases including ERK1/2, JNK, and p38MAPK pathways which leads to expression of pro-inflammatory factors such as Interleukin 6 and 8 (IL-6 and IL-8), tumor necrosis factor α (TNF- α), which act positively and enhance P38MAPK activation. On the other hand, it is shown that cysteine residues changes in both TRPV1 and TRPV4 channels by ROS products, phosphorylation via CaMKII, phosphoinositide 3-kinase (PI3K) and p38MAPK lead to activation of these channels directly (Ho et al. 2012; Pires and Earley 2017) (all linked pathways are summarized in Fig. 8 schematically).

Fig. 5 TRPV1 and TRPV4 immunohistochemical stains: Brain sections were prepared and the immunohistochemical technique was used in the brain map-indicated regions including the amygdala, caudate-putamen, cortex, entopeduncular nucleus, hypothalamus, thalamus, and cerebellum. Brown stained regions indicate the expression of TRPV1 (in the left set) and TRPV4 (in the right set). In both TRPV1 and TRPV4 immunostaining, the most stained regions are accompanied by cerebellum and entopeduncular regions respectively and the lowest stained regions are accompanied by hypothalamus in control group. But during heat stress, this ratio is changed so that, TRPV4 staining density in the cerebellum is lower than caudate-putamen, thalamus, and entopeduncular nucleus and also, TRPV1 staining density in the hypothalamus is not lowest in 14 days heat stress group

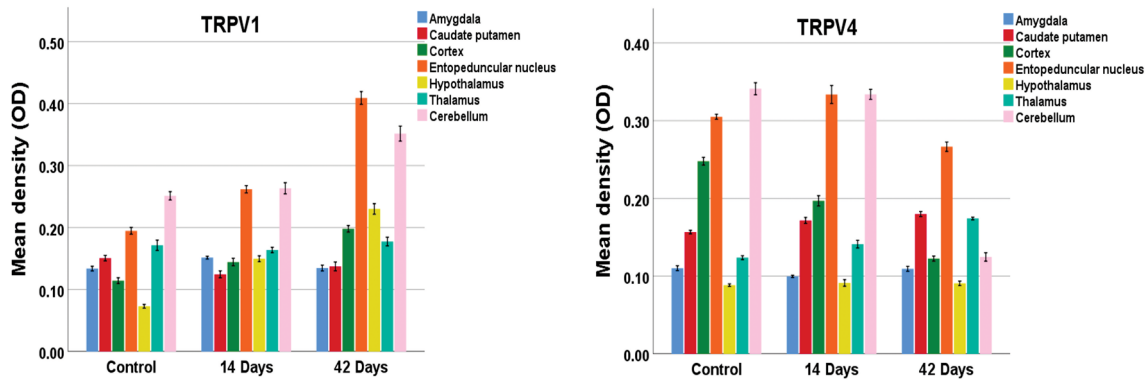
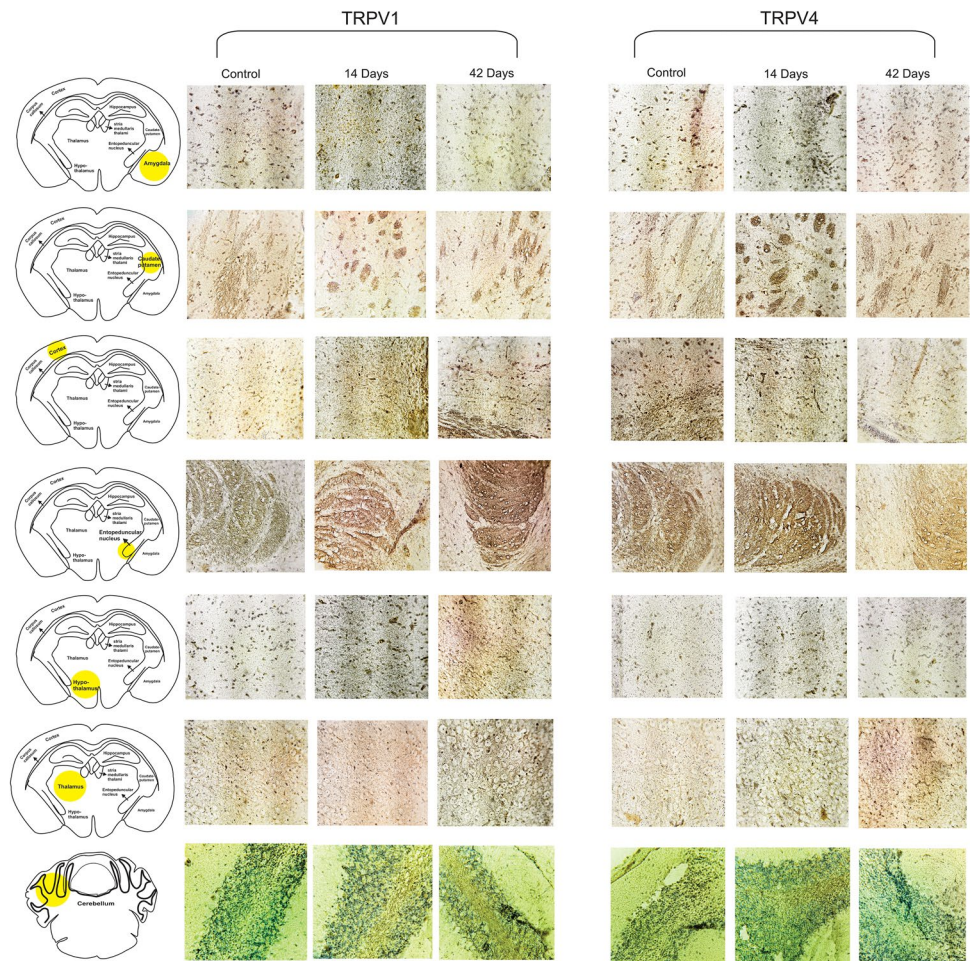


Fig. 6 Image J analyses of immunohistochemical stain: Evaluating TRPV1 and TRPV4 protein levels displayed that expression means of TRPV4 in the control group is more than TRPV1. In heat-stressed groups TRPV1 is increased in time-dependent manner in cortex,

cerebellum, hypothalamus, and entopeduncular nucleus, whereas TRPV4 shows time-dependent decrease in cortex and cerebellum [(n=3 in each group)]

In the proposed heat model, it was observed that an increase in the protein levels of TRPV1 that was time-dependent, being significant after 21 days of heat stress without changes in mRNA levels that corroborates previous

findings, in which TRPV1 protein expression is enhanced without alteration in mRNA levels through activation of P38MAPK pathway. previous findings indicated that activated P38MAPK pathway by the nerve growth factor (NGF),

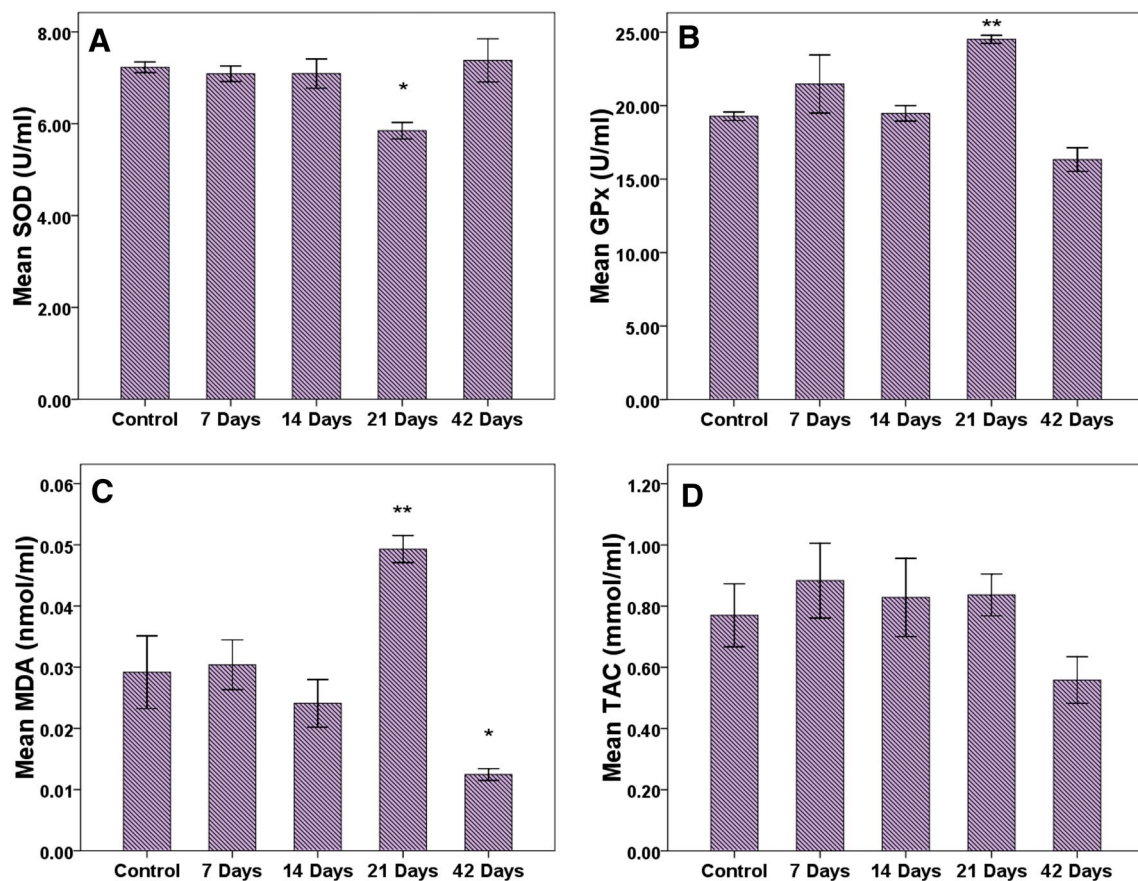


Fig. 7 Effects of heat stress on stress oxidative markers changes ($n=6$ in each group): **a** SOD levels decreased significantly in the 21-day group compared to control ($p=0.015$). **b** GPx levels increased significantly in the 21-day group compared to control ($p=0.009$). **c**

MDA levels showed a significant increase then decrease in 21 and 42 days groups ($p=0.008, 0.036$) respectively. **d** TAC levels showed no significant alteration among the groups. * $p < 0.05$ and ** $p < 0.01$ as compared to control group

Table 3 Levels of stress oxidative markers: the values are presented as mean (SEM), $n=6$ in each group)

Markers	Control	7 Days	14 Days	21 Days	42 Days
SOD (U/mL)	7.23 (0.12)	7.08 (0.17)	7.09 (0.32)	5.85 (0.18)*	7.38 (0.47)
GPx (U/mL)	19.3 (0.29)	21.5 (1.98)	19.5 (0.53)	24.5 (0.28)**	16.3 (0.80)
MDA (nmol/mL)	0.03 (0.006)	0.03 (0.004)	0.02 (0.004)	0.05 (0.002)**	0.01 (0.001)*
TAC (mmol/mL)	0.77 (0.10)	0.88 (0.12)	0.83 (0.13)	0.84 (0.07)	0.56 (0.08)

Data are expressed as mean (SEM). There is no significant alteration in TAC levels in all treated groups (p value > 0.05)

SOD superoxide dismutase, GPx glutathione peroxidase, MDA malondialdehyde, TAC total antioxidant capacity

* $p < 0.05$ as compared to control group, ** $p < 0.01$ as compared to control group

reasons activation of MAPK interacting kinase (MNK), which reacts with eIF4G (as initiation factor of translation) and phosphorylates IF4E (another initiation factor) then eventually, simplifies the translation of specific mRNAs such as TRPV1 (Ji et al. 2002; Puntambekar et al. 2005; Constantin et al. 2008; Roux and Topisirovic 2012). Our findings highlight that there may be a considerable link

between heat stress and activation of these signaling pathways which can result in TRPV1 over translation. Moreover, we concluded that TRPV4 is modulated upon heat stress, as we observed a time-dependent decrease of both its mRNA and protein levels. It remains unclear how heat stress can reduce TRPV4 expression, but it seems that the production of pro-inflammatory factors during heat stress can play a

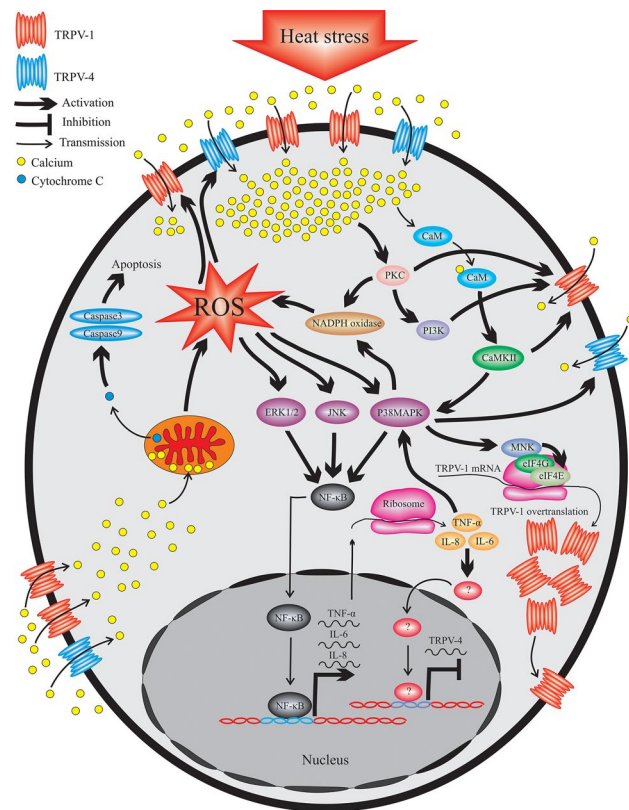


Fig. 8 Schematic diagram for the linkage between TRPV1 and TRPV4 expression levels, ROS generation, and involved pathways

crucial role in this process. As evidence proved that pre-inflammatory factors such as IL-8 and TNF- α can decrease TRPV4 mRNA and protein levels (Fusi et al. 2014; El Karim et al. 2015). On the other hand, general MAPK pathway activation via increased Ca^{2+} influx and TRPV1 noxious stimulation results in enhanced release of pro-inflammatory factors such as IL-6 and IL-8 (Zhang et al. 2007). Taking these facts into consideration do a possible explanation in which heat stress through the p38MAPK pathway regulates both channel's expression reversely (Fig. 8). This is compatible with our findings in which there is a reverse correlation between protein levels of TRPV1 and TRPV4 at different time points of thermal stress, pointing to an inverse participation of both channels in the adaptation to thermal heat. Nevertheless, further studies should be done to clarify their precise role in mediating the heat response.

It is reported that TRPV1 first was identified in both dorsal root ganglion (DRG) and trigeminal ganglion (TG), and abundantly expresses in primary sensory neurons which play a pivotal role in pain generation but function, distribution, and expression of TRPV1 channel in the brain is not vivid and it still remains unknown (Huang et al. 2014). Based on previous studies, it seems that TRPV1 expression is low in the brain generally (Kunert-Keil et al. 2006). TRPV4 is

widely expressed in the brain especially in the pyramidal neurons of hippocampal but, there are no data which present functional expression of TRPV4 (Lipski et al. 2006; Cao et al. 2009; Bai and Lipski 2010). Therefore, to investigate TRPV1 and TRPV4 protein level changes contribution in different brain regions and cell types during thermal stress, we performed immunohistochemistry assay in mice brain for the first time. Based on our findings we observed the most abundant expression of TRPV1 and TRPV4 in entopeduncular nucleus followed by caudate-putamen. We also examined their expression changes during heat stress in cortex, entopeduncular nucleus and caudate-putamen. Our results showed that time-dependent heat stress enhances TRPV1 protein expression which confirms western blot analysis in whole-brain lysate. Although the expression of TRPV1 is increased in the cerebellum, entopeduncular nucleus the hypothalamus and cortex, the expression of TRPV4 is downregulated in the cerebellum and cortex, whereas there isn't a noticeable change in the other regions. Expression of TRPV4 is downregulated in caudate-putamen and is upregulated in entopeduncular nucleus whereas there isn't a noticeable change in the cortex. These results reveal that heat stress contributes to an increase of TRPV1 protein levels without change in mRNA levels. But for TRPV4 the case is complicated. It assumes that TRPV4 is most effected by inflammatory factors pathways and has not only similar but also distinct pathways with TRPV1. Moreover, for the first time, we also measured ROS markers including SOD, GPx, MDA, and TAC to highlight the significance of ROS generation in the brain during heat stress and attempt to find a connection between both TRPV1 and TRPV4 expression changes and ROS. We found that tangible alterations in SOD, GPx, and MDA levels occur in 21 days of heat stress which show a concordant pattern with the onset of TRPV1 and TRPV4 expression changes. But surprisingly in the 42-day group, alterations are reversed dramatically compared to the 21-day group except for MDA, their alteration in the 42-day group is not notable compared to the control group. Furthermore, their correlation between together is strong and significant. It is shown that heat stress causes SOD mRNA and its cytoplasmic protein levels to decrease, whereas increases MDA level (El-Orabi et al. 2011; Belhadj Slimen et al. 2014). These findings are pursuant to our findings. Hyperthermia converts superoxide anions to hydrogen peroxide (H_2O_2), it seems that as this conversion increases and reduced superoxide anions as SOD enzyme substrates which can decrease SOD level because there is no further need to the production of an enzyme during thermal stress. H_2O_2 causes the reversible covalent alterations in cysteine residues of specific proteins such as TRPV channels and changes the activation (Belhadj Slimen et al. 2014; Pires and Earley 2017). We found the anti-parallel pattern of GPx change with comparison to SOD, probably because of its

role as a natural antioxidant enzyme in the conversion of H_2O_2 to H_2O (Belhadj Slimen et al. 2014). But the answer to this question that why these effects seem to be reversed during 42 days of heat exposure is ambiguous and would need further studies. Nevertheless, it could be a cellular adaptation to protect from ROS-related cell death. Last but not least, reports show under the effect of heat stress and ROS generation, TNF- α induces SOD which conforms resistance against hyperthermia and cytotoxicity by scavenging of ROS products (Li and Oberley 1997) This mechanism prevents the cell from being apoptotic during hyperthermia and may be the probable reason for the ways in which SOD increases in the 42-day group compared to 21. SOD recovery in longer periods of heat stress probably acts as a natural barrier against cytotoxicity and cell death.

Conclusion

Heat stress as a major extracellular stimulation and an inducer of many cellular responses causes a significant increase in TRPV1 protein levels and a considerable decrease in the expression in TRPV4, both in mRNA and protein levels. Our results show that these channels are correlated inversely dependently on the duration of the thermal stress. Considering this, it is plausible to think that both channels participate in the neuronal thermal response, probably through related pathways. Further studies on the cellular mechanisms linking these channels to heat exposure and oxidative stress could contribute us to identify potential therapeutic targets to treat heat-related disease in the future.

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Author Contributions MAHF, LMF conceived and designed the study. AA, MG carried out the experiments, acquired the results, MG designed used primers. AA, LMF, MG analyzed and explicated results and drafted the manuscript. LR prepared tissue sections and analyzed the immunohistochemistry results. Each named author reviewed and approved the manuscript. All authors confirm that this manuscript has not been previously published and is not currently under consideration by any other journal.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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