

Laboratorinė medicina.
2019, t. 21, Nr. 2(82), p. 51–55.

Circulating Microvesicles Association with Oxidative and Chronic Stress in Healthy and Coronary Artery Disease Affected Male Populations

Vytautas Žėkas^{1,2}
Rėda Matuzevičienė^{1,2}
Dovilė Karčiauskaitė^{1,2}
Dalius Vitkus^{1,2}
Mantas Radzevičius^{1,2}
Aušra Janiulionienė²
Aušra Linkevičiūtė^{1,2}
Sandra Kutkienė³
Zita Kučinskienė^{1,2}

Summary

Introduction. Endothelial microvesicles (EMV) could be potential biomarkers for early diagnosis of myocardial infarction yet their release in post myocardial infarction and association with oxidative stress largely not studied. The objective was to determine the differences in EMV numbers in patients after myocardial infarction and their association with oxidative stress in comparison with healthy controls

Materials and Methods. 15 post myocardial infarction (MI) patients and 81 healthy controls were included into the study. Blood plasma samples tested using BD Fortessa cytometer (BD, San Jose). Three EMV populations were examined: 1) CD144+, CD42a-, CD61-, 2) CD144+, CD42a+, CD61-, 3) CD105+, CD42a-, CD61- and percentage of EMV, expressing CD62e was determined. Malondialdehyde (MDA) concentration determined by a Nexera X2 UHPLC system (Shimadzu, Japan).

Results. Individuals participating in the study grouped into three groups: 1) younger healthy group (25–39 year old), 2) elder healthy group (40–60 year old) and 3) patients (40–60 year old). CD62e expression on EMV membrane was higher in patients group in comparison to control younger and elder groups. MDA concentration median was higher in patients group. EMV populations and their activation levels were associated with MDA concentration only in the control groups at different age intervals.

Conclusions. Activation of EMV was higher in post MI group in all populations of endothelial microvesicles except for CD144+ and CD42a-. Counts of circulating EMV populations and their CD62e expression showed associations with oxidative and chronic stress markers.

Keywords: microvesicles, flow cytometry, oxidative stress.

¹Department of Physiology, Biochemistry, Microbiology and Laboratory Medicine, Institute of the Biomedical Sciences, Faculty of Medicine, Vilnius University, M. K. Čiurlionio str. 21/27, LT-03101 Vilnius

²Center of Laboratory Medicine, Vilnius University Hospital Santaros Clinics, Santariškių str. 2, LT-08661 Vilnius

³Clinic of Cardiac and Vascular Diseases, Institute of Clinical Medicine, Faculty of Medicine, Vilnius University, Santariškių str. 2, LT-08661 Vilnius

INTRODUCTION

In the past decade, extracellular vesicles (ECV) were understood as the potential players of intercellular communication. Now these vesicles are intensively investigated in the range of pathological processes in hopes to use them as biomarkers for clinical di-

agnostics. This heightened interest is not surprising since current biomarkers have left unmet needs in the early diagnosis of several diseases. Atherosclerosis and its clinical manifestations such as coronary heart disease is one good example out of many. Oxidative stress is implicated in the pathogenesis of athero-

sclerosis [1] and is caused by production of excess reactive oxygen species (ROS). ROS are formed naturally and act as regulators of various cell functions and biological processes. The oxidative stress processes are important in the pathogenesis of myocardial infarction and post-stroke survival [2]. Malondialdehyde (MDA) concentration in blood plasma is proposed as an effective oxidative stress biomarker. Most of this substance is produced during reaction process from decomposition of products of lipid peroxidation and it helps to evaluate oxidative stress level in vascular system [3]. Since uncontrolled production of reactive oxygen species is implicated in vascular injury [4], the association of ECV numbers with MDA is important. Fundamental studies describe their involvement in oxidative stress and inflammation affecting angiogenesis [5]. Yet we still need further evaluation of chronic inflammation and sustained stress involvement in the recurrence of coronary stenosis and cardiac failure [6]. Cortisol is considered a stress hormone. Produced in adrenal gland this hormone primarily regulates metabolism by increasing blood sugar and aiding in the metabolism of other substances like fats or proteins [7]. It also suppresses immune system [8] and it is essential for maintenance of normal blood pressure [9]. Cortisol could be detected in saliva human hair or serum [10]. At least one study showed a prospective association between cortisol stress reactivity and progression of sub-clinical atherosclerosis [11]. Recent studies found that elevated cortisol response to mental stress linked to higher cardiac troponin T levels [12]. These findings show importance of psychosocial stress in subclinical atherosclerosis. Microvesicles and exosomes could participate in this process and give valuable information about the stress level. It was found that exosomes could be enriched by heat shock protein 70 (HSP70) and modulated by stress and cortisol in rainbow trout (*Oncorhynchus mykiss*) [13], but clinical studies are lacking in this field.

MATERIAL AND METHODS

Study subject inclusion criteria

Test group included fifteen patients who were treated for myocardial infarction in Vilnius University Hospital Santaros Clinics three months prior

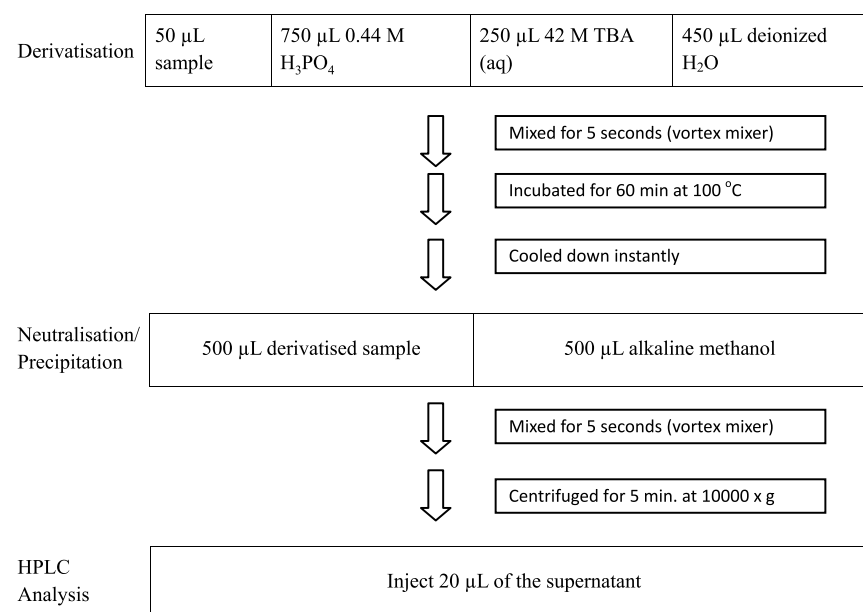


Fig. Flow diagram for derivatization and HPLC method for the determination of malondialdehyde in serum

the inclusion. 81 healthy individuals who were examined between December 2015 and July 2017 have formed a control group. Control subjects had no prior history of acute cardiovascular disease and were not under any treatment for cardiovascular diseases. For determining depression level in the control group the Beck questionnaire was used. Study protocol was approved by local Vilnius university Bioethics committee (No.: 158200-18-990-495 and 158200-15-807-319). Control group individuals were placed into two groups: 1) younger healthy group (25–39 year old) (n=52) and 2) elder healthy group (40–60 year old) (n=29).

Blood sampling and testing

For enumeration of EMV 4 mL of blood was drawn from the arm vein into the citrated Vacutainer tubes. To produce platelet free plasma the tubes then subjected to differential centrifugation. First they were centrifuged at 3000 g rcf for 10 minutes and three quarters of recovered plasma was transferred into a sterile tube and centrifuged again at 10000 g rcf for 30 minutes. The resulting pellet was reconstituted with 1 ml of serum free RPMI medium (Biological Industries, Israel) and analyzed using LSR Fortessa (BD, California) flow cytometer.

The method was based on the scientific article published by Khoshsorur GA and his colleagues in 2000 [14]. The frozen patient serum was thawed at room temperature.

50 L of thawed serum was mixed with 750 L 0.44 M phosphoric acid solution, 250 L of 42 mM thiobarbituric acid solution and 450 L of deionised water. The prepared samples were incubated in a thermostate at 100 °C for 60 minutes. After incubation, the samples were rapidly cooled in an ice bath. In the next step 500 L of the sample was taken and added 500 L of methanol solution (1:1). The sample was mixed by shaking and centrifuge for 3 min at 10000 g. After centrifugation 500 L of the centrifugate was added to the chromatographic vial and analyzed the sample via high performance liquid chromatography method based on method described in literature [14]. The received measurement data was processed by LabSolutions software (USA, Atlanta).

Flow cytometry

For this experiment, we used the protocol described in our previous publication [15]. All samples were labeled with anti CD144-FITC (anti VE-cadherin), anti CD105-BV421 (anti endoglin), anti CD42a-PerCP (anti glycoprotein IX), anti CD62e-PE (anti e-selectin) and anti CD61-APC (anti Integrin beta-3) (BD, San Jose, California). All events were gated on a forward vs. side-scattered light dot plot according to their forward scatter intensity (size). Each analysis included 1,000,000 events. Total absolute counts of EMV populations were quantified by flow cytometry and expressed as microvesicles/ L. CD62e expression was calculated as percent-

Table 1. Demographic and clinical characteristics of the population studied. The data values are presented as means (SD) or as medians and interquartile range (IQR) depending on variable distribution. NS indicates not significant.

Variables	Median (IQR) or means (SD)			P
	Healthy 25–40 year old (n=52)	Healthy 40–60 year old (n=29)	Post MI group 40–60 year old (n=15)	
Age, years	Means (SD): 30.31 (0.49)	Means (SD): 47.28 (0.84)	Means (SD): 51.93 (1.54)	<0.001
Systolic blood pressure	Median (IQR): 131.5 (12)	Median (IQR): 131 (18.5)	Median (IQR): 120 (15)	0.021
Diastolic blood pressure	Median (IQR): 78.5 (8)	Median (IQR): 82 (12.5)	Median (IQR): 80 (6)	NS
Heart rate	Median (IQR): 71.5 (14.75)	Median (IQR): 71 (17.5)	Median (IQR): 64 (12)	NS
Malondialdehyde concentration	Median (IQR): 92.17 (30.98)	Median (IQR): 101.61 (34.85)	Median (IQR): 154.72 (8.21)	<0.001
Cortisol concentration	Median (IQR): 0.23 (0.09)	Median (IQR): 0.2 (0.11)	Not evaluated	NS

Table 2. Characteristics of the EMV (endothelial microvesicles) populations presented as medians and interquartile range (IQR). CD62e expression is calculated as a percentage from total microvesicles count. Also are given P values for median differences between groups.

EMV population	Microvesicles count (microvesicles/ L) (Median±IQR)				CD62e expression (%) (Median±IQR)			
	Healthy 25–40 year old	Healthy 40–60 year old	Patients (40–60 year old)	P	Healthy 25–40 year old	Healthy 40–60 year old	Patients (40–60 year old)	P
CD105+ EMV	22.89±75.2	10.93±53.81	33.68±205.47	NS	1.75±4.58	1.4±5.05	14.8±17.6	<0.001
CD 144+, CD42a+ EMV	322.79±232.64	173.37±426.84	215.56±133.67	NS	5.55±6.13	7±5.95	1.8±6	0.03
CD 144+, CD42a- EMV	254.68±185.15	304.73±210.64	73.29±49.23	<0.001	41.25±87.8	63.7±79.45	98.9±5.8	0.002
PMV	257.14±353.43	186.92±307.86	338.99±375	NS	–	–	–	

Table 3. Association between atherosclerosis risk factors, MDA concentration and total count of EMV populations, and their CD62e expression

Groups	Younger healthy control group			Elder healthy control group			
	CD144+ CD42a- EMV	CD144+ CD42a- and CD62e+ EMV	CD105+ and CD62e+ EMV	PMV	CD144+ CD42a+	CD105+ and CD62e+ EMV	CD105+ EMV+
MDA concentration in serum	R=0.28 P=0.04	NS	NS	R=0.64 P<0.001	R=0.49 P=0.007	P=0.008 R=0.49	R=0.38 P=0.04
Depression level	NS	R=0.33, P=0.018	NS	NS	NS	NS	NS
Age	NS	NS	R=-0.54 P<0.001	NS	NS	NS	NS

age from total count of respective endothelial microvesicles populations.

Cortisol concentration in serum analysis

For ELISA analysis, ELISA Kit (LDN®, Germany) was used. To the 96-well plate 20 L of blood serum were added. Than 200 L of conjugate were added to each well and incubated for 1 hour at room temperature. 3 cycles of washing were performed using 400 L of wash solution per well per cycle. Then 100 L of substrate were added to each well and the plate was incubated for 15 minutes at room temperature. 100 L of the stopping solution were added to all wells, mixed and scanned at 450±10 nm. The standard densities of the optical densities of the standard substances provide a calibration curve to determine the concentration of serum cortisol (ng/mL). Gemini automatic analyzer (Strattec Biomedical, Germany) was used based on manufacturer's recommendations

Statistical analysis

Results were presented as median and interquartile range if not stated otherwise. Variables were tested by the non-paired non-parametric Wilcoxon test and Spearman test for correlation coefficient. The confidence level for the test was set at 0.05. Statistical analysis was carried out using SPSS version 21 (IBM).

RESULTS

Clinical characteristics

Demographic and clinical data are displayed in Table 1.

Characteristics of all four populations of microvesicles and their CD62e expression are presented in Table 2.

Three different populations of endothelial microvesicles were detected: 1) CD105+, CD42a- and CD61- EMV; 2) CD144+, CD42a- and CD61- EMV; 3) CD144+, CD42a+ and CD61- EMV.

Each subpopulation had one endothelial cell marker CD105 (endoglin) or CD144 (VE-cadherin) that could possibly reflect their function according to the association with the marker.

Groups' characteristics

Patients had significantly elevated concentration of MDA (P<0.001). There was a mean age difference (Table 1) between the two groups, since there were more post myocardial infarction (MI) individuals between 50–60 years of age and there were more controls between 40 and 50 years old (d=-4.83±1.62, P=0.005). In the younger healthy group, 9 individuals were smoking at the time of blood collection and one individual smoked before. In the elder healthy group four individuals were smoking. In the patients' group, no one was smoking at the time of blood collection, but all of them smoked before the cardiac event. Eleven individuals from elder control group and nine individuals from young

ger control group were smoking at the time of blood collection.

Numbers of circulating endothelial microvesicles

Between groups there was median difference between total count of VE-cadherin EMV with thrombocytic markers (322.79 (younger control group) vs 173.37 (elder control group) vs 215.56 (patients), $P<0.001$).

When comparing CD62e expression on EMV membrane between controls and patients all EMV populations expressed CD62e differently on their membranes. There was also median difference between control groups when comparing EMV activation levels: CD105+ EMV (1.75 vs 1.4, $P<0.001$) and CD144+ and CD42a-EMV (5.55 vs 7, $P=0.002$) expressed CD62e differently.

Association between systolic blood pressure, depression level smoking and malondialdehyde concentration and total count of EMV populations and their CD62e expression

Associations between MDA concentration, depression level and age is shown in Table 3.

In post MI group total count of CD105+ EMV positively correlated with systolic blood pressure ($R=0.54$, $P=0.038$). CD62e+ level in CD144+ and CD42a- EMV negatively correlated with systolic blood pressure ($R=-0.54$, $P=0.037$); CD62e+ level in CD144+ and CD42a+ EMV negatively correlated with systolic blood pressure ($R=-0.53$, $P=0.04$). Heart rate and diastolic blood pressure had no association with endothelial microvesicles in all three groups.

In the younger control group only the total count of CD144+ and CD42a-EMV positively correlated with malondialdehyde concentration ($P=0.04$, $R=0.28$). In the elder control

group MDA concentration positively correlated with total count of PMV ($P<0.001$, $R=0.64$), total count of CD144+ and CD42a- EMV ($P=0.007$, $R=0.49$), total count of CD105+ EMV ($P=0.04$, $R=0.38$) and with CD62e expression in CD105+ EMV population ($P=0.008$, $R=0.49$). In post MI group there was no association between MDA concentration and total counts of EMV or their activation levels.

Smoking as influencing factor on microvesicles counts and oxidative stress was also investigated. Only in the elder healthy group smoking individuals had higher activation level in the CD144+ and CD42a+ population (smoking 96.8% vs nonsmoking 48.5%, $P=0.025$) and also lower cortisol concentration in serum (0.15 nonsmoking vs 0.21 smoking, $P=0.043$).

Since we have observed association between MDA and microvesicles counts in two healthy groups, serum cortisol in these groups was measured in order to assess this phenomenon. In a young healthy group serum cortisol concentration negatively correlated with MDA concentration ($R=-0.31$, $P=0.028$). No correlation was found between cortisol concentration and microvesicles counts as well as between the groups there was no cortisol concentration median difference.

DISCUSSION

The objective of our study was to determine the association of endothelial microvesicles numbers with oxidative stress in healthy individuals and patients with coronary heart disease. MDA concentration was used as an oxidative stress marker and cortisol was used as a chronic stress marker. Two main markers used to describe EMV in our study were CD105 (endoglin) and CD144 (VE-cadherin) which led to detection of two EMV subpopulations. To characterize the portion of activated EMV in each subpopulation, CD62e (e-selectin) was used as an activation marker. In this study, we did not find any remarkable difference between groups when looking into EMV

numbers but activation level was different for almost all EMV populations. MDA concentration was also found to be different between all groups and was increased in patients. Interestingly MDA concentration was associated only with the increase of EMV concentration and activation level in a control group and not in patients. This could mean that oxidative stress is associated with higher release of EMV in relatively healthy population. Cortisol was then used to evaluate if the association between oxidative and chronic stress could be seen in association with EMV numbers. Cortisol could be used as a chronic stress marker [16] and monitoring it could bring a benefit for stress assessment. In literature, the connection between MDA and cortisol concentrations was observed and tied to depression [17]. In the study, association between cortisol and MDA concentration, but not association between microvesicle numbers and cortisol concentration was found. The association between hair cortisol and microvesicle numbers was seen in our previous publication [15], but not here. This shows that momentary changes in cortisol concentration probably do not affect the microvesicles numbers. On the other hand we found that smoking influenced CD144+, CD42a+ and CD62e+ microvesicles numbers and cortisol concentration. Seemingly, smoking people had lower cortisol concentration. This was not the case for younger healthy group. We suspect age is an important factor and different triggers could be working at different age intervals for generation and release of EMV.

In conclusion, activation of EMV was higher in post MI patients group in all populations of endothelial microvesicles except for VE-cadherin without thrombocytic markers EMV. Counts of circulating endothelial microvesicles populations and their CD62e expression showed associations with oxidative and chronic stress markers. ♦

Gauta:	2019 06 04
Priimta spaudai:	2019 06 28

Santrauka

CIRKULIUOJANČIŲ MIKRODALELIŲ RYŠYS SU OKSIDACINIŲ IR LĖTINIŲ STRESU SVEIKOJE IR KORONARINĖ ŠIRDIES LIGA SERGANČIŲ VYRŲ POPULIACIJOSE

Vytautas Žėkas, Rėda Matuzevičienė, Dovilė Karčiauskaitė, Dalius Vitkus, Mantas Radzevičius, Aušra Janilionienė, Aušra Linkevičiūtė, Sandra Kutkienė, Zita Kučinskienė

Įvadas. Endotelio mikrodalelės (EMD) galėtų būti miokardo infarkto ir koronarinės širdies ligos biožymenys, tačiau jų gamyba ir išsiskyrimas po miokardo infarkto ir sąsajos su oksidaciniu stresu nėra iki galo aiškios. Šio tyrimo tikslas buvo nustatyti EMD kieki sveikų asmenų ir miokardo infarkto prieš tris mėnesius patyrusių pacientų periferiniame kraujyje bei galima ryšį tarp oksidacinio streso ir EMD kiekio.

Tiriamieji ir metodai. Į tyrimą buvo įtraukta 15 pacientų po miokardo in-

farcto (MI) ir 81 sveikas asmuo. Kraujo plazmos mėginiai buvo tiriami naudojant „BD Fortessa“ citometrą (BD, San Jose). Buvo tiriamos trys EMD populiacijos: 1) CD144+, CD42a-, CD61-, 2) CD144+, CD42a+, CD61-, 3) CD105+, CD42a-, CD61-. Kiekvienoje EMD populiacijoje buvo nustatyta procentinė dalis mikrodalelių, turinčių CD62e ant savo membranos (aktyvacijos lygis). Malono dialdehido (MDA) koncentracija buvo nustatyta naudojant „Nexera X2 UHPLC“ sistemą (Shimadzu, Japonija).

Rezultatai. Tyrimo dalyviai suskirstyti į tris grupes: 1) jaunesnių sveikųjų grupę (25–39 metų), 2) vyresnio amžiaus sveikųjų grupę (40–60 metų) ir 3) miokardo infarktą patyrusių pacientų (40–60 metų). CD62e ant savo membranos turinčių EMD populiacija buvo didesnė pacientų grupėje, palyginti su kontrolinėmis grupėmis. MDA koncentracijos mediana buvo didesnė pacientų grupėje. EMD populiacijų kiekiai ir jų aktyvacijos lygiai buvo susiję su MDA koncentracija tik kontrolinėse grupėse.

Išvados. EMD aktyvacijos lygis pacientų grupėje buvo didesnis visose endotelio mikrodalelių populiacijose, išskyrus CD144 + ir CD42a-. Cirkuliuojančių EMD populiacijų kiekiai ir jų CD62e raiška parodė ryšį su oksidacinio ir lėtinio streso žymenimis.

Reikšminiai žodžiai: endotelio mikrodalelės, tėkmės citometrija, oksidacinis stresas.

REFERENCES

1. Stocker R, Keaney JF. Role of oxidative modifications in atherosclerosis. *Physiol Rev* 2004; 84: 1381–478.
2. Filippa CD, Cuzzocrea S, Rossi F, Marfella R, D'Amico M. Oxidative stress as the leading cause of acute myocardial infarction in diabetics. *Cardiovasc Drug Rev* 2006; 24: 77–87.
3. Lee R, Margaritis M, Channon K, Antoniadou C. Evaluating oxidative stress in human cardiovascular disease: methodological aspects and considerations. *Curr Med Chem* 2012; 19(16): 2504–20.
4. Mittal M, Siddiqui MR, Tran K, Reddy SP, Malik AB. Reactive oxygen species in inflammation and tissue injury. *Antioxid Redox Signal* 2014; 20(7): 1126–67.
5. Bodega G, Alique M, Bohórquez L, Morán M, Magro L, Puebla L, et al. Young and especially senescent endothelial microvesicles produce NADPH: The fuel for their antioxidant machinery. *Oxid Med Cell Longev* 2018; 2018: ID3183794.
6. Wang J, Liu J, Zhu X, Zhang M, Wang S, Zheng Z. Nonculprit lesion progression in patients with ST elevation myocardial infarction after primary percutaneous coronary intervention. *International Heart Journal* 2014; 55: 48–52.
7. McEwen BS. Central effects of stress hormones in health and disease: understanding the protective and damaging effects of stress and stress mediators. *Eur J Pharmacol* 2008; 583(2–3): 174–85.
8. Morey JN, Boggero IA, Scott AB, Segerstrom SC. Current directions in stress and human immune function. *Curr Opin Psychol* 2015; 5: 13–7.
9. Kelly JJ, Mangos G, Williamson PM, Whitworth JA. Cortisol and hypertension. *Clinical and Experimental Pharmacology and Physiology* 1998; 25(S1): S51–6.
10. Wright KD, Hickman R, Laudenslager ML. Hair cortisol analysis: a promising biomarker of HPA activation in older adults. *Gerontologist* 2015; 55 (Suppl 1): S140–5.
11. Hamer M, Endrighi R, Venuraju SM, Lahiri A, Steptoe A. Cortisol responses to mental stress and the progression of coronary artery calcification in healthy men and women. *PLoS One* 2012; 7(2): e31356.
12. Lazzarino AI, Hamer M, Gaze D, Collinson P, Steptoe A. The association between cortisol response to mental stress and high-sensitivity cardiac troponin T plasma concentration in healthy adults. *J Am Coll Cardiol* 2013; 62(18): 1694–701.
13. Faught EL, Henrickson L, Vijayan MM. Plasma exosomes are enriched in Hsp70 and modulated by stress and cortisol in rainbow trout. *J Endocrinol* 2017; 232(2): 237–46.
14. Khoschrorur GA, Winkhofer-Roob BM, Rabl H, Auer Th, Peng Z, Schaur RJ. Evaluation of a sensitive HPLC method for the determination of malondialdehyde, and application of the method to different biological materials. *Chromatographia* 2000; 52(3–4): 181–4.
15. Žėkas V, Matuzevičienė R, Karčiauskaitė D, Mažeikienė A, Burokienė N, Radzevičius M, Janilionienė A, Linkevičiūtė A, Kučinskienė ZA. Chronic and oxidative stress association with total count of endothelial microvesicles in healthy young male plasma. *Adv Clin Exp Med* 2019; 28(5): 683–92.
16. Lee DY, Kim E, Choi MH. Technical and clinical aspects of cortisol as a biochemical marker of chronic stress. *BMB Reports* 2015; 48(4): 209–16.
17. Islam MR, Islam MR, Ahmed I, Moktadir AA, Nahar Z, Islam MS, et al. Elevated serum levels of malondialdehyde and cortisol are associated with major depressive disorder: a case-control study. *SAGE Open Med* 2018; 6: 2050312118773953.