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Research Article

The Effect of Chrysin on the Distribution of Extracellular Matrix Proteins around Leydig Cells under Heat Stress Injury

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Objective. In the testicular tissue stroma, matrix proteins such as laminin and fibronectin play a critical role in transmitting signals under normal and stressful conditions to Leydig cells. Heat stress is considered one of the stressful conditions in the testicular tissue. This study has investigated the protective effects of chrysin on the distribution of laminin and fibronectin around Leydig cells under heat stress. *Methods*. Rats were divided into four groups, including sham (A), heat stress (B), heat stress with chrysin treatment (50 mg/kg) (C), and heat stress with chrysin treatment (75 mg/kg) (D). At the end of the treatment period, on the 21st day, testosterone serum levels were evaluated, and the testes were also assessed to determine the changes in gene expression and distribution of laminin, fibronectin, and caspase-3 proteins. *Results*. Exposure to heat stress in Group B resulted in increased levels of caspase-3, decreased activity, height, and diameter of seminiferous tubules, and altered distribution of laminin and fibronectin proteins around the Leydig cells when compared to the sham group ($p < 0.05$). Treatment with chrysin 75 resulted in a significant decrease in caspase-3 levels, improved testosterone levels, and increased distribution of laminin and fibronectin around Leydig cells compared to group B ($p < 0.05$). In addition, the group treated with chrysin 75 showed improved seminiferous histological activity markers in comparison to group B ($p < 0.05$). *Conclusion*. Following heat stress, concurrent with disrupted steroidogenesis, an evident change happens in the distribution pattern of the extracellular matrix (ECM) proteins of the testis, which may indicate the transmission of stress signals from the ECM to the Leydig cells. Treatment of chrysin in 75 mg/kg dose can reduce the damage caused by heat stress on the ECM and Leydig cells.

1. Introduction

Testicular stroma is the heart of interactions and communications in the steroidogenesis process due to having two main components, including Leydig cells and extracellular matrix (ECM) [1]. By carrying out steroidogenesis, Leydig cells are the most salient somatic cells in the testicular stroma. Since these cells have no basement membrane, their active interactions with ECM components considerably help regulate their division, differentiation, morphology, and function [2]. Laminin and fibronectin are among the most significant ECM components influencing steroidogenesis. Moreover, laminin and fibronectin can transmit oxidative stress, hypoxia, and growth factor signals between ECM components [3]. These proteins contribute to the matrix and Leydig cells' cross talk and play a role in the seminiferous tubules-Leydig cell interaction [4].

Spermatogenesis is a temperature-dependent process in testicular tissue. Any increase in temperature above the normal scrotum temperature range may affect male reproductive performance, possibly through the induction of ROS (reactive oxygen species) [5, 6]. In this study, the retractile testis (RT) model was designed to induce heat stress. The RT model is traditionally defined as a normal testis that

returns to the supra-scrotal position for various reasons, including the cremasteric reflex [7]. Research has shown that the testis is exposed to heat stress, which leads to damaged germ and somatic cells and also disrupts steroidogenesis of the testis Leydig cells by the oxidative stress-related response [8, 9]. However, the heat damage to the ECM in the testis is not evident. Since by affecting matrix proteins, heat stress causes improper deposition of these proteins and, finally, matrix separation [10], and then, changes in the laminin and fibronectin proteins following heat stress in the testicular tissue will not be unexpected. In this case, the first line of treatment is to eliminate heat stress by returning the testis to the scrotum and then by antioxidant treatment [11].

Chrysin is one of the most active natural flavonoids, exerting its protective effects through antitumor and anti-inflammatory antioxidant activities [12]. This flavonoid has shown protective effects against oxidative stress in damaged testes by inhibiting apoptosis [13]. Chrysin is recognized as an antiapoptotic substance in tissues under stress by reducing caspase-3 as a matrix modulation affecting metalloproteinases [14, 15]. Furthermore, steroidogenic effects of this substance have also been observed in Leydig cells under oxidative stress [16]. In the current study, we aimed to investigate the distribution of changes in ECM proteins (laminin and fibronectin) and caspase-3 as an apoptosis marker surrounding Leydig cells under heat stress damage and also to measure testosterone levels.

2. Materials and Methods

2.1. Animals. At the beginning of the experiment, 32 Wistar rats from the Animal House of the Mashhad University of Medical Sciences were provided. The eight-week-old rats weighed approximately 250–300 g and were kept in the standard conditions of the animal house. These conditions included 12/12 h light/dark cycles, the temperature at $23 \pm 2^{\circ}$C, and the humidity at 60%–70%. Rats in all groups had free access to food and water. Ethical considerations were considered according to the regulations of the Vice Chancellor for Research and Technology in the Mashhad University of Medical Sciences (No. 971786). Rats were randomly divided into four experimental groups:

Group 1: as a control group, a scrotum incision and suture were performed without any treatment or injury (sham group)

Group 2: the retractile testis procedure was performed for 120 hours to induce heat stress (HS group)

Group 3: heat stress was induced along with chrysin treatment at a dose of 50 mg/kg for 21 days (HS + CH50 group)

Group 4: heat stress was induced along with chrysin treatment at a dose of 75 mg/kg for 21 days (HS + CH75 group)

2.2. Surgical Procedure. At the beginning of surgery, rats were anesthetized with 50 mg/kg ketamine and 10 mg/kg xylazine. To induce retractile testis (RT), the following was carried out: the testicles were exposed after longitudinal incisions into the left scrotum. Then, the testicle and its spermatic cord were separated from the surrounding fascia. The testicle was moved from the scrotum into the abdominal cavity. They were fixed to the abdominal wall with 6.0 silk sutures. Afterward, the scrotum was sealed with a 5.0 silk suture. In the HS group (Group 2), the testicle was maintained in the abdominal cavity for 120 hours (one of the limitations of the experiment was the loss of a testis after 120 hours due to necrosis).

After 120 hours [17], the testicles were returned to the scrotum. Next, the scrotum was sutured with 5.0 silk sutures. In the treatment groups, rats were treated with daily oral chrysin at doses of 50 mg/kg (Group 3) and 75 mg/kg (Group 4) for 21 days [18]. All surgeries and anesthesia methods (except RT induction) in the sham group were carried out. Finally, at the end of treatment on the twenty-first day, the rats were anesthetized and underwent orchiectomy. The right testes of the rats in each group were finally fixed in formalin for conducting histological and immunohistochemical (IHC) studies. The left testes of the rats in each group were employed for real-time studies. Simultaneously with orchiectomy, blood samples were obtained for biochemical studies [19].

Postoperative care: animals and surgical wounds were observed daily until full recovery. After the daily monitoring, the surgical team documented any occurrence of common problems such as hypothermia, hypoglycemia, and dehydration. They also noted if there was any abscess, infection, or skin repair at the site of the surgical wound. All of this information was recorded in the surgical file.

2.3. Seminiferous Tubular Activity Evaluation. In this study, the seminiferous tubular diameter and epithelial height were measured in stages 7 and 8 of spermatogenesis. To determine the diameter of the seminiferous tubules, we randomly selected round cross-sections. Using Image J software, the diameter of each tubule was calculated from one side of the base membrane to the other side of the tube. The height of the seminiferous epithelium was measured at 400x magnification using Image J software from the basal membrane to the seminiferous epithelium's apical surface.

The spermatogenesis index was evaluated by using Johnsen scoring. To evaluate pathological lesions, 30 seminiferous tubules were examined in cross-section and scored on a scale of 1–10 based on Johnsen's score as follows: 10: complete spermatogenesis and perfect tubules, 9: many spermatozoa are present but disorganized spermatogenesis, 8: only a few spermatozoa are present, 7: no spermatozoa but many spermatids present, 6: only a few spermatids are present, 5: no spermatozoa or spermatids are present but many spermatocytes are present, 4: only a few spermatocytes are present, 3: only spermatogonia is present, 2: no germ cells present, and 1: neither germ cells nor Sertoli cells present [20].

2.4. Immunohistochemical Staining. On the 21st day after the end of the treatment period, the scrotum was sectioned, and the right testis was removed. To perform the IHC technique,

the tissue was first fixed in formalin 10%. The steps of dehydration with ascending ethanol and clarification with xylene and liquid paraffin were implemented. After molding with paraffin, the samples with a thickness of 4 μm were cut using a microtome. Then, the obtained slides were hydrated. The sample antigens were recovered using the thermal method in a water bath at a temperature of 100°C. Also, to inhibit the activity of peroxidases, the samples were immersed in a 3% H_2O_2 solution. Then, to inhibit the activity of nonspecific antigens, the slides were placed adjacent to a 10% goat serum solution. Slides were coated with primary antibodies at a temperature of 4°C overnight; after that, the slides were incubated with secondary antibodies for 2 hours at room temperature. By exposing the slides to the diaminobenzidine (DAB) solution, the reactions with the secondary antibody were observed in brown. To clarify the cell nucleus, the samples were stained with hematoxylin. After dewatering and pasting the coverslip, the stained slides were observed using an optical microscope with a magnification of 400. The protein expression intensity was quantified by using Image J software [19]. In this study, the primary antibodies used in the immunohistochemical technique are as follows: fibronectin: ab268020, laminin: ab11575, and caspase-3 *p*17 (D-12): sc-373730. The characteristics of the secondary antibodies used are as follows: m-IgGκ BP-HRP: sc-516102, and goat antirabbit IgG-HRP.

2.5. Real-Time Polymerase Chain Reaction Evaluation. After conducting orchiectomy, testicular samples were homogenized using a homogenizer. The total RNA of the testicular tissue was extracted according to the kit protocol. After that, RNA purity was determined on agarose gel by electrophoresis. Reverse transcription of RNA was made according to the cDNA synthesis kit protocol. Also, real-time PCR (RT-PCR) was performed on an ABI PRISM®48 device. The main components of the RT-PCR mixture included 1 μl of cDNA template, 0.2 μm of forward primers, 0.2 μmol of reverse primer, 3.6 μl of sterilized water, and 5 μl of SYBR green. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as the internal control gene. The ΔΔCT method was used to measure the relative changes in gene expression. Gene augmentation was carried out by the following protocol in this way: for 10 minutes at 95°C, 40 two-step replicates for 15 seconds at 95°C, for 30 seconds at 58°C, for 30 seconds at 72°C, for 15 seconds at 95°C, and 1 hour at 55°C [21]. Oligonucleotide primers used in RT-PCR are as follows: GAPDH (forward primer: *CCAATGTATCCGTTGTGGATC*/reverse primer: *ATTGTC ATACCAGGAAATGAGC*), laminin (forward primer: *GGTCAGGTGACTCGCTTTG*/reverse primer: *GCTCTT AACGTGCCGTCTGT*), fibronectin (forward primer: *GTGGCTGCCTTCAACTTCTC*/reverse primer: *AGTCCT TTAGGGCGGTCAAT*), and caspase-3 (forward primer: *TGTATTCTTACTCTACCGCAC*/reverse primer: *GCACAA AGTGACTGGATGAAC*).

2.6. Statistical Analysis. Statistical analysis was performed in SPSS 20 software (IBM; USA). The Kolmogorov–Smirnov test was used to determine the normal distribution of the data. All data were presented as mean ± standard deviation. One-way analysis of variance (ANOVA), followed by Tukey's post hoc test, was used to compare the IHC values. The independent *t*-test was used to compare and analyze the data of the RT-PCR technique. The statistical significance level was determined at $p \leq 0.05$.

3. Results

3.1. The Spermatogenesis Index of Seminiferous Tubular. Johnson scoring system was used to determine the seminiferous tubular activity. The obtained result regarding the mean score in the sham group was 9.68. Statistical evaluations showed a significant decrease in this score in the heat stress group compared to the sham group. Although chrysin treatment increased Johnson scores in both HS + CH50 and HS + CH75 groups compared to the HS group, there was a significant increase in this score only in the HS + CH75 group ($p \leq 0.05$) (Table 1) (Figure 1).

3.2. Seminiferous Tubular Diameter and Epithelial Height. In the current study, the seminiferous tubular diameter and epithelial height significantly reduced in the HS group than in the sham group. However, after chrysin treatment in HS + CH50 and HS + CH75 groups, amelioration in seminiferous diameter and epithelium height index was observed. Amelioration in the above indices only in the HS + CH75 group was statistically significant ($p \leq 0.05$) (Table 1) (Figure 1).

3.3. Evaluation of the Distribution of Caspase-3 Protein Leydig Cells. The caspase-3 protein IHC analysis revealed the uniform distribution of this protein in the cytoplasm of some Leydig cells in sham groups. Based on the data analysis, the difference in the intensity of the reaction to caspase-3 protein was significantly higher in the HS group than in the sham group. After chrysin treatment in the HS + CH75 group, compared to the HS group, the decrease in the intensity of the reaction to these proteins was significant. No significant difference was observed in the reaction of the mentioned protein intensity in the HS + CH50 group compared to the HS group ($p \leq 0.05$) (Figure 1 and 2).

3.4. Evaluation of the Distribution of Caspase-3 Protein Spermatogonia Cells. In the sham group, the caspase-3 protein distribution was observed in the cytoplasm of some spermatogonial cells. The distribution of this protein in the HS group showed significantly higher distribution intensity than in the sham group. In the HS + CH50 and HS + CH75 groups, the intensity of caspase-3 distribution decreased compared to the HS group, but it was not significant ($p \leq 0.05$) (Figures 1 and 2).

Table 1: Comparison of the seminiferous histological marker in all experimental groups.

	Sham	HS	HS + CH50	HS + CH75
Modified Johnsen score	9.68	3.05*	5.03	8.61**
Diameter, μm (mean ± SD)	231 ± 3.2	146.02 ± 24.63*	152.01 ± 11.12	192.00 ± 19.03**
Epithelium thickness, μm (mean ± SD)	81 ± 2.27	23.51 ± 4.67*	39.11 ± 2.22	61.01 ± 3.88**

Evaluation of testicular modified Johnsen score, seminiferous tubule diameter, and epithelium height following the induced heat stress and chrysin treatment. Values are mean ± SD. $p < 0.05$ are significant, $^{*}p < 0.05$ vs sham, and $^{**}p < 0.05$ vs HS group.

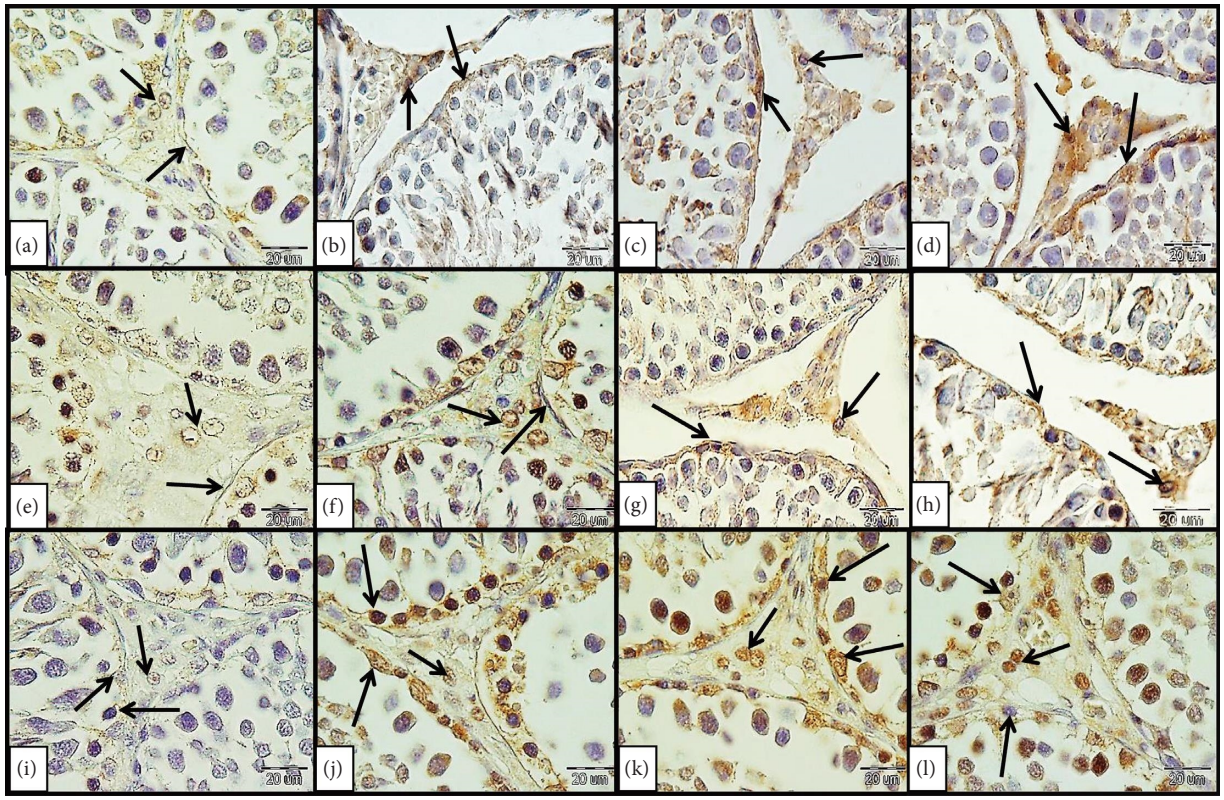

Figure 1: Evaluation of chrysin treatment on distribution intensity of laminin and fibronectin surrounding Leydig cells and seminiferous tubular under heat stress injury. Representative light microscopy images of laminin in sham (a), HS (b), HS + CH50 (c), and HS + CH75 (d) and fibronectin in sham (e), HS (f), HS + CH50 (g), and HS + CH75 (h). Evaluation of chrysin treatment on distribution intensity of caspase-3 proteins in Leydig, Sertoli, and spermatogonia cells under heat stress injury. Representative light microscopy images of caspase-3 in sham (i), HS (j), HS + CH50 (k), and HS + CH75 (l).

3.5. Evaluation of the Distribution of Caspase-3 Protein Sertoli Cells. In the caspase-3 protein IHC analysis, the distribution of this protein was observed in the cytoplasm of a small number of Sertoli cells in sham groups. After the induction of heat stress in the HS group, it was found that the intensity of the reaction to caspase-3 protein was significantly higher than in the sham group. After chrysin treatment in the HS + CH75 group, the distribution of this protein was significantly reduced compared to HS groups. However, no significant decrease was observed in the distribution of caspase-3 protein in the HS + CH50 group than in the HS group ($p \leq 0.05$) (Figures 1 and 2).

3.6. Immunohistochemical Analysis of Laminin and Fibronectin around Leydig Cells. In the IHC analysis of laminin and fibronectin, we observed the distribution of this protein around the membrane of Leydig cells as dashed and dark lines in the sham group. Our data indicated that the intensity of the reaction to antilaminin and fibronectin antibodies significantly reduced surrounding the Leydig cell membrane in the HS group than in the sham group. Chrysin treatment in the HS + CH75 group significantly increased the intensity of the reaction to laminin and fibronectin proteins than in the HS group. Furthermore, no significant change was observed in the distribution of this protein in chrysin treatment in the HS + CH50 group than in the HS group ($p \leq 0.05$) (Figures 1 and 3).

3.7. Evaluation of the Distribution of Laminin and Fibronectin Protein around Seminiferous Tubular. In this study, the distribution intensity of laminin and fibronectin proteins in the bases of seminiferous tubules was observed as dark lines in the sham group. After the induction of heat stress, the intensity distribution of these proteins was significantly

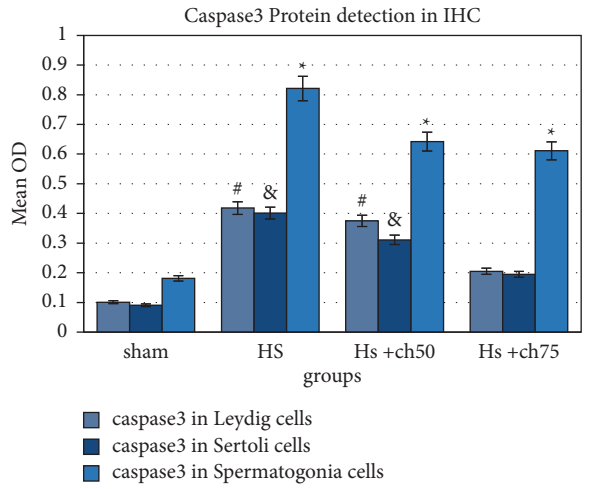

Figure 2: Evaluation of chrysin treatment on the density of caspase-3 proteins in Leydig, Sertoli, and spermatogonia cells under heat stress injury. The reported optical density of caspase-3 proteins in all experimental and sham groups. The exhibited caspase-3 proteins in spermatogonia cells (*), Leydig cells (#), and Sertoli cells (&) of experimental groups compared with sham. Values are mean ± SD. $p < 0.05$ are significant.

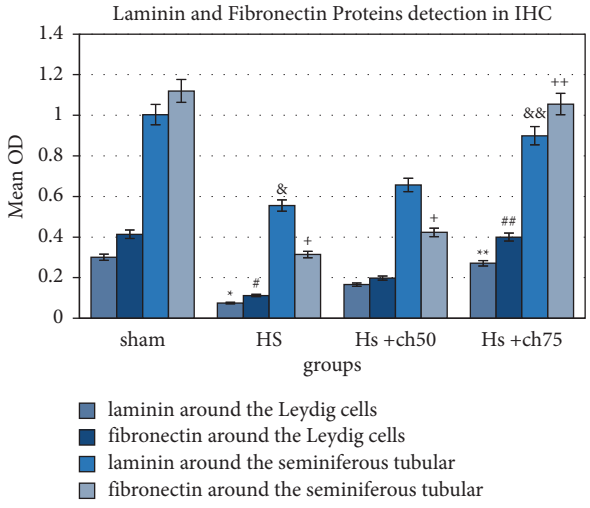

Figure 3: Evaluation of chrysin treatment on the density of laminin and fibronectin around Leydig cells and seminiferous tubular under heat stress injury. The reported optical density of laminin and fibronectin proteins in all experimental and sham groups. *Laminin around the Leydig cells of experimental groups compared with sham. **Laminin around the Leydig cells of experimental groups compared with the HS group. #Fibronectin around the Leydig cells of experimental groups compared with sham. ##Fibronectin around the Leydig cells of experimental groups compared with the HS group. &Laminin around the seminiferous tubular of experimental groups compared with sham. &&Laminin around the seminiferous tubular of experimental groups compared with the HS group. +Fibronectin around the seminiferous tubular of experimental groups compared with sham. ++Fibronectin around the seminiferous tubular of experimental groups compared with the HS group.

reduced in the HS group than in the sham group. It was also observed that in chrysin treatment in the HS + CH75 group, the distribution intensity of these proteins around the tubules significantly increased compared to the HS group. In addition, no significant changes were observed in the distribution of these proteins around the seminiferous tubules of the HS + CH50 group compared to the HS group ($p \leq 0.05$) (Figures 1 and 3).

3.8. Analysis of Caspase-3 Real-Time Polymerase Chain Reaction. In the analysis of caspase-3 mRNA expression as an apoptosis marker, it was observed that the testicular tissue had a normal level of expression of this gene in the sham group. Compared to the sham group, the caspase-3 mRNA level significantly increased in the HS group. Chrysin treatment in the HS + CH50 group could reduce the caspase-3 level compared to the HS group, but it was not significant. However, in the HS + CH75 group, the caspase-3 mRNA level showed a significant decrease compared to the HS group ($p \leq 0.05$) (Figure 4).

3.9. Analysis of Laminin and Fibronectin Real-Time Polymerase Chain Reaction. In this study, the results of mRNA, laminin, and fibronectin levels in the total testicular tissue were such that a normal level of expression of the mentioned genes was observed in the sham group. Induction of heat stress in the HS group resulted in a significant decrease in laminin and fibronectin mRNA expression compared to that in the sham group. Chrysin treatment in both HS + CH50 and HS + CH75 groups could significantly increase mRNA, laminin, and fibronectin levels compared to the HS group ($p \leq 0.05$) (Figure 4).

3.10. Biochemical Findings. According to testosterone biochemical analysis performed on the serum of the investigated mice, it was observed that after the induction of heat stress, the level of this hormone significantly decreased in the HS group than in the sham group. Chrysin treatment in both HS + CH50 and HS + CH75 groups resulted in a relative improvement in the level of this hormone compared to that in the HS group. In this study, the levels of caspase-3, laminin, and fibronectin mRNA were analyzed based on the RT-PCR results in the total testicular tissue ($p \leq 0.05$) (Figure 5).

4. Discussion

The present study highlighted the effects of chrysin on the distribution of laminin and fibronectin around Leydig cells, focusing on the spermatogenesis and steroidogenesis performance of testes exposed to heat stress. Reciprocal interactions between Leydig cells and seminiferous tubules are influenced by the distributional pattern of ECM proteins, including laminin and fibronectin that are deemed necessary for the normal functioning of spermatogenesis and

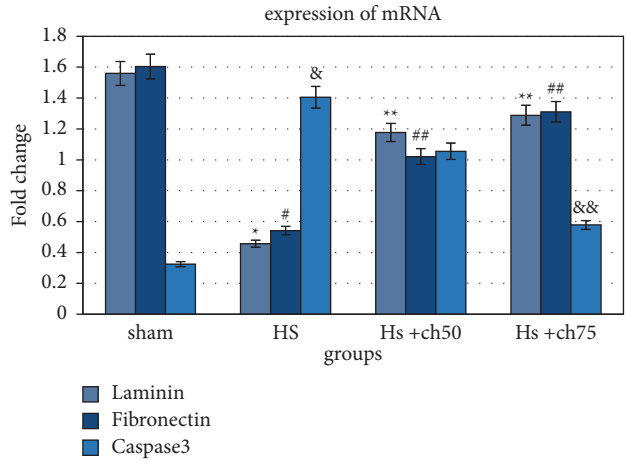

Figure 4: Evaluation of chrysin treatment on laminin, fibronectin, and caspase-3 mRNA expression genes in the testicular tissue of all groups following the heat stress injury as measured by the real-time PCR. Values are mean ± SD. $p < 0.05$ are significant. *Laminin of experimental groups compared with sham. **Laminin of experimental groups compared with the HS group. #Fibronectin of experimental groups compared with sham. ##Fibronectin of experimental groups compared with the HS group. &Caspase-3 of experimental groups compared with sham. &&Caspase-3 of experimental groups compared with the HS group.

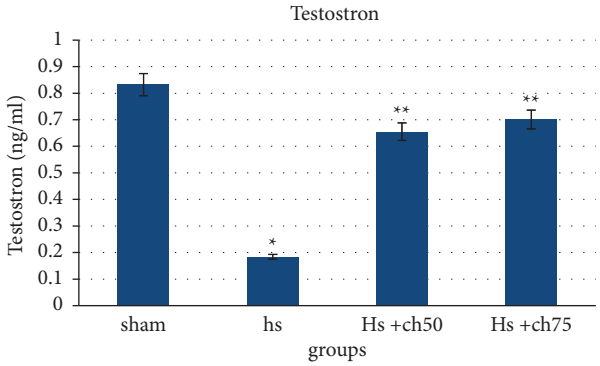

Figure 5: Evaluation of chrysin treatment on the testosterone level in sham and experimental groups following the heat stress injury. Values are mean ± SD. $p < 0.05$ are significant. * $p < 0.05$ as compared with sham. ** $p < 0.05$ as compared with the HS group.

steroidogenesis [4, 22]. ECM proteins surrounding Leydig cells not only regulate steroidogenesis but also play key roles in transmitting vital or oxidative stress signals [2]. Our previous studies showed that the intensity of these protein distributions is altered in the stroma of testicular tissue during oxidative stress caused by the ischemia-reperfusion of the testes [23]. Leydig cells are among the most resistant cells against oxidative stress in the testicular stroma. However, the exposure of these cells to stress may ensure dysfunctional steroidogenesis [24].

Heat stress is among the insults that can disrupt the interaction between Leydig cells and seminiferous tubules, leading to dysfunctional spermatogenesis [25] and steroidogenesis [26]. In this regard, Dolatkhah et al. demonstrated that varicocele-induced heat stress can result in the destruction of spermatogenesis by inducing reactive oxygen species (ROS) [27]. However, the effects of heat stress on the testicular ECM are not fully understood. Therefore, in this study, testicular cells and ECM were exposed to heat stress using an RT model. The IHC technique results of this study revealed an increase in the intensity of caspase-3 distributions as an apoptotic marker, in many spermatogonial cells, and some Sertoli and Leydig cells following the heat stress.

In this regard, studies have shown that testicular germ cells are temperature-sensitive, while Leydig and Sertoli cells may become merely dysfunctional in response to heat stress [28].

In addition, the histological and biochemical findings of this study demonstrated a decline in testosterone levels (0.184 ng/ml) and the Johnsen score (3.05) of the seminiferous tubules, confirming dysfunctional steroidogenesis and spermatogenesis. Heat stress has been reported to be associated with the destruction of integrity and detachment of ECM [29]. In a study by Danker et al., it was noted that heat shock led to the abnormal distribution of ECM proteins, including laminin and fibronectin [30]. In another study, Matuszczak et al. suggested that the activation of matrix metalloproteinases following heat shock stress leads to ECM protein destruction [31]. Since ECM proteins have a regulatory role in the apoptosis and autophagy processes, destroying these proteins will endanger the normal function of spermatogenesis and steroidogenesis [23]. The present study also shows a decrease in the distribution of laminin and fibronectin not only around Leydig cells but also around seminiferous tubules. Considering the abnormal distributional pattern of laminin and fibronectin, which coincided with dysfunctional spermatogenesis and steroidogenesis, this hypothesis can be put forward that the cause of dysfunction of Leydig cells may be the result of the degradation of ECM proteins and disruption of the interaction between Leydig and seminiferous tubules. In the same way, Aldahhan RA et al. argued that heat stress did not directly interfere with the function of Leydig cells, but seminiferous tubules damaged under heat stress transmit signals that interfere with steroidogenesis by these cells [8]. When the testicular tissue is affected by heat stress, the first line of treatment is to obviate the heat shock, followed by the administration of antioxidants [32], such as chrysin as suggested in this study, to curtail the damage caused by heat stress. Chrysin has been shown to improve the steroidogenic activity of Leydig cells exposed to oxidative stress [16] and to suppress caspase-3 under stress conditions, indicating its antiapoptotic function [18]. In the present study, biochemical and histological findings revealed that treatment with chrysin decreased apoptosis in spermatogonia, Leydig cells, and Sertoli cells. These observations, along with the improved spermatogenesis activity of tubules and improved steroidogenesis of Leydig cells, suggested an antiapoptotic role for chrysin under heat stress. In this study, the results of IHC revealed an increase in the density of ECM proteins around Leydig cells after treatment with chrysin. In line, some studies have declared that chrysin can modulate ECM proteins under stress, an activity attributed to its regulatory effects on MMPs [15]. Considering that ECM proteins play a role in steroidogenesis regulation, it seems that chrysin's

protective effects on laminin and fibronectin improved steroidogenesis (0.701 ng/ml). The mechanism of the chrysin effect on the ECM protein under heat stress is probably due to the modulating of matrix metalloproteinase function, followed by the regulation of apoptosis and autophagy. In this study, the evaluation of seminiferous tubules showed an improvement in spermatogenesis activity (8.61). However, we noticed that only at a high dose (75 mg) chrysin was able to alleviate heat stress-induced damage.

Overall, the results of this study showed that chrysin could improve the intensity of ECM protein distributions, spermatogenesis, and steroidogenesis. These findings suggested that this compound could re-establish the interactions between seminiferous tubules and Leydig cells by improving the distributional pattern of laminin and fibronectin within the testicular stroma. It is suggested that future studies investigate other ECM proteins including type IV collagen and growth factors involved in the cross talk between Leydig cells and seminiferous tubules.

Patients with retractile testes are at risk of heat stress-induced damage to steroidogenesis and spermatogenesis, as well as testicular carcinoma [33]. The data from this study indicate that changes in the distribution pattern of ECM proteins around Leydig cells and seminiferous tubules are likely responsible for the outcome observed in patients with retractile testis. Modulating the ECM through chrysin treatment can be promising in treating retractile testis outcomes in patients.

Data Availability

All data generated or analyzed during this study are included within the article and are available from the corresponding author upon request.

Ethical Approval

Ethical considerations were taken into account according to the principles of the Vice Chancellor for Research and Technology of Mashhad University of Medical Sciences (No. 971786).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Surgical procedure (induction of retractile testis) was performed by Malihe Soltani. Immunohistochemical analysis was performed by Mohammad Reza Nikravesh. Real-time analysis was performed by Majid Rahmati. Statistical analysis performed by Shahin Saeedi Nejat. Testicular histology analysis was performed by Mahdi Jalali.

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