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Original Article

The Effect of Sericin on Heat Shock Proteins 60 and 90, and TLR2 and TLR4 Induced by Chronic Heat Stress in Male Mice

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Abstract

Background: Heat stress followed by heat stroke, which is caused by an increase in body temperature to more than 40 °C, is a medical emergency that affects many people in work situations and weather conditions and results in irreversible neurological and cognitive damage.

Methods: In general, 75 male albino mice in the weight range of 28-30 g were used in the present study. The mice were divided into 5 groups of 15, including the control group, heat stress (HS) group, HS group + sericin 100, HS group + sericin 150, and HS group + sericin 200. Toll-like receptor 2 (TLR2) and Toll-like receptor (TLR4) expression levels were measured using the real-time polymerase chain reaction (RT-PCR), and the expression of hsp60 and hsp90 proteins was measured using a western blot.

Results: The expression level of TLR2 and TLR4 receptors increased significantly with the passage of time as a result of stimulation. Western blot results showed that the expression levels of HSP60 and HSP90 increased as well.

Conclusion: The results of RT-PCR revealed that HS significantly increases the expression of innate immune system receptors such as TLR2 and TLR4.

Keywords: Heat stress, TLR2, TLR4, Mice

Introduction

Global temperature has significantly risen in recent decades and is expected to rise further.[1] It can result in heatstroke, which is the most threatening condition in a spectrum of illnesses developing from heat exhaustion to heatstroke, in which hyperthermia is the most common finding.[2] Heat-stress-induced hyperthermia above 40 ˚C is life-threatening and kills more than half of heat victims in a short period of time.[3] Hence, the ability of organisms to deal with these elevated temperatures will be vital for maintaining their functions.[1]

According to the evidence, the brain is a vulnerable organ to high-temperature exposure, in which heat stress (HS) can result in cognitive impairment and memory loss, probably through the destruction of the brain's structure and impaired functions. More precisely, HS can affect the structure and function of the hippocampus, a crucial assembly involved in the management of memory and learning processes.[4,5] Previous studies revealed that rats exposed to whole-body hyperthermia for 4 hours at 38 ˚C showed profound neuronal, glial, and axonal injuries in the cerebral cortex, thalamus, hypothalamus, hippocampus, and cerebellum in a specific manner at light microscopy.[6]

Several mechanisms have been suggested through which HS causes cognitive impairment, including increasing the amount of hypothalamic-pituitary axis activity and the production of glucocorticoids, including cortisol,[4,7] a rise in the expression of pro-inflammatory cytokines such as *interleukin 6* (IL-6), *tumor necrosis factor-alpha*, and IL-1β in the hippocampus region, triggering of inflammatory reactions in the central nervous system, oxidative stress,[4,8] synaptic changes,[4] apoptosis,[9] and destruction of the blood-brain barrier.[10]

All living organisms react to environmental stresses involving hyperthermia by producing a set of proteins originally called heat shock proteins (HSPs).[11] According to their molecular weights, mammalian HSPs are categorized into various families involving HSP105/110, HSP90, HSP70, HSP60, HSP40, and other small HSPs. HSPs appear as molecular chaperones, supporting the folding, assembly, and disassembly of other proteins.[12,13]

Toll-like receptors (TLRs) are the main components of the innate immune system that identify a wide diversity of pathogen-associated molecular patterns.[14] The TLR family is made up of 13 members, and TLRs 1-9 are expressed in both mice and humans.[15,16] Lately, some evidence suggests that TLRs are significant mediators of brain injury.[17]

TLR2 and TLR4 are mainly expressed in microglia, astrocytes, neurons, and endothelial cells and play a significant role in inflammatory cascades.[14] Hence, understanding TLRs and their correlation with cerebral disease is becoming increasingly valuable to basic and clinical scientists.[14]

The silk cocoon made from the silkworm, *Bombyx mori*, involves two main proteins, fibroin and sericin. Sericin contributes to about 20%-30% of the whole cocoon weight and residual fibroin.[18] Sericin has anti-bacterial, antioxidant, wound healing, cell proliferation, anti-tumor activity, and UV protection properties; it is also thought to act as a potential neuroprotective compound against Alzheimer's disease in a widely accepted animal model, namely, the rat.[19] Additionally, the cerebral anti-apoptotic effect of sericin has been illustrated in chronic restraint stress-evoked anxiety and depressive-like behaviors.[20] Considering the antioxidant and anti-apoptotic effects of sericin, this agent may play a part in the response to HS.

Therefore, this study aimed to examine the effect of sericin on HSP60, HSP90, and TLR2, and TLR4, induced by chronic HS in male mice.

Materials and Methods

Animals

Seventy-five healthy adult male albino mice (weighing 28-30 g) were used in this study. The absence of any disease and abnormal behavior was considered as the inclusion criterion, while the occurrence of any disease or injury during the study period was considered the exclusion criterion.

Experimental Protocol

The animals were randomly divided into five groups of 15, including control, HS, HS + sericin100, HS + sericin150, and HS + sericin200 groups. Mice in control groups were not treated with sericin and only transferred to the HS induction machine for 15 minutes daily, after which they received normal saline with a volume of 10 mL per kg of body weight orally. Mice in the second group were only subjected to HS and administered normal saline with a volume of 10 mL/kg of body weight orally. Mice in the remaining groups (III to V) were subjected to HS and administered sericin at doses of 100, 150, and 200, respectively, orally. Sericin doses were selected based on previous studies.[26]

Induction of Heat Stress

Mice were placed into cages and moved to a temperature-controlled chamber maintained at 43 ˚C for 15 minutes once a day for 14 days. Then, they returned to their cages at room temperature and received their respective treatments according to the intended grouping.

Sampling

Forty-eight hours after the last treatment, the animals were anesthetized by the intra-peritoneal injection of ketamine (80 kg/mg) and xylazine (15 kg/mg). The hippocampal tissue was then isolated and stored in liquid nitrogen.

Real-Time Polymerase Chain Reaction

To extract RNA, 20 mg of the isolated tissue was homogenized with 1 mL of Tri-Pure reagent based on the related extraction protocol. The Rotor-Gene™ 6000 (Corbett) machine was used for all RT-PCRs.

The heating schedule of the device was set in three stages. The first step, which leads to the denaturation of cDNA molecules, was at 95 ˚C for 5 minutes; the second stage was at 95 ˚C for 10 seconds for annealing, 56 ˚C for 10 seconds for annealing, and 60 ˚C for 24 seconds for extension in 40 consecutive cycles. In the final stage of drawing the melting curve, the temperature was increased by 1 degree every 5 seconds from 50 ˚C to 99 ˚C.

These reactions were performed in duplicate in 0.1 mL microtubes with a final volume of 10 microliters. The ingredients of each reaction included 5 microliters of 2X QuantiFast SYBR Green PCR Master Mix and 0.5 µL of each of the reverse primers. With a concentration of 10 pM, 2 µL of RNase-free water and 2 µL of cDNA were the template. To draw the standard curve, one of the control cDNAs with the appropriate concentration was selected, and then the respective dilutions of 51-55 were utilized to draw the standard curve. The standard curve was drawn based on the logarithm of the cDNA concentration on the horizontal axis and the threshold cycle on the vertical axis for each gene. The PCR efficiency was determined based on the standard curve for each gene. A sample without template cDNA (No template control) was used as a negative control for each RT-PCR. An amplification curve was drawn accordingly.

Western Blot

Electrophoresis was performed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. About 34 µg of each protein sample were mixed with the sample buffer at a ratio of 1.1 and used after performing the Bradford test. In addition, a molecular weight marker was utilized to determine the position of hsp60 and hsp90 proteins. Further, the beta-ACTIN antibody was employed for loading control. After using electrophoresis to transfer proteins from the gel to the sandwich model membrane using Whatman sheets, the nitrocellulose membrane and gel were prepared and placed in the transfer tank containing buffer. After blockage with 5 mL of a 3% bovine serum albumin (BSA) blocking solution and washing with a Tris-salt buffer solution containing Tween for 1 hour, the membrane was washed with 2 mL of Abcam and rabbit anti-4 primers. HSP90 and HSP60 antibodies were diluted in 3% BSA at a ratio of 1:1,000 and then incubated for 30 minutes in phosphate-buffered saline/BSA secondary antibodies with a concentration of 1:1000 in ABC Staining Kits. Then, the filter was exposed to ABC-reagent AP (Laboratory Vector) for 30 minutes. Finally, Vector Blue-Alkaline Phosphate Substrate Solution was poured on the filter. The substrate solution was removed and the filter was washed after the appearance of blue protein bands. After drying, the membrane was photographed with a Cannon Japan 11 camera. Finally, ImageJ software was used for band densitometry.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 7 software based on an analysis of variance and the Student's *t* test. *P* values greater than 0.05 were considered statistically significant.

Results

Expression of Toll-like Receptors 2 and 4

The results of the study demonstrated that the expression of both TLR2 and TLR4 decreased with an increase in the dose of sericin, which was significant in all groups compared to the control group ($P<0.0001$). This reduction was remarkable for the dose of 200 mg of sericin .(Figure 1A, 1B).

Expression of HSP60 and HSP90

Based on the results of immunoblotting, the levels of the protein expression of HSP90 and HSP60 were different at different doses of sericin, and there was a significant decrease in the 200 mg and 150 mg doses as compared with the control group (Figure 1C).

Discussion

HSPs are instantly produced in neuronal and glial cells in different stressful conditions and play a protective role beyond molecular chaperones.[21]

HSPs have been revealed to provide neuronal rescue and neuroprotective mechanisms.[22] In contrast, numerous studies have recently established the glial expression of small HSPs (HSP32, 27, and 47) under various neuropathological conditions in the brain.[23-27] Glial expression of these HSPs may play a significant role in the metabolic alterations that happen throughout the glial response to neural injury. However, less is known about the production of the large-molecular-weight HSP90 family in glial cells after neuropathological conditions. HSP90 is a member of the HSP90 family, which is fundamentally expressed even under normal conditions, and whose expression is enhanced to differing levels under stressful conditions.[28,29] The in vitro generation of HSP90 in macrophages throughout phagocytosis, heat shock exposure, and viral infection has been associated with the activation of macrophages, indicating that HSP90 functions by behaving as a molecular chaperone and by protecting the macrophage against the auto-oxidative damage correlated with respiratory bursts.[30-34]

Recently, it has been found that the HSP90 protein was activated after traumatic brain injury (TBI), and the inhibition of this protein decreased the disruption of the blood-brain barrier. However, it could improve the neurobehavioral scores in a mouse model of TBI across the action of 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DA), which inhibited reactive oxygen species production and adjusted matrix metalloproteinases MMP-2, MMP-9, nuclear factor kappa B, and caspase-related pathways. Therefore, blocking the HSP90 protein may be a potential therapeutic strategy for TBI.[35]

HSP60 functions as an immunomodulatory molecule and a mitochondrial chaperone, thus it can activate the antigen-presenting cells of the immune system, including an auto-immunogen, at the site of inflammation.[36,37] Additionally, it becomes upregulated in reaction to mitochondrial impairment and is believed to be an indicator of mitochondrial stress.[38]

Moreover, HSP60 controls endogenous IL-1β production by stimulating mitochondrial stress and triggering the nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3) inflammasome pathway in microglia. Thus, HSP60 is enhanced in microglia in reaction to harmful stimuli and sequentially stimulates the inflammasome complex, causing consecutive microglial activation. According to these findings, it has been suggested that HSP60 can be a potential therapeutic target for the improvement of various neuroinflammatory and neurodegenerative diseases.[38]

TLR2 and TLR4 are two main components of the brain's innate immune system, and these receptors are mostly expressed in microglia, neurons, astrocytes, and endothelial cells.[14] It was recently revealed that TLR2 and TLR4 are fundamental for sterile organ damage involving ischemic brain injury.[39,40] Additionally, TLR2 and TLR4 were recognized as being significant in the pathological evolution of cerebral ischemia and reperfusion.[41,42] However, a scarcity of studies has examined the role of TLRs in neonatal ischemic brain injury.[43,44] In one study, it was found that both TLR2 and TLR4 are enhanced in the hippocampus of neonatal rats after hypoxia-ischemia, suggesting the opportunity to treat neonatal brain injury by inhibiting TLR2 and TLR4.[14]

Along with these studies, our study showed that sericin can reduce the expression of TLR2 and TLR4, as well as HSP90 and HSP60. Likewise, it was demonstrated

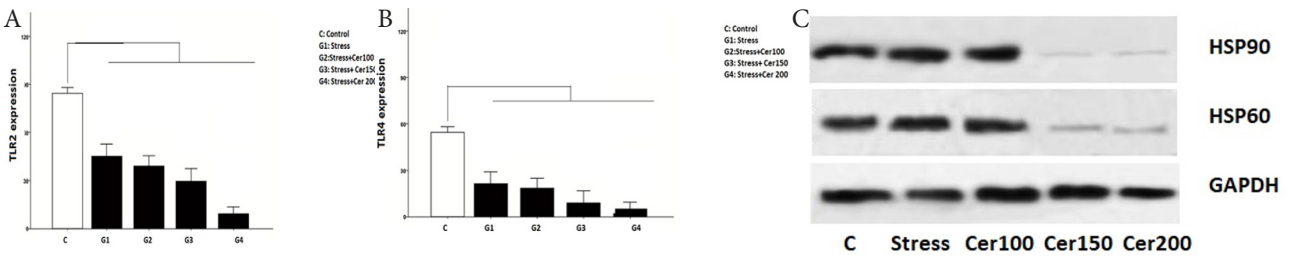

Figure 1. The Real Time PCR and Western blot analysing results. A: The TLR2 expression B: The TLR4 expression C: western blot results with drugs treatment

that sericin can affect cerebral edema and increase the stimulation of calcium channels in the brain.

Conclusion

TLR2 and TLR4 expression decreased significantly with an increase in the sericin dose, andHSP90 and HSP60 levels.

Authors' Contribution

Conceptualization: Mina Deljavan Ghodrati.
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Writing–review & editing: Mobina Belalzadeh.

Competing Interests

None.

Ethical Approval

The study was Approved by Ethics Committees of Vice-Chancellor in Research Affairs - Tabriz University of Medical Sciences (IR.TBZMED.VCR.REC.1400.475).

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