Title: Title: The PCSK9 Protein is not necessarily a Risk Factor for MDD

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Abstract

Major depressive disorder (MDD) is ne of the common psychiatric disorders that is characterized by abnormal neurobiological responses. Proprotein convertase subtilisin/kexin 9 (PCSK9) have a important role in cholesterol homeostasis. This study was aimed to investigate PCSK9 level and oxidative stress with MDD disease.The study included 30 with MDD and 30 healthy controls. The. Blood was collected in sterile tubes and the serum PCSK9 concentration, SOD and GPx activity were determined by ELISA kits. TAC, TOS, MDA and copper concentration were determined manually.There was a significant increase in PCSK9 level in the patient group (p<0.05). ROC curve had sensitivity of 57% and specificity of 52% and 0.928 (95% CI, 0.86-0.996) for PCSK9, in the patient group (p <0.001). It was found that MDA (p = 0.036) level was higher in MDD group, but TAC (p = 0.445) level, SOD (p = 0.148), GPx (p = 0.019) activities and copper concentration were lower in the patient group in compare with the control group.In conclusion, this study results confirm the relationship of oxidative stress and major depressive disorder and also suggest a link between PCSK9 and MDD disease.

Keywords: Oxidative stress, Copper, Major depressive disorder and PCSK9.

Highlights:

1-In MDD individuals there is a significant increase PCSK9 level.

2- TAC, SOD and GPx activities and copper concentration level were lower in the MDD group.

3- TOS and MDA were significantly higher in MDD group.

4- The oxidative stress has role the pathogenesis in major depressive disorder.

1. Introduction

Major depressive disorder (MDD) is a common psychiatric disorder in the worldwide. It has an prevalence rate of more than 35% in the population of most of countries (Liu et al., 2021). It was reported that MDD due to its progressive nature, commonly causes disability in subjects which are associated with low activity, few social function and high disease burden (Grases et al., 2019). MDD affects sleep patterns, appetite, perception, emotion, energy balance and mood changes, even suicidal thoughts (Michel et al., 2012). Many studies support that oxidative stress and elevated inflammation are underlying pathophysiology of MDD and are commonly found in depressed individuals (Echeverria et al., 2016). The mechanisms by which imbalance between oxidant/antioxidant may be lead to the depressive symptoms are yet to be clarified, although previously reported that the brain cells are very sensitive to the elevated oxygen free radicals (Liu et al., 2015). Increasing the oxidant agents to the antioxidant defense system may contribute to the oxidative damage in brain cells and necrosis (Van der Vliet et al., 1999). It was also found that elevated Reactive oxygen species (ROS) generation in brain cells with a weakened antioxidant defense system causes a major damage of the cell membrane lipids, proteins and DNA that it leads to cellular dysfunction and illness (Hasler et al., 2007). Oxidative stress has been found to be connected to several factors in brain cells receptors for example; serotonin, Gamma amino butyric acid (GABA), and Glutamate (Ten Have et al., 2018). Notably, one of the proteins found to be associated with oxidative stress is the protein factor PCSK9 (proprotein convertase subtilisin / kexin type 9). PCSK9 was expressed in various tissue such as central nervous system (CNS), kidney, pancreas and intestine (Seidah et al 2014; Seidah et al., 2003).

Accordingly, PCSK9 as an intracellular factor plays an essential role in cholesterol metabolism through regulating Low Density Lipoprotein receptors (LDLR) in the membrane (Stoekenbroek et al., 2019). The LDLR is the key cell surface receptor for cholesterol trafficking and uptake through intaking Low Density Lipoprotein cholesterol LDL-C and mediating its metabolism. LDLR gene expression is tightly regulated via cholesterol availability which functions through a negative feedback mechanism (Schlüter et al., 2017). The PCSK9 with an enzymatic function effectively regulates the posttranscriptional of LDLR and possibly overexpressed PCSK9 might be induced cholesterol accumulation. Moreover, large-scale population studies revealed that PCSK9 is commonly associated with inflammatory and neurodegenerative diseases (Kurata et al., 2013).

Accumulated LDL as a result of PCSK9 overexpression in neurons was usually linked to the generation of ox-LDL, thereby oxidative damage in neurons. As a result, damage in neurons contributes to mental disorders including MDD (Need et al., 2012). Although, many studies have suggested a potential link between PCSK9 and several mental diseases including schizophrenia and bipolar disorder, the possible link between PCSK9 and MDD has not yet been investigated (Shi et al., 2008). Therefore, the present study was designed to provide an appropriate answer for the question of whether PCSK9 is associated with MDD disease.

2. Material and Methods

2.1. Participants and Sample collection

The number 30 healthy individuals and 30 individuals with MDD were selected after assessment the characteristics of depression by Hamilton Depression Rating Scale (HDRS) in Iran. Participants were received both written and oral information about the study and matched in terms of age, BMI and sex in two groups. Inclusion criteria for participants with MDD new case to be diagnosed by a psychiatrist and have a HAM-D (Hamilton Depression Rating Scale) index above 17 in patients, ages between 18-65, non-use of SSRIs (Selective serotonin reuptake inhibitors), no smoking, alcohol and chronic physical illness such as epilepsy, Autoimmune diseases, neoplasia and infectious diseases. Exclusion criteria were taking for lipids-balance medications, ethanol and drug addiction, hyperlipidemia, nephritic syndrome, infectious diseases, mental disorders, and taking antioxidant and vitamin medications from the previous month. From all of control and MDD individuals in fasting state (12 h overnight) 10 ml of blood were obtained. Then serum samples separated inside sterile tubes and stored at -20 °C.

2.2. Lipid profile

The total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C) of serum were measured by Hitachi 7150 automated analyzer (Hitachi, Japan). LDL-C concentration was calculated by this formula: LDL-C = TC - [HDL-C + TG/ 5] and for VLDL-C calculation Nobert formula was used (VLDL-C = TG/5)

2.3. Measurement of PCSK9 protein concentration

For measure the serum PCSK9 protein concentration, the ELISA method was used according to commercial kit (ZellBio GmbH, Germany) protocol and by Elisa reader (synergy HTX, Biotech, USA).

2.4. Measurement of Total Antioxidant Capacity (TAC)

TAC measurement was performed by ferric reducing antioxidant power assay (FRAP). In this method, the antioxidant reduced ferric ion to ferrous ion . At first, FRAP reagent, including acetate buffer (25 ml, 300 mM, pH 3.6) with 16 ml acetic acid was prepared. To obtain 2,4,6-tripyridyl-s-triazine (TPTZ) (2.5-ml), TPTZ (10 mM), HCl (40 mM) and $FeCl_3 \cdot 6H_2O$ (2.5 ml) were used. Then, 300µL reagent was added per 10 µL of samples and incubated at 37 °C for 10 min. The maximum absorption of the complex among Fe^{2+} and TPTZ was measured by Spectrophotometer (Bell-Italy) at a wavelength of 532nm was used (Mohseni et al., 2020).

2.5. Measurement of Total Oxidant Status (TOS)

TOS measurement was assayed by ferrous oxidation in xylenol orange. After oxidation, the ferric ions can react with xylenol orange and exist a colored dye in acidic medium. Finally, the absorption of complex was measured at a wavelength of 560 nm (Asadi et al., 2019).

2.6. Measurement of Malondialdehyde level

This method based on the thiobarbituric acid (TBA) (Merck, Germany) reaction with malondialdehyde (MDA) as a production of lipid peroxidation. First, 1.5 ml of 20% acetic acid and 0.2 ml of 8.1% SDS were added for 50 µl of the plasma sample, and mixed gently. After addition of TBA and distilled water (4 ml), the solution was incubated in a boiling water at 90 °C for 60 min. Then, 3 ml of n-butanol was added and centrifuged for 15 min at 3000g. Finally, the absorbance of organic layer was measured by fluorometric assay (Jasco, Japan). The intensity of fluorescence is measured at the stimulus wavelength of 515nm and the wavelength of 553nm emission (Mazloomi et al., 2020).

2.6. Superoxide dismutase (SOD) enzyme activity

Serum SOD enzyme activity was measured by the ZellBio kit (ZellBio GmbH, Germany). In this method, under enzymatic reaction superoxide radicals is converted to hydrogen peroxide and

oxygen. Finally, the colored compounds resulting from this enzymatic reaction were measured at 420 nm according to U/mg protein unite.

2.7. Glutathione peroxidase (GPx) enzyme activity

Serum GPx enzyme level was measured by the ZellBio kit (ZellBio GmbH, Germany). In this method, the GPx enzyme as an electron donor, reduce the selenocysteine in the active site by the glutathione. The enzyme converts GSH to GSSG, and the remaining GSH in the reaction medium can regenerate DTNB and produce a yellow color. The yellow complex has absorbtion at 412 nanometers according to the instruction. Color production is inversely related to enzyme activity. Finally, the GPx activity was reported as U/mg protein unite.

2.8. Measurement of Copper concentration

First, standard copper solutions were made and incubated for 1 hour at 20 °C. Then, 1ml of lanthanum chloride solution (Sigma-Aldrich Co, Steinheim, Germany) was added to per each 10 ml of standard solution for eliminate the interference of anions such as sulfate, nitrate and phosphate. To ionization process, the pH of the solutions was reduced to 2 by HCL. The regulatory specifications of the British atomic absorbing device (Thermo Fisher Scientific, Paisley, UK) were adjusted based on the cathode ray tube, acetylene flammable gas, air oxidizer and wavelength of the emission equal to 422.7 nm, which eventually led to the reduction of copper. The samples are pH = 2 before measurement and then filtered by a 0.22 micrometer filter and the absorption of the samples is measured and quantified by drawing a standard curve (Maduray et al., 2017).

Statistical analysis

Statistical analysis was performed by using the statistical software SPSS 16 (SPSS Inc. Chicago, USA) and Based on the results of the Kolmogorov-Smirnov test, t-test followed to compare differences between groups. ROC curve analysis was performed to investigate the PCSK9 factor. Pearson analysis were used to investigate the relationship between the factors. Results were expressed as mean ± SD. The significance differences of all tests was considered $p< 0.05$.

3. Results

3.1. Demographic characteristics of patients

The baseline clinical and biochemical characteristics are shown in Table 1. The MDD group and control group were similar in term of mean age (p = 0.423), as were the gender and BMI ratios between two groups (p = 0.147, p =0.085 respectively). TC and LDL-C levels were increased in the MDD group (p = 0.026, p =0.019 respectively). But there were no significant difference in TG and HDL-C level between two groups (p = 0.312, p =0.148 respectively).

3.2. Assessment of PCSK9 serum levels

In Fig 1, the serum level of PCSK9 protein increased significantly in the patient individuals (p <0.001). ROC curve analysis was showed PCSK9 protein factor could be considered as a diagnostic factor for MDD. The area under curve (AUC) of PCSK9 in MDD group and control group were obtained as [0.928 (95% CI, 0.86-0.996)] (Fig 2).

3.3. Evaluation of TAC, TOS and MDA

As shown of analysis, TAC significantly don't had difference among the study groups (p = 0.445). In addition The TOS level in MDD group was not increased significantly in comparison of the control group (p = 0.207) (Table 2). A significant increase in MDA as a most common indicator of oxidative stress were observed in the MDD group (p = 0.036).

3.4. SOD and GPx Activity

The results of SOD and GPx enzyme activity are illustrated in the table 2. As can be seen, the SOD activity levels between MDD and control groups were not significantly reported despite the decrease in the patient group (p = 0.148). It was also found that GPx enzyme activity was significantly decreased in MDD group (p = 0.019).

3.5. Copper

The mean serum concentrations of copper ions in study groups showed that Cu were significantly associated with diagnosis. In order that the serum concentrations of copper ions were significantly lower in the patient group (p = 0.012) (Table 2).

3.6. Association analysis of PCSK9 with LDL-C, SOD, GPx, TOS and TAC serum levels

The association analysis showed that in the control group (as shown in Figure 2), the PCSK9 had a significant negative association with SOD (*p*-value = 0.029, β = -0.706), GPx (*p*-value = 0.044, β = -0.002), Copper (*p*-value = 0.044, β = -0.738) and TAC (*p*-value = 0.024, β = -0.615). While, there was a significant positive association between PCSK9 and LDL-C (*p*-value = 0.019, β = 0.816), MDA (*p*-value = 0.033, β = 0.576) and TOS (*p*-value = 0.010, β = 0.473). In addition, in the patient group it found a significant negative association between PCSK9 with SOD (*p*-value = 0.029, β = -0.676), GPx (*p*-value = 0.037, β = -0.015), Copper (*p*-value = 0.034, β = -0.726) and TAC (*p*-value = 0.026, β = -0.685). But in this group, there was a significant positive association between PCSK9 and LDL-C (*p*-value = 0.023, β = 0.634), MDA (*p*-value = 0.029, β = 0.791), TOS (*p*-value = 0.044, β = 0.399 (Figure 3).

4. Discussion

It was examined serum levels of PCSK9, as an oxidative stress indicator, in individuals with major depressive disorder. In addition, oxidative stress factors including TAC, TOS, MDA, and the activity of antioxidant enzymes SOD and GPx were assessed, as well as copper ion concentration. Association between oxidative stress and MDD has been confirmed in several studies (Moylan et al., 2013, Gałecki et al., 2009). In the present study, TAC was reduced in MDD group compared to the control group, but the change was not statistically difference. Also, TOS index increased in the patient group compared to the control group without significant difference. In addition, a significant increase in MDA levels was observed in the patient group when compared to the controls, which was in parallel with lipid peroxidation. Inconsistent with our finding, Santoshi R et al. reported a significant decrease in TAC levels. They also indicated that TOS and MDA levels increased significantly in serum of the MDD patients comparing with the control group (Cumurcu et al., 2009). On the other hand, Sofic et al. reported that there was no significant change in serum antioxidant capacity in the patients with MDD compared with the healthy group (Sofic et al., 2002). Previous studies have demonstrated that the activities of SOD and GPx enzymes significantly decreased in the serum of MDD patients compared to the control group (Stefanescu et al., 2012; Herken et al., 2005; Srivastava et al., 2002). We found that, GPx activity was significantly lower in the patient group. Moreover, SOD activity in the patient group was lower than that of the control group without significant difference.

It has been reported that copper as an essential component, plays a critical role in enzyme activity. It is the main cofactor for some enzymes including monoamine oxidase, therefore, copper-related deficiencies can lead to major disorders like depression. In addition, copper as a cofactor is considered to be essential for some antioxidant enzymes for example lysine oxidase and superoxide dismutase and its deficiency may result in the decreased activity of these enzymes (Maes et al., 2011). Some studies have also shown that copper is essential for the development and functioning of the CNS and low copper concentrations can result in inappropriate development of CNS, while excess levels maybe dangerous (Styczeń et al., 2016). Study on brain tissue has shown that brain is copper-rich which suggest that copper may play an essential role in brain cells function and mood disorders (Scheiber et al., 2014). Many studies also showed that the induction of oxidative stress pathways in major depression is associated with the activation of the inflammatory response and acute phase proteins (AP). The changes in serum sepsis (key protein in copper storage) among the depressed patients could be considered as another reason for the possible role of copper ions in depression (Etebary et al., 2010; Siwek et al., 2013; Lee et al., 2018; Russo ., 2011). We found that patients with MDD had lower copper concentration as compared to the healthy subjects. In line with our observation, some studies have also shown that copper as a trace metal may be involved in the pathophysiology of depression. Styczeń research team found lower serum copper level in depression than that in the normal group (Styczeń et al., 2016). Furthermore, Twayej and et al observed a significant reduction in copper concentration, accompanied with upregulation of all cytokines, in depressed individuals, when compared to the healthy ones (Twayej et al., 2019).

By investigating of the serum PCSK9 concentration in the patients with MDD it was observed a significant increase in this group. ROC curve analysis was showed that PCSK9 protein factor could be considered as a diagnostic factor for MDD. PCSK9 protein has been identified as an important mediator in LDL-plasma cholesterol controlling through playing a role in mediating LDL receptor degradation (Seidah et al., 2003). Moreover, this protein is expressed as a secretory protein in the liver, small intestine, kidneys, and also brain tissues such as the hippocampus and cerebellum. Some studies have shown that mutations in the functional areas of this protein have led to some diseases such as hypercholesterolemia and atherosclerosis. Based on studies, those mutations leading to loss of function in this protein, are associated with the decreased LDL-plasma cholesterol levels (Costet et al., 2006; Cohen et al., 2006; Cohen et al., 2005). As indicated by

previous studies, increasing the serum level of PCSK9 can result in elevated serum ox-LDL levels as well as overexpression of LOX1 in the vessel wall, which it can lead to an increase PCSK9 expression at later stages (Shapiro et al., 2017). Oxidized LDL then causes inflammation by increasing NFkB and other inflammation factors (Bossù et al., 2009; Ogłodek ., 2017). Since PCSK9 plays a functional role in raising serum ox-LDL level, (Since PCSK9 acts as a factor that raise serum ox-LDL level), it can also be considered as an important factor in causing inflammation. In this regard, in a study by Zhihan Tang, siRNA of PCSK9 was used to demonstrate the effect of this factor on controlling the inflammation caused by ox-LDL through the NFkB pathway (Tang et al., 2012). On the other hand, oxidized LDL was known as a factor that induces antigen-providing cells. Several studies have shown the importance of the MAPK pathway in depression (Kumamaru et al., 2011). A study found that a change in the P38MAPK factor in the cerebral cortex -which is the site of production and location of serotonin receptors- may ultimately cause depression (Bruchas et al., 2011; Rasakham et al., 2012). P38MAPK activity increases the expression of HNF1α nuclear factor, which subsequently leads to PCSK9 gene expression (overexpression) (Cui et al., 2016). These results, which considered inflammation as a factor in increasing PCSK9 level, can be used for confirming the present study results. It is important that, according to the results, the relationship between PCSK9 and LDL-C levels, as MDA and TOS, is significantly positive in two groups It confirms our thesis that by increase the PCSK9 variable, increase LDL-C as an oxidative factor and due to for the increase of MDA and oxidative stress. So, PCSK9 plays an effective role in the progression of depression by creating oxidative stress and inflammation and it can be claimed that this factor can be considered as an important factor in the diagnosis or even treatment of depression. The limitation of this study is the low new samples, the time consuming collection time and the cost of performing tests related to the protein PCSK9 metabolic pathway.

Conclusion

For this study, inclusion criteria for participants with MDD new case to be diagnosed by a psychiatrist and have a HAM-D and serum samples were analysis for related tests**.** In general, the results suggest that PCSK9 protein as a factor involved in oxidative stress, was effective through certain signaling pathways in the occurrence of MDD. These findings help us to gain an insight to

use PCSK9 as a diagnostic factor or even achieve therapeutic goals by PCSK9 inhibition. However, our results are needed to be further studies.

Data Availability Statement

The authors confirm that the data supporting the findings of this study are available within the article.

Conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Contributorship

GS, EH and SK researched literature and conceived the study .MD and LJ selected and diagnosed of individuals with MDD and normal. EH and SK were involved in blood collection and laboratory protocols. JK and GS wrote the first manuscript draft. All authors reviewed and approved the final version of the manuscript.

Ethical approve

This research was approved by the Ethics Committee of Hamadan University of Medical Sciences (ethics committee code: IR.UMSHA.REC.1397.525).

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