RESEARCH ARTICLE

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Age- and Sex- Dependent Changes in Serum Levels of TAS, TOS, TLR2, TLR4, HSP60, HSP90, and HMGB1

ABSTRACT

Objective: Cellular and physiological functions may be affected in an age- and sex-specific manner. The aim of this study is to investigate sex- and age-specific differences in the serum levels of Total Antioxidant Status (TAS), Total Oxidant Status (TOS), Oxidative Stress Index (OSI), Toll-Like Receptor 2 (TLR2), Toll-Like Receptor 4 (TLR4), Heat Shock Protein 60 (HSP60), Heat Shock Protein 90 (HSP90), and High Mobility Group Box 1 (HMGB1) as well as to examine the correlation between them.

Method: Four groups of mice, each including seven animals, were used in the present study: young males and females (6 months old); old males and females (24 months old). Blood samples were taken from the heart and serum was used to assay the levels of TLR2, TLR4, HSP60, HSP90, HMGB1, TAS and TOS.

Results: HGMB1, TOS and OSI were higher in old females than in young females (p<0.05). TLR2 and TLR4 levels were higher in young females than in young males; however, HSP60 was lower in young females than in young males (p<0.01). HSP60 was lower in old males than in young males (p<0.05). Positive correlations were present between TLR2, TLR4 and HMGB1 (p=0.001, r=0.096; p=0.012, r=0.867; p=0.002, r=0.935, respectively) as well as between HMGB1 and HSP60 (p=0.049, r=0.756) in young females. A negative correlation was detected between HSP90 and TLR4 in young males (p=0.000, r=-0.982), and between HSP60 and TLR2, OSI in old males (p=0.014, r=-0.856; p=0.042, r=-0.772, respectively).

Conclusion: The results of present study indicated that age and sex may be important factors for serum levels of TLR2, TLR4, HSP60, HSP90, HMGB1 and OSI as well as the correlation between them.

Keywords: Sex, Age, High Mobility Group Box 1, Heat Shock Proteins, Toll-Like Receptors, Oxidative Stress.

TAS, TOS, TLR2, TLR4, HSP60, HSP90 ve HMGB1 Serum Düzeylerinde Yaşa ve Cinsiyete Bağlı Değişiklikler

ÖZET

Amaç: Hücresel ve fizyolojik fonksiyonlar yaşa ve cinsiyete özgü bir şekilde etkilenebilir. Bu çalışmanın amacı, Toplam Antioksidan Durumu (TAS), Toplam Oksidan Durumu (TOS), Oksidatif Stres İndeksi (OSI), Toll-Benzeri Reseptör 2 (TLR2), Toll-Benzeri Reseptör 4 (TLR4), Isı Şok Proteini 60 (HSP60), Isı Şok Proteini 90 (HSP90) ve Yüksek Mobilite Grup Kutusu 1 (HMGB1) serum seviyelerindeki cinsiyete ve yaşa özgü farklılıkları ve aralarındaki korelasyonu incelemektir.

Gereç ve Yöntem: Bu çalışmada her biri yedi hayvan içeren dört fare grubu kullanıldı: genç erkekler ve dişiler (6 aylık); yaşlı erkekler ve dişiler (24 aylık). Kan örnekleri kalpten alındı ve TLR2, TLR4, HSP60, HSP90, HMGB1, TAS ve TOS seviyelerini değerlendirmek için serum kullanıldı.

Bulgular: HGMB1, TOS ve OSI yaşlı dişilerde genç dişilere göre daha yüksekti (p<0,05). TLR2 ve TLR4 seviyeleri genç dişilerde genç erkeklerden daha yüksekti; ancak HSP60 genç dişilerde genç erkeklere göre daha düşüktü (p<0,01). HSP60 yaşlı erkeklerde genç erkeklere göre daha düşüktü (p<0,05). Genç dişilerde TLR2, TLR4 ve HMGB1 arasında (sırasıyla p=0,001, r=0,096; p=0,012, r=0,867; p=0,002, r=0,935) ve HMGB1 ile HSP60 arasında (p=0,049, r=0,756) pozitif korelasyonlar mevcuttu. Genç erkeklerde HSP90 ile TLR4 arasında (p=0,000, r=-0,982), yaşlı erkeklerde HSP60 ile TLR2 ve OSI arasında negatif korelasyon saptandı (sırasıyla p=0,014, r=-0,856; p=0,042, r=-0,772).

Sonuç: Bu çalışmanın sonuçları, TLR2, TLR4, HSP60, HSP90, HMGB1 ve OSI'nin serum düzeyleri ve aralarındaki korelasyon için yaş ve cinsiyetin önemli faktörler olabileceğini göstermiştir.

Anahtar Kelimeler: Cinsiyet, Yaş, Yüksek Mobilite Grup Kutusu 1, Isı Şok Proteinleri, Toll Benzeri Reseptörler, Oksidatif Stres.

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INTRODUCTION

The issue of age- and sex- related differences plays a vital role in our understanding of differences in physiological and pathophysiological mechanisms. In recent years, researchers have shown an increased interest in age- and sex-specific changes in several diseases, including cancer, cardiovascular, metabolic, coronavirus disease, and neural diseases (1-4). To date, however, cellular mechanism(s) underlying age- and sex-related changes in several diseases has not been fully elucidated. In addition, under normal physiological conditions age- and sex- specific variability in serum biomarkers and/or proteins such as oxidative stress markers, High Mobility Group Box 1 (HMGB1), Heat Shock Proteins (HSPs), and Toll-Like Receptors (TLRs) are likely to be present. In fact, it should be stated that little is known regarding differences in them depending on age and sex.

Oxidative stress can be called as a vital signaling factor in various cellular pathways, by which apoptosis or survival is achieved. Oxidative stress is observed when the antioxidant defense system is submerged by oxidants. Excess oxidative stress can alter cellular signaling transduction by which it can take a part in the process of several diseases. Oxidative stress degree has linked with age and sex. It increases with aging (5), and males have higher oxidative stress than age-matched females (6).

HMGB1 and HSPs, which are members of the protein family known as damage-associated molecular pattern molecule (DAMP), are released out of the cell in the presence of intracellular stress such as oxidative stress. In response to oxidative stress, activation and/or release of HMGB1 and HSPs are important to whether a cell goes to apoptosis or survive (7, 8). HMGB1 and HSPs as inducers, sensors, and mediators of stress can mediate their effects via binding to TLRs on the cell membrane (9, 10). For example, HMGB1 and HSPs could induce inflammatory cells to active through TLR2 and TLR4 (11). In addition, according to the intracellular pathway activated in this binding, cell death pathway, inflammation, or cell survival pathways are activated. Of the TLR receptors, TLR2 is generally associated with cell survival or regeneration, while TLR4 is reported to be associated with cell death pathways (12).

With advance age, circulating levels of DAMP become elevated, which is proposed that they are likely to be crucial biomarkers to predict or evaluate the risk for disease (10). However, whether levels of oxidative stress, TLR2, TLR4, HSP60, HSP90, and HMGB1 vary with age and/or sex, and whether there is a correlation between them is unclear under normal physiological processes. Overall, the aim of this study is to explore age- and sex-specific differences in the circulating levels of TAS, TOS, TLR2, TLR4, HSP60, HSP90, and HMGB1.

MATERIAL AND METHODS

Experimental Animals: Experimental protocols were approved by the Local Animal Ethics Committee of Düzce University, Düzce, Türkiye (Approval no: 2021/03/06). Animals were exposed to a 12 h- light/dark cycle, and they were fed with a standard pellet food and water *ad libitium*. Four groups of mice (*M. musculus*), each including seven animals, were used in the present study: young males and females (6 months old); old males and females (24 months old).

Biochemical Analyses

Blood Sample: Experimental animals were weighted and then anesthetized with a mixture of ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). A blood sample was collected by cardiac puncture and allowed to clot at room temperature for 30 min. The samples were then centrifugated at 4000 rpm for 10 min at +4°C. Samples were stored at -80 until analysis. The serum was used to assay the levels of TLR2, TLR4, HSP60, HSP90, HMGB1, TAS and TOS, following the manufacturer's instructions.

Total Antioxidant Status (TAS): Total antioxidant status (TAS) was measured using commercially available kits (Rel Assay Diagnostic, Ankara, Turkey) with Mindray's BS-300 auto chemistry analyzer according to the manufacturer's instructions. The results were expressed as mmol Trolox equivalent/L (13).

Total Oxidant Status (TOS): TOS levels were measured using commercially available kits (Rel Assay Diagnostic, Ankara, Turkey) with Mindray's BS-300 auto chemistry analyzer according to the manufacturer's instructions. The assay was calibrated with hydrogen peroxide and the results were expressed in terms of micromolar hydrogen peroxide equivalent per liter (μmol H_2O_2 equivalent/L) (14).

Oxidative Stress Index (OSI): The ratio of TOS to TAS was accepted as the oxidative stress index (OSI) (15, 16). For calculation, the resulting unit of TAS was converted to μmol/L, and the OSI value was calculated according to the following Formula: OSI (arbitrary unit) = TOS (μmol H_2O_2 equivalent/L) / TAS (μmol Trolox equivalent/L).

Measurement of Circulating TLR2, TLR4, HSP60, HSP90 and HMGB1: TLR2, TLR4, HSP60, HSP90, HMGB1 levels were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Elabscience, USA) with Biotek ELx800 microplate reader according to protocols provided by the manufacturer.

Statistical Analyses: Data was analyzed with either the Statistical Package for Social Sciences (SPSS: version 21.0; SPSS Inc., IL, USA)

or GraphPad Prism (Version 9.3.1, La Jolla, CA). Comparisons between groups for the serum levels of TLR2, TLR4, HSP60, HSP90, HMGB1, TAS, TOS and OSI were performed using two-way ANOVA with age, sex as main factors, and age-by-sex interactions. A Bonferroni post-hoc. test was used when the effect of factor(s) and/or interactions on the dependent variables were significant. Relationships between TLR2, TLR4, HSP60, HSP90, HMGB1, TAS, TOS, and OSI in young males and females, and old males and females, separately were analyzed with Pearson's *r* test for correlation. Results were expressed as the mean ± standard deviation (mean ± SD). *p* values less than 0.05 were considered as statistically significant.

RESULTS

TAS, TOS and OSI: Although the overall effects of sex and age on TAS were significant, with no significant sex-by-age interaction effects (Table 1; $p < 0.05$), the individual comparisons between sexes and ages were not. This means that TAS did slightly higher in young females (1.23 ± 0.12 mmol/L) than in young males (1.11 ± 0.19 mmol/L), and it did slightly lower in old males (0.88 ± 0.18 mmol/L) and females (1.07 ± 0.12 mmol/L) when compared to young groups (1.11 ± 0.19 mmol/L and 1.23 ± 0.12 mmol/L, respectively; Figure 1A). There was also a significant age-specific difference in TOS and OSI (Table 1; $p < 0.001$) such that TOS and OSI was significantly higher in old females (20.32 ± 9.97 µmol/L for TOS, and 1.65 ± 0.75 for OSI) than in young females (5.90 ± 2.77 µmol/L for TOS, and 0.50 ± 0.22 for OSI; Figure 1B and C; $p < 0.01$). TOS and OSI tended to be higher in old males than in young males; however, it did not reach statistically a significant level. Taken together, these results indicate that oxidative stress was markedly observed in old females.

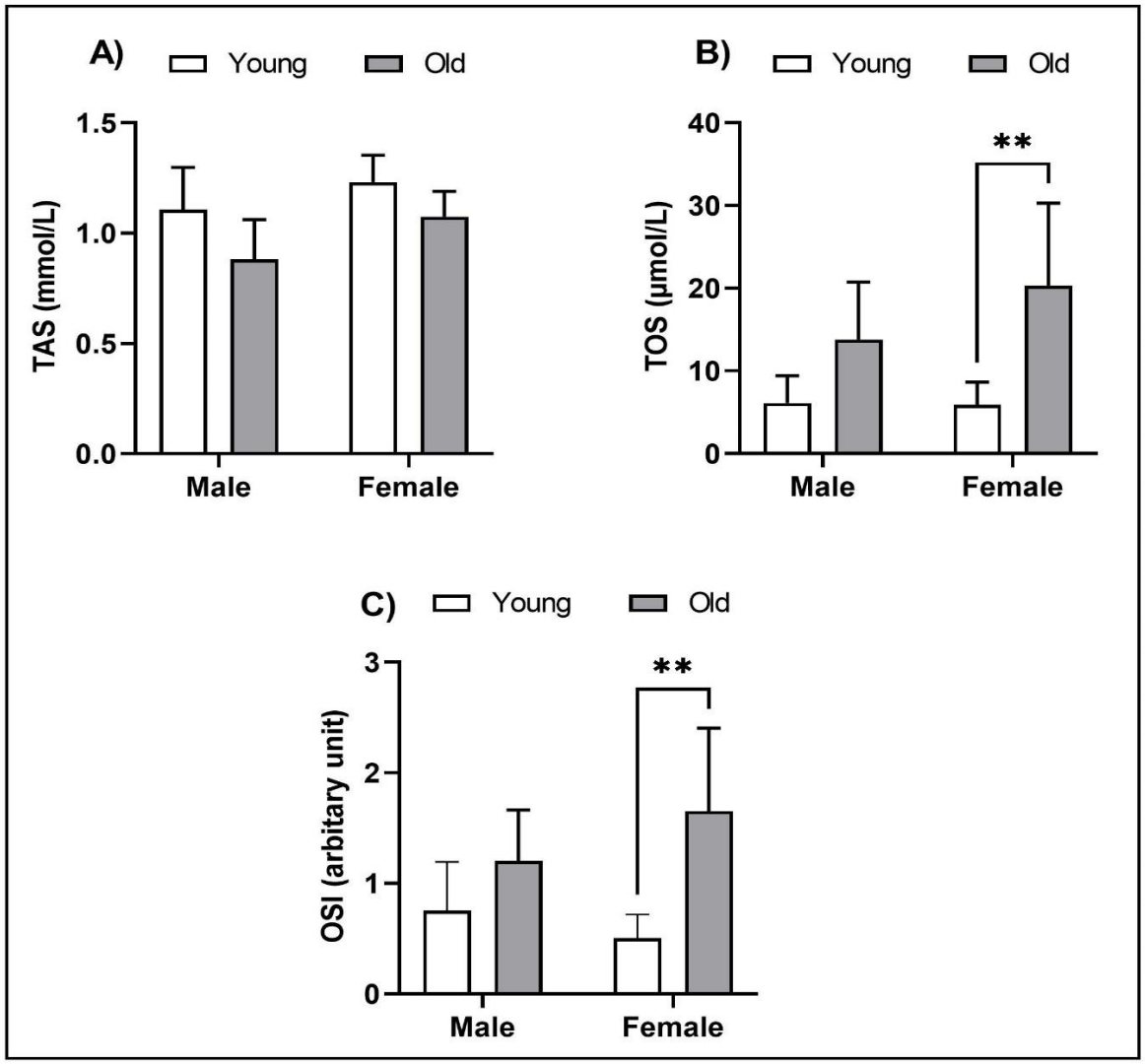

Figure 1. TAS, TOS and OSI in young and old males and females. (Abbreviations: TAS, Total Antioxidant Status; TOS, Total Oxidant Status; OSI, Oxidative Stress Index. Young male, n = 7; old male, n = 7; young female, n = 7; old female, n = 7. Values were expressed as mean ± SD (**p <0.01) and analyzed with two-way ANOVA with a Bonferroni post-hoc. test.)

Table 1 Effects of sex, age and sex-by-age interaction on parameters.

Parameters	*p* value		
	Sex	Age	Sex x Age
TAS	**0.0130**	**0.0035**	0.5511
TOS	0.2454	**0.0001**	0.1471
OSI	0.6094	**0.0003**	0.0784
TLR2	**0.0294**	**0.0035**	0.0762
TLR4	**0.0038**	0.2964	**0.0160**
HSP60	**0.0020**	0.3583	**0.0025**
HSP90	0.4542	0.9946	**0.0091**
HMGB1	0.5269	0.0965	**0.0167**

Abbreviations: TAS, Total Antioxidant Status; TOS, Total Oxidant Status; OSI, Oxidative Stress Index; TLR2, Toll-like Receptor 2; TLR4, Toll-Like Receptor 4; HSP-60, Heat Shock Protein 60; HSP90, Heat Shock Protein 90; HMGB1, High Mobility Group Box 1. Bold values denote significance at $p < 0.05$.

TLR2, TLR4, HSP60, HSP90, and HMGB1: A significant effect of sex and age, with no sex-by-age interaction effect was observed on TLR2 (Table 1; p <0.05), where serum TLR2 level was significantly higher in young females (0.47 ± 0.05 ng/ml) than in young males (0.41 ± 0.04 ng/ml; p <0.05; Figure 2A). In addition, old females had significantly low level of TLR2 (0.39 ± 0.03 ng/ml) when compared to young females (0.47 ± 0.05 ng/ml; p <0.01; Figure 2A). There were a significant effect of sex (p <0.01) and sex-by-age interaction (p <0.05) on TLR4 levels (Table 1), such that TLR4 level was significantly higher in young females (0.54 ± 0.02 ng/ml) than in young males (0.51 ± 0.01 ng/ml; p <0.01; Figure 2B). There was also a significant sex-specific effect on HSP60 level, with a significant sex-by-age interaction effect (Table 1; p <0.01). HSP60 level was significantly lower in young females (0.39 ± 0.10 ng/ml) than in young males (1.08 ± 0.47 ng/ml; p <0.001; Figure 2C). Besides, although there was no significant difference in HSP60 level between young and old females, HPS60 level was significantly lower in old males (0.64 ± 0.17 ng/ml) than in young males (1.08 ± 0.47 ng/ml; p <0.05).

Although the overall effect of sex-by-age interaction on HSP90 was significant (Table 1; p <0.05), post-hoc analyses did not show a significant difference in the individual sex and age groups. This means that HSP90 level was slightly lower in old males (82.90 ± 34.21 pg/ml) than in young males (117.40 ± 31.05 pg/ml); however, it was slightly higher in old females (108.10 ± 35.69 pg/ml) than in young females (73.75 ± 26.74 pg/ml; Figure 2D). In addition, young females had a modest low level of HSP90 when compared to young males.

The overall sex- and age- related differences in serum HMGB1 level were no significant; however, a significant sex-by-age interaction was observed (Table 1; p <0.05), where serum HMGB1 level was significantly higher in old females (45.64 ± 15.07 pg/ml) than young females (30.71 ± 6.77 pg/ml; p <0.05; Figure 2E). By contrast, serum HMGB1 level was slightly lower in old males (34.48 ± 5.60 pg/ml) than in young males (37.41 ± 5.76 pg/ml; p <0.05), with not statistically significance.

Correlation Analysis: Pearson Correlation Analysis was used to determine the relationship between TLR2, TLR4, HSP60, HSP90, HMGB1, TAS, TOS, and OSI. The investigated correlation may be of different size and shape in different subgroups. If the factors such as age and sex were not separately examined in the correlation analysis, a high correlation would be obtained. Therefore, the correlation analysis was performed separately in young males and females, and in old males and females.

In young males, the results of the correlation analysis are set out in Figure 3A. A strong negative (r = -0.982) and significant (p <0.01) correlation were found between TLR4 and HSP90 in young males. This means that while TLR4 level increased, HMGB1 level decreased or vice versa. Besides, there was a strong positive correlation with high significance between TOS and OSI (r =0.940; p <0.01).

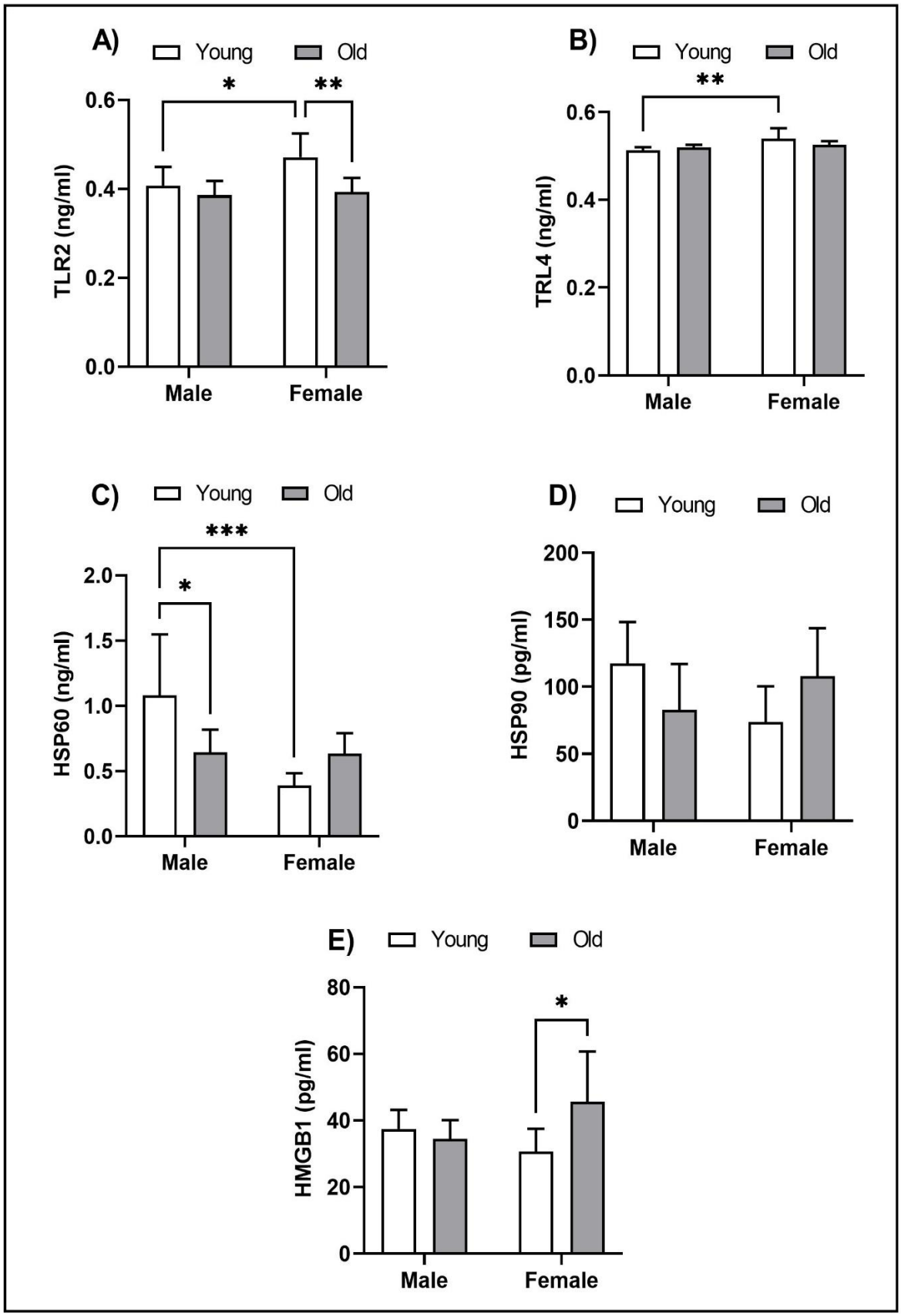

Figure 2. Serum levels of TLR2, TLR4, HSP60, HSP90, HMGB1 in young and old males and females. (Abbreviations: TLR2, Toll-like Receptor 2; TLR4, Toll-Like Receptor 4; HSP60, Heat Shock Protein 60; HSP90, Heat Shock Protein 90; HMGB1, High Mobility Group Box 1. Young male, n = 7; old male, n = 7; young female, n = 7; old female, n = 7. Values were expressed as mean ± SD (*p <0.05; **p <0.01; ***p <0.001) and analyzed with two-way ANOVA with a Bonferroni post-hoc. Test).

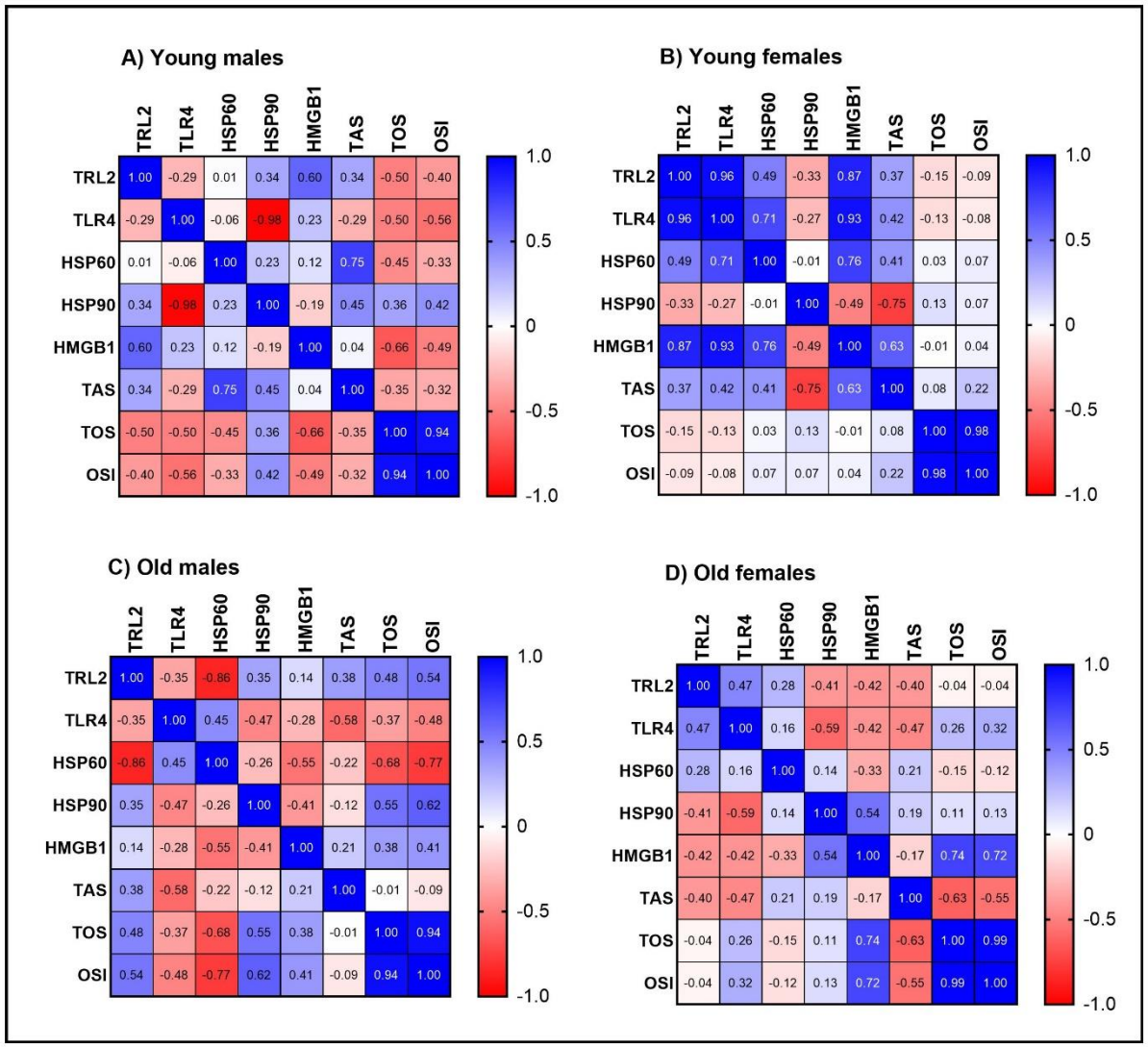

Figure 3. The Pearson correlation matrix of variables. (Abbreviations: TAS, Total Antioxidant Status; TOS, Total Oxidant Status; OSI, Oxidative Stress Index; TLR2, Toll-like Receptor 2; TLR4, Toll-Like Receptor 4; HSP60, Heat Shock Protein 60; HSP90, Heat Shock Protein 90; HMGB1, High Mobility Group Box 1. Young male, n = 7; old male, n = 7; young female, n = 7; old female, n = 7. The color scale bar shows the Pearson r value: the more positive the correlation (closer to 1), the darker the shade of blue; the more negative the correlation (closer to −1), the darker the shade of red).

In young females, the results of the correlation analysis are presented in Figure 3B. The results displayed that a strong positive correlation with high significance was present between TLR2 and TLR4 in young females (r =0.960; p <0.01), which means that TLR2 increased when TLR4 increased or vice versa. Similarly, there were significantly strong correlations between HMGB1 and TLR2 (r =0. 960; p <0.05), TLR4 (r =0.935; p <0.01), and HSP60 (r =0.756; p <0.05). In addition, there was a strong positive correlation with high significance between TOS and OSI (r =0.979; p <0.01), like observed in young males.

In old males, the results of the correlation analysis are shown in Figure 3C. A strong negative (r = 0.856) and significant (p <0.05) correlation was found between TLR2 and HSP60 in old males. This data demonstrates that TLR2 level increased with HSP60 or vice versa. In addition, there was significantly a high correlation between HSP60 and OSI (r =0.772; p <0.05). This finding shows that HSP60 level increased with an increase in OSI. Like data obtained in young males and females, there was significantly a strong correlation between TOS and OSI in old males (r =0.942; p <0.01).

In old females, the results of the correlation analysis are displayed in Figure 3D. A strong correlation with high significance was only observed between TOS and OSI in old females (r =0.986; p <0.01).

DISCUSSSION

To investigate age- and sex-specific changes in possible biomarkers or differences in cellular signaling pathways under physiological and pathophysiological processes is vitally important to

modulate and/or develop new therapeutic approaches. To this end, this study set out to assess changes in circulating levels of TLR2, TLR4, HSP60, HSP90, HMGB1, TAS, and TOS as well as to explore the correlation between them in different sex and age groups. The main findings of the study were (I) TOS, OSI, and HGMB1 were higher in old females than in young females, (II) TLR2 and TLR4 levels were higher in young females than in young males, (III) HSP60 and HSP90 levels were lower in young females than in young males, (IV) HSP60 was lower in old males than in young males, (V) Positive correlations was present between TLR2, TLR4, and HMGB1 as well as between HMGB1 and HSP60 in young females (VI) A negative correlation between HSP90 and TLR4 was found in young males, and also a negative correlation between HSP60 and TLR2, OSI was detected in old males.

Age- and sex-related differences in oxidative stress and the role of such differences in the mechanism underlying the physiological and pathophysiological processes take great attention for a long time (5, 6). In the present study, the findings showed that oxidative stress increased with aging, markedly in the females, evidenced by the fact that TOS and OSI was higher in the old females than in the young females. Contrary to expectations, this study did not find a significant difference TAS, TOS and OSI in the young and old females when compared to age-matched males, which means that oxidative stress is likely to be independent of sex in normal physiologic conditions. It is important to mention that a significant difference in TOS and OSI was observed only between young and old females, rather than in the males, suggesting that the effect of aging on oxidative stress may be more dominate or evident in the females. In accordance with the present results, a previous study indicated that no differences in antioxidant barrier efficacy and oxidative index in rats as well as between age-matched males and females from healthy subjects were obtained, however, oxidative stress in females, but not in males was reported to increase as age increased (17). However, it should be stated that there are some experimental and clinical studies showing a marked significant alternation in oxidative stress between males and females, which are contrary to the results of present study and the mentioned investigation. This conflicting evidence may partly be explained by differences in oxidative stress measurements, methods, markers studies, and samples (18).

HMGB1 is highly conserved protein, which exists from yeast to human. It has been known that circulating level of HMGB1 has changed in several physiological dysfunctions such as in stress induced depression (19), epilepsy (20), and cardiovascular dysfunctions (21). However, very little was present in the literature on the question of does serum HMGB1 levels changes depending on sex and age as a normal biological process. In a recent clinical study, it has been indicated that HMGB1 levels show differences depending on sex, age, and race, such as its levels are higher in females than in males, and in Blacks than in White. In addition, HMGB1 levels rise with increasing age (22). Similarly, the findings of the present study indicated that circulating levels of HMGB1 was affected by sex-by-age interaction, such as its level was higher in old females than in young females. In addition, it should be indicated that there was no difference in HMGB1 between young and old males, unlike females. On the contrary, there are conflicting evidence in the literature as to age- and sex- specific changes in the circulating levels of HMGB1. Data from a previous study stated that circulating level of HMGB1 exerted age-dependent change, indicated by the fact that serum HMGB1 levels was lower in healthy old human when compared to healthy young human (23). However, another clinical study has showed that HMGB1 levels do not show sex- and age-specific differences in healthy control (24).

HSPs are critical chaperon proteins induced by several factors, such as oxidative stress, inflammation, which play a crucial role in homeostasis, cell survival etc. Among HSPs, HSP72, HSP27, HSP90 and HSP60 are of great interest in extracellular compartment as alarmins. That means they take a part in the transmission of signal about alarm (25). Their cellular functions show differences; for example, HSP90 may exert anti-apoptotic features although HSP60 may be important in pro-apoptotic process (26). Their circulating levels may be assessed to be a potential marker for some diseases, including diabetes (27) and acute lymphoblastic leukemia (28). Therefore, it is important to identify age- and sex-specific alternation in circulating levels of HSPs for clarifying their role in health and disease. For example, as organism is getting older, decreased levels of HSPs may make maintaining homeostasis to difficult, resulting in biological dysfunctions-cancer and cell senescence etc. (29). In addition, several cellular process such as cell differentiation and cell cycle regulate HSPs synthesis under normal physiological function (30). In aging, circulating HSP70 became decrease in healthy population and with advance in age, it was found a positive association between HSP70, inflammation and frailty in older patients (30). A previous study indicated that circulating levels of HSP60 decreased as getting older (31), which supports available data emerging from the present study. In young males, circulating HSP60 levels were higher than in old males, and young females also had lower HSP60 levels than in young males. These results indicate that an effect of sex and sex-by-age interaction on circulating HSP60 levels exists in the current study, wherein circulating levels of HSP60 declined as

males got older. What is curious about this result is that age-related changes in circulating HSP60 was not observed in females. Akin to HSP60, a sex-by-age interaction had a significant effect on circulating HSP90, which indicated that circulating HSP90 appeared to decrease with age in males although it tended to increase with age in females. It should be emphasized that pair-wise comparison between groups did not reach a statistically significant level. Similarly, circulating HSP60 levels become increase after menopause in healthy women and no correlation between HSP60 and age as well as with how much years pass following menopause was observed. In addition, HSP60 levels were found to be increased in the situation of estrogen deprivation or deficiency following ovariectomy (32, 33). In peripheral blood cells, age-specific increase in the basal levels of HSP90 was obtained under normal physiological conditions (30). Circulating HSP90 levels showed a significant positive correlation with age in healthy subject while HSP90 showed a weak negative correlation with age in patients subjects regardless of sex (27). However, controversy data are present. In healthy young age-matched men and women, circulating levels of HSP60, HSP70 and oxidative stress markers such as reduced glutathione and thiobarbituric acid reactive substance did not indicate sex-dependent differences (34). Although tissue specific differences in the protein expression of HSP90 and HSP60 have been obtained (35), age- and sex-specific alteration in circulation HSP90 levels in healthy conditions has not yet been exactly clarified. As far as the author knows, it is the first study to explore this issue.

TLRs, found in almost all cells, are crucial proteins that recognize DAMPs. Based on their location in the cell, TLRs can be divided into two types, including cell membrane TLRs (TLR1, TLR2, TLR4, TLR5, and TLR10) and intercellular TLRs (TLR3, TLR7, TLR8, and TLR9). A surge in their expression results in excess activation of the inflammatory process (36). TLR2 and TLR4 have been suggested as a biomarker for many diseases, such as breast cancer (37), colorectal cancer (38), multiple sclerosis (39), acute aortic dissection (40), and diabetes (41). TLR2 and TLR4 are known as the soluble form of TLRs, which may act as an inhibitor for excessive TLR activation (36). In addition, signaling pathways initiated by TLRs activation might be negatively regulated by soluble form of TLRs found in the circulation. In other words, circulating TLRs or soluble TLRs act as a receptor for ligands which induces activation of TLRs, thereby limiting binding of the ligands to TLRs (42). This means that the inflammatory process mediated by TLRs might be prevented by soluble TLRs in the circulation. In the skin sample, the expression profile of TLRs has shown differences depending on age, indicated that TLR2 and TLR4 expression in adult skin specimen was lower than in embryonic and fetal one (43). Salivary concentration of TLR2 decreased with age, as evidenced by healthy individuals aged 30-39 years having lower TLR2 levels than those aged 6-15 years (44). It should be emphasized that it was not focused on sex-related changes in salivary concentration of TLR2 in the mentioned study. Overall, these results state importance of age and sex in the levels of TLR2 in different body fluid. In support of these results, the result of the current study indicated that a sex- and age-specific differences in TLR2 and a sex- and age-by-sex interaction in TLR4 levels was observed, wherein circulating TLR2 level was lower in old females than in young females, and TLR2 and TLR4 in young females was high compared to young males. Interestingly, age-related decrease in TLR2 and TLR4 were not markedly observed in males. Females have higher TLRs expression on macrophage, estrogen deficiency following ovariectomy results in decreased expression of TLR2 and TLR4 on macrophages (45). In another study, it has been showed that expression of TLRs on platelet was higher in women than in men (46). In contrast, conflicting evidence is also present on sex-specific differences in circulating TLR4 level, indicated by a clinical study reported that serum TLR4 levels did not exert difference between males and females from control groups (40).However, it has been unknown whether estrogen levels can modulate cleavage and/or ectodomain shedding of surface TLRs, resulting in increased serum TLRs levels. Although serum estrogen levels were not measured in the present study, it may be speculated that estrogen may be responsible for higher circulating levels of TLRs because 24 months old female mice had lower serum TLR2 than 6 months old female mice. Further study is required to assess the role of estrogen level in circulating TLRs and their association in health and disease state. In addition, age-specific changes in the function of TLRs are not exactly clarified (47); however, changes in circulating levels of TLRs may be given as a possible explanation for such alteration in their function.

The cellular mechanism underpinning HMGB1 release and/or activity have not yet exactly clarified. Oxidative stress may be an important factor for the functions of HMGB1 (48, 49). Treatment of experimental animals with lipopolysaccharide resulted in increased reactive oxygen generation, which in turn induced the release of HMGB1 into circulation. That means a link between oxidative stress and HMGB1 release (50). In old females, there was a likely positive correlation between TOS and HMGB1. However, the observed difference between TOS and HMGB1 in this study was not significant (r = 0.740, p = 0.057). HMGB1 is also crucial in depressive behavior. Serum levels of HMGB1 increased in stress induced depression. It has been proposed that

the underlying mechanism of HMGB1 in depressive behavior might be mediated via TLR4, which led to cytokine induction (19). In epilepsy patients, there was a positive correlation of HMGB1, TLR4 with epilepsy seizure as well as higher serum HMGB1 and TLR4 levels than healthy subjects (20). In addition, a current study has been set out to determine whether circulating levels of HGMB1 is an important factor in epilepsy patients' resistance to drugs (51). With respect to the question, it is found that serum levels of HGMB1 increase in patients with drug resistance although there is no association between HMGB1 and seizures, by which it is suggested that targeting circulating HMGB1 may be a good point for evaluating the therapy efficacy in the epilepsy treatment. It should be mentioned that age- and sex-specific differences in this correlation were not evaluated in these clinical studies. In reviewing the literature, no data was found on the association between HMGB1 and age and sex under normal physiological conditions. In the present study, a significant positive correlation between HMGB1 and TLR2, TLR4 and HSP60 only in young females was observed. In accordance with a previous study, there was a significant correlation between TLR2 and HMGB1 levels and a trend towards a positive correlation between TLR4 and HMGB1 with no reaching significant levels (52). HSPs can circulate throughout the body via bloodstream, and they exert cellular effects via cell surface receptors. HSPs are known as endogenous ligands for TLRs, especially TLR2 and TLR4 (53). For example, HSP60 induced NF-κB signaling pathway by binding to TLR2 or TLR4, resulting in production of cytokines and chemokines (54, 55). In addition, HSP60 induced expression of TLR2 (33). In old males, a negative correlation was found between HSP60 and TLR2 in the present study. In addition, a negative correlation was found between HSP90 and TLR4 in young males. In contrast, it was found that HSP60 was positively correlated with TLR4 and a correlation between TLR2 and HSP60 levels with no reaching significant levels (52). The negative correlation between HSP60 and OSI in old males is interesting because general knowledge is that oxidative stress result in expression of HSPs, especially HSP90 and HSP60. The reason for such controversy may arise from that in response to oxidative stress, circulating levels of HSPs may differently change when compared to their protein expression in a cell or tissue.

In the current study, the mechanism by which age and sex leads to changes in the serum levels of mentioned parameters was not addressed here but remains an interesting avenue for further study. Both transcriptional and post-transcriptional mechanisms should be investigated. Circulating levels of HMGB1, HSPs and TLRs with aging and sex differences. It should be kept in mind that the presence or absence of correlation does not explain causality. Therefore, further works should be designed to elucidate the causal mechanisms underlying associations between these parameters based on sex and age. The findings of this study provide important insights into the role of age and sex in circulating levels of parameters studied and make contributions to the current literature. Therefore, the present study lays the groundwork for future research on these subjects.

CONCLUSION

Scientists are increasingly researching age-related cellular changes and the physiological dysfunctions caused by these changes to produce new treatment approaches and solutions. The results indicated that age-related increases in oxidative stress varied between sexes and tended to be greater in females, as demonstrated by higher levels of TOS and OSI in old females. TLR2, TLR4 and HSP60 indicated differences between sexes and females had higher levels of TLR2 and TLR4, and lower level of HSP60 in circulation. Moreover, HMGB1 increased in the circulation as females, but not males, got older. These data might provide the basis information on the role of sex and age in variation of circulating levels of HSPs, TLRs and HMGB1. An understanding of age- and sex-related differences in HMGB1, HSPs and TLRs as well as their interaction under normal biological process as biomarkers is critical to develop new therapies to ameliorate disease and/or to clarify differentiation in the cellular pathways associated with aging and biological sex.

Ethics Committee Approval: All experiments were performed with the permission of the Local Animal Ethics Committee of Düzce University, Düzce, Türkiye (Approval no: 2021/03/06).

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REFERENCES

1. Ghimire A, Howlett SE. An acute estrogen receptor agonist enhances protective effects of cardioplegia in hearts from aging male and female mice. Exp Gerontol. 2020;141:111093.
2. Rubin JB, Lagas JS, Broestl L, et al. Sex differences in cancer mechanisms. Biol Sex Differ. 2020;11(1):17.

3. Brand BA, de Boer JN, Sommer IEC. Estrogens in schizophrenia: progress, current challenges and opportunities. Curr Opin Psychiatry. 2021;34(3):228-37.
4. Connelly PJ, Azizi Z, Alipour P, et al. The Importance of Gender to Understand Sex Differences in Cardiovascular Disease. Can J Cardiol. 2021;37(5):699-710.
5. Luo J, Mills K, le Cessie S, Noordam R, van Heemst D. Ageing, age-related diseases and oxidative stress: What to do next? Ageing Res Rev. 2020;57:100982.
6. Kander MC, Cui Y, Liu Z. Gender difference in oxidative stress: a new look at the mechanisms for cardiovascular diseases. J Cell Mol Med. 2017;21(5):1024-32.
7. Tang D, Kang R, Zeh HJ, 3rd, Lotze MT. High-mobility group box 1, oxidative stress, and disease. Antioxid Redox Signal. 2011;14(7):1315-35.
8. Szyller J, Bil-Lula I. Heat Shock Proteins in Oxidative Stress and Ischemia/Reperfusion Injury and Benefits from Physical Exercises: A Review to the Current Knowledge. Oxid Med Cell Longev. 2021;2021:6678457.
9. Tsan MF. Heat shock proteins and high mobility group box 1 protein lack cytokine function. J Leukoc Biol. 2011;89(6):847-53.
10. Huang J, Xie Y, Sun X, et al. DAMPs, ageing, and cancer: The 'DAMP Hypothesis'. Ageing Res Rev. 2015;24(Pt A):3-16.
11. Ooboshi H, Shichita T. [DAMPs (damage-associated molecular patterns) and inflammation]. Nihon Rinsho. 2016;74(4):573-8.
12. Li X, Jiang S, Tapping RI. Toll-like receptor signaling in cell proliferation and survival. Cytokine. 2010;49(1):1-9.
13. Erel O. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. Clin Biochem. 2004;37(4):277-85.
14. Erel O. A new automated colorimetric method for measuring total oxidant status. Clin Biochem. 2005;38(12):1103-11.
15. Kosecik M, Erel O, Sevinc E, Selek S. Increased oxidative stress in children exposed to passive smoking. Int J Cardiol. 2005;100(1):61-4.
16. Yumru M, Savas HA, Kalenderoglu A, et al. Oxidative imbalance in bipolar disorder subtypes: a comparative study. Prog Neuropsychopharmacol Biol Psychiatry. 2009;33(6):1070-4.
17. Brunelli E, Domanico F, La Russa D, Pellegrino D. Sex differences in oxidative stress biomarkers. Curr Drug Targets. 2014;15(8):811-5.
18. Sanchez-Rodriguez MA, Mendoza-Nunez VM. Oxidative Stress Indexes for Diagnosis of Health or Disease in Humans. Oxid Med Cell Longev. 2019;2019:4128152.
19. Lian YJ, Gong H, Wu TY, et al. Ds-HMGB1 and fr-HMGB induce depressive behavior through neuroinflammation in contrast to nonoxid-HMGB1. Brain Behav Immun. 2017;59:322-32.
20. Kan M, Song L, Zhang X, Zhang J, Fang P. Circulating high mobility group box-1 and toll-like receptor 4 expressions increase the risk and severity of epilepsy. Braz J Med Biol Res. 2019;52(7):e7374.
21. Raucci A, Di Maggio S, Scavello F, et al. The Janus face of HMGB1 in heart disease: a necessary update. Cell Mol Life Sci. 2019;76(2):211-29.
22. Chen L, Zhu H, Su S, et al. High-Mobility Group Box-1 Is Associated With Obesity, Inflammation, and Subclinical Cardiovascular Risk Among Young Adults: A Longitudinal Cohort Study. Arterioscler Thromb Vasc Biol. 2020;40(11):2776-84.
23. Fu GX, Chen AF, Zhong Y, Zhao J, Gu YJ. Decreased serum level of HMGB1 and MyD88 during human aging progress in healthy individuals. Aging Clin Exp Res. 2016;28(2):175-80.
24. Umit EG, Baysal M, Bas V, et al. Value of Extracellular High Mobility Group Box 1 (HMGB1) in the Clinical Context of Immune Thrombocytopenia. Journal of Clinical and Experimental Investigations. 2019;10(2).
25. Giuliano JS, Jr., Lahni PM, Wong HR, Wheeler DS. Pediatric Sepsis - Part V: Extracellular Heat Shock Proteins: Alarmins for the Host Immune System. Open Inflamm J. 2011;4:49-60.
26. Purandhar K, Jena PK, Prajapati B, Rajput P, Seshadri S. Understanding the role of heat shock protein isoforms in male fertility, aging and apoptosis. World J Mens Health. 2014;32(3):123-32.
27. Ocana GJ, Sims EK, Watkins RA, et al. Analysis of serum Hsp90 as a potential biomarker of beta cell autoimmunity in type 1 diabetes. PLoS One. 2019;14(1):e0208456.
28. Pawlik-Gwozdecka D, Gorska-Ponikowska M, Adamkiewicz-Drozynska E, Niedzwiecki M. Serum heat shock protein 90 as a future predictive biomarker in childhood acute lymphoblastic leukemia. Cent Eur J Immunol. 2021;46(1):63-7.
29. Murshid A, Eguchi T, Calderwood SK. Stress proteins in aging and life span. Int J Hyperthermia. 2013;29(5):442-7.
30. Njemini R, Lambert M, Demanet C, Kooijman R, Mets T. Basal and infection-induced levels of heat shock proteins in human aging. Biogerontology. 2007;8(3):353-64.
31. Rea IM, McNerlan S, Pockley AG. Serum heat shock protein and anti-heat shock protein antibody levels in aging. Exp Gerontol. 2001;36(2):341-52.

32. Kim YS, Koh JM, Lee YS, et al. Increased circulating heat shock protein 60 induced by menopause, stimulates apoptosis of osteoblast-lineage cells via up-regulation of toll-like receptors. Bone. 2009;45(1):68-76.
33. Koh JM, Lee YS, Kim YS, et al. Heat shock protein 60 causes osteoclastic bone resorption via toll-like receptor-2 in estrogen deficiency. Bone. 2009;45(4):650-60.
34. Benini R, Nunes PRP, Orsatti CL, Portari GV, Orsatti FL. Influence of sex on cytokines, heat shock protein and oxidative stress markers in response to an acute total body resistance exercise protocol. Journal of Exercise Science & Fitness. 2015;13(1):1-7.
35. Voss MR, Stallone JN, Li M, et al. Gender differences in the expression of heat shock proteins: the effect of estrogen. Am J Physiol Heart Circ Physiol. 2003;285(2):H687-92.
36. El-Zayat SR, Sibaii H, Mannaa FA. Toll-like receptors activation, signaling, and targeting: an overview. Bulletin of the National Research Centre. 2019;43(1):187.
37. El-Kharashy G, Gowily A, Okda T, Houssen M. Association between serum soluble Toll-like receptor 2 and 4 and the risk of breast cancer. Mol Clin Oncol. 2021;14(2):38.
38. Paarnio K, Tuomisto A, Vayrynen SA, et al. Serum TLR2 and TLR4 levels in colorectal cancer and their association with systemic inflammatory markers, tumor characteristics, and disease outcome. APMIS. 2019;127(8):561-9.
39. Hossain MJ, Morandi E, Tanasescu R, et al. The Soluble Form of Toll-Like Receptor 2 Is Elevated in Serum of Multiple Sclerosis Patients: A Novel Potential Disease Biomarker. Front Immunol. 2018;9:457.
40. Li T, Jing JJ, Yang J, et al. Serum levels of matrix metalloproteinase 9 and toll-like receptor 4 in acute aortic dissection: a case-control study. BMC Cardiovasc Disord. 2018;18(1):219.
41. Liu S, Wang X, Kai Y, et al. Clinical significance of high mobility group box 1/toll-like receptor 4 in obese diabetic patients. Endocr J. 2021.
42. Liew FY, Xu D, Brint EK, O'Neill LA. Negative regulation of toll-like receptor-mediated immune responses. Nat Rev Immunol. 2005;5(6):446-58.
43. Iram N, Mildner M, Prior M, et al. Age-related changes in expression and function of Toll-like receptors in human skin. Development. 2012;139(22):4210-9.
44. Staller S, Lindsay AK, Ramos ED, Thomas P, Srinivasan M. Changes in salivary microbial sensing proteins CD14 and TLR2 with aging. Clin Oral Investig. 2020;24(7):2523-8.
45. Scotland RS, Stables MJ, Madalli S, Watson P, Gilroy DW. Sex differences in resident immune cell phenotype underlie more efficient acute inflammatory responses in female mice. Blood. 2011;118(22):5918-27.
46. Koupenova M, Mick E, Mikhalev E, et al. Sex differences in platelet toll-like receptors and their association with cardiovascular risk factors. Arterioscler Thromb Vasc Biol. 2015;35(4):1030-7.
47. Shaw AC, Panda A, Joshi SR, et al. Dysregulation of human Toll-like receptor function in aging. Ageing Res Rev. 2011;10(3):346-53.
48. Yu Y, Tang D, Kang R. Oxidative stress-mediated HMGB1 biology. Front Physiol. 2015;6:93.
49. Chen R, Kang R, Tang D. The mechanism of HMGB1 secretion and release. Exp Mol Med. 2022;54(2):91-102.
50. Abdulmahdi W, Patel D, Rabadi MM, et al. HMGB1 redox during sepsis. Redox Biology. 2017;13:600-7.
51. Walker LE, Sills GJ, Jorgensen A, et al. High-mobility group box 1 as a predictive biomarker for drug-resistant epilepsy: A proof-of-concept study. Epilepsia. 2022;63(1):e1-e6.
52. Devaraj S, Dasu MR, Park SH, Jialal I. Increased levels of ligands of Toll-like receptors 2 and 4 in type 1 diabetes. Diabetologia. 2009;52(8):1665-8.
53. Calderwood SK, Mambula SS, Gray PJ, Jr. Extracellular heat shock proteins in cell signaling and immunity. Ann N Y Acad Sci. 2007;1113:28-39.
54. Hori M, Nishida K. Toll-like receptor signaling: defensive or offensive for the heart? Circ Res. 2008;102(2):137-9.
55. Milani A, Basirnejad M, Bolhassani A. Heat-shock proteins in diagnosis and treatment: an overview of different biochemical and immunological functions. Immunotherapy. 2019;11(3):215-39.