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Investigation Of The Relationship Between Mirnas And Neurohormones In Hospitalized Covid-19 Patients

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Abstract

microRNAs (miRNAs) are master regulators of gene expression that involve in immunomodulation and neuromodulation process. Several lines of evidences demonstrate that miRNAs participate in pathogenesis of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus. Since the miRNAs are potent in regulation of multiple pathways, infected patients showed different types of neurological abnormalities also possibly due to dysregulation of neurotransmitters and neurohormones. Herein, we aimed to evaluate cross talk between the neurohormones and miRNAs that participate in both of the nervous and immune systems. For this purpose, 60 hospitalized COVID-19 patients and 40 control were selected and the level of neurohormones and miRNAs level were determined by using ELISA kits and qRT-PCR method respectively. Results showed that dopamine and oxytocin levels are reduced in patients significantly. COVID-19 induces miRNAs network disruption by mediation of miR-124, miR-218 and miR-337 down-regulation. SARS-CoV-2 infection also upregulated circular level of miR-101 and miR-802. Increased miR-802 causes oxidative stress and hyperactivation of nuclear factor (NF)-κB that lead to interleukins and cytokines overproduction which could be referred to cytokine storm. Reduced amount of oxytocin, as antioxidant neuromodulator, is in same line that led to liver injuries. Diminished content of miR-124 and miR-218 that accompanied with reduced dopamine level could affect synaptic plasticity and cause cognitive impairments and pain syndrome which observed in about 60 % of hospitalized COVID-19 patients. Accordingly, there are a mutual regulatory relationship between miRNAs and neurohormones that should be considered as a therapeutic target. This study emphasizes the importance of dopamine and

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oxytocin as immunomodulator which could affect the cytokine storm and help to reduce neurological side effects of SARS-CoV-2 infection.

Keywords: *miR-802; Oxytocin; Dopamine; Neuromodulation; Oxidative stress; Liver damages; cytokine storm*

1. Introduction

Coronavirus disease 2019 (COVID-19) is a pneumonia-like syndrome caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been started as a pandemic in China and spread to everywhere around the world since December 2019 (Shereen et al., 2020). Coronavirus belongs to the Coronaviridae family and Nidovirales order that could transmit between human very fast (Shereen et al., 2020). The most common symptoms between patients are mild fever, dry cough, nasal congestion and exhaustion (Shereen et al., 2020). While pre-existing health problems like cardiovascular, kidney and liver disease, obesity, hypertension, cancer, respiratory disorders and psychiatric failures cause more severe symptoms in COVID-19 patients (Flaherty et al., 2020). According to the previous reports, SARS-CoV-2 infection could make some neurological irregularities may be due to inflammatory cascades in body or imperfect oxygen supply to the central nervous system (CNS) (Balcom et al., 2021). Some of symptoms related to CNS like muscle aches, headaches, dizziness, changed taste and smell have been reported in most of infected patients and a dramatic state of confusion that was called delirium observed in rare number of sufferers (Balcom et al., 2021). Unknown events also rarely cause seizures, major strokes, muscular weakness and nerve injury in COVID-19 patients (Balcom et al., 2021). Several reports have pointed out that neurological symptoms of SARS-CoV-2 infection are likely

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due to the immune response overactivation rather than the virus directly infecting the brain or neural cells (Balcom et al., 2021). According to the previous experiments, micro RNAs (miRNAs) play crucial role in CNS functions, development and disease (Cao et al., 2016). They are a group of endogenous noncoding RNAs (ncRNAs) that are responsible for gene expression regulation through attach to the complementary target mRNAs (Cao et al., 2016). These single stranded ncRNAs have been known to regulate both innate and adaptive immune systems by controlling the expansion and activation of immune cells (Lu and Liston 2009). miRNAs are master regulators could participate in more than one pathway, they could regulate expression of more than one gene and could affected by different regulators also (Ben-Hamo and Efroni 2015). Recently, it has been known that some of the miRNAs such as miR-802, miR-124. miR-181 and miR-155 are effective player in both of the nervous system and immune system (Zhou et al., 2021). miR-124 block the inflammation process by down-regulating proinflammatory cytokines and previous study manifested its reduced expression during multiple sclerosis (MS) development (Yousuf and Qurashi 2021). miR-802 has the potential to dampen the nuclear factor kappa B (NF-κB)-repressing factor (NRF) expression, so causes hyperactivation of NF-κB factor. NF-κB is a transcription factor that mainly regulate inflammatory cascade in all mammalian cells, so miR-802 is a pro-inflammatory agent (Sun et al., 2019). This miRNA plays crucial role in neurodevelopmental disease such as Rett syndrome (Kuhn et al., 2010). miR-218 regulates synaptic plasticity and affects learning related behaviors through targeting AMPA signaling (Schell et al., 2022). While according to the published reports, miR-218 could block the respiratory inflammation through targeting the catenin, chemokines and also matrix metalloproteinase-9 (MMP-9) expression (Guo et al., 2019, Liang et al., 2020). miR-101 could regulate cytokines expression and play role in initiation and development of autoimmune disease

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and also involved in neurogenesis and neurodevelopment process (Lippi et al., 2016, Zhao et al., 2021). miR-9 that inhibits the NF-κB pathway, motivates the CNS development and neural differentiation primarily (Radhakrishnan and Anand 2016, Shen et al., 2021). Overall, the nervous system and immune system are closely related to each other and could affected by similar miRNAs network. While, the miRNAs regulate biosynthesis and release of the neurohormones such as vasopressin, oxytocin and dopamine so could affect CNS indirectly (Cao et al., 2018) that refer to a reciprocal relationship between them. The neurohormones produce a coordination between nervous system and peripheral organ's function (Taub 2008) that possibly was influenced by SARS-CoV-2 infection.

In light of these considerations, this study was designed to evaluate the circular contents of miR-802, miR-124, miR-218, miR-337, miR-9 and miR101 in hospitalized adult patients with COVID-19 who are at high risk of progressing to severe disease. Since the neurohormones have immunomodulatory effects (Taub 2008) so possibly influence the initiation and progression of COVID-19 disease. Therefore, we tried to produce a reasonable relationship between miRNAs dynamics and dopamine and oxytocin circular content. This kind of researches could help to clarify some of the ambiguities in COVID-19 symptoms and also explain the molecular events take place in viral infections.

2. Material and methods

2.1. experimental design

This study is a cross sectional evaluation has been done on 60 severe COVID-19 patients (30 males and 30 female) and 40 control subjects (20 males and 20 female) were hospitalized in al-

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Zahra hospital in kut, Iraq from February 2021 to march 2021 Informed and written consent is obtained from participants. Consent was obtained from each patient and their relatives before the participant enters the research, and there is no undue influence on participants to consent. Inclusion criteria for patients are: Age>25 years old, respiratory distress, respiratory rates ≥ 30 breaths/minute, oxygen saturation ≤ 90 % at a rest state, arterial oxygen tension (PaO2) over inspiratory oxygen fraction (FIO2) ratio ≤ 300 mmHg (1 mmHg = 133 Pa) and the lesion in multiple pulmonary lobes is more than 50 % progression in 24–48 h on imaging. The control group has, Age >18 years old, no heart and liver disease, no diabetes (type 1 and type 2), no thyroid disorder, no diabetic nephropathy, no history of neurodegenerative disease and using of iron supplement. COVID-19 disease was permitted by expert physician according to physiological symptoms, computerized tomography (CT) scan imaging and real-time reverse transcriptase-polymerase chain reaction (RT-PCR) methods. Because, the diagnosis of SARS-CoV-2 infection is often confused with that of influenza and seasonal upper respiratory tract viral infections. Then about 10 ml of venous blood were collected in an anti-coagulated tubes containing EDTA and the second plain tube for serum will be taken from all participant and blood serum will be separated by centrifugation at 3000rpm for 10 min. Prepared samples were separated to small aliquots, and stored at -20 °C until its use for different analysis.

2.2. Biochemical parameters evaluation

Use an automatic biochemistry analyzer (flexor xl Elitech, Netherlands) for determination of glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), alkaline phosphatase (alp), urea, creatinine and human Elisa kits D-Dimer (Abcam, ab260076) also were assessed by using specific kits according to company advice. Dopamine and oxytocin

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neurohormones were measured by using Elisa kit were prepared from My BioSource Company, USA (MBS494471 and MBS2700454 respectively).

2.3 miRNA content analysis in blood samples by using real time PCR (RT-PCR)

miRNAs level in plasma samples of COVID-19 and control samples have been evaluated by using the RT-PCR technique and in this process U6 noncoding RNA was considered as internal control (Yu et al., 2021). For this purpose, the total RNA was isolated from plasma samples using TRIzol LS reagent (Invitrogen, Life Technologies, Paisley, UK) following the manufacturer's instructions. Quality control of extracted RNA and its concentration were evaluated by using NanoDrop UV/Vis spectrophotometer. Then the complementary DNA (cDNA) was synthesized from total RNA by using TaqMan Reverse Transcription Kit (Applied Biosystems, Foster City, USA). Subsequently, cDNA was used as template in qRT-PCR that was conducted using SYBR Green PCR Kit (Toyobo, Tokyo, Japan) on ABI Prism 7500 Detection System (Applied Biosystems). Specific primers were obtained from Sangon Biotech (Shanghai, China) (Table 1). U6 was served as an internal reference for miRNAs because its expression is constant. Normalization of data against U6 count reduce technical variations effects and increase data accuracy. The relative expression of miRNAs was evaluated with the $2^{-\Delta\Delta Ct}$ method after normalization (Yu et al., 2021).

2.4 Statistical analysis

Statistical assessment was done by using the analysis of variance with Bron-Forsite correction to detect statistical differences between results related to SARS-CoV-2 infected group and healthy control. Tukey's multiple comparison test was applied to compare two experimental groups with each other and significant differences were showed in each plot by indication of star symbol

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according to the P value. All statistical analyses were performed with the GraphPad Prism software (version 9.5, GraphPad Software, Inc. San Diego, CA, USA).

3. Results

The highly approved evidences suggest that most COVID-19 patients manifested a mild or moderate level of neurological abnormalities while some of them showed severe neural damages possibly due to some alterations in miRNAs expression (Keikha et al., 2021). Here we aimed to evaluate the possible variations of neuroactive miRNAs and hormones in COVID-19 patients in comparison with age-match control. Age distribution in two groups is the same and there is not significant difference according to one-way ANOVA (P=0.13, ns). General characteristics and parameters with diagnostic value in COVID-19 patients and control cases were joined this study are shown in table 2. Based on the obtained biochemical results, circular content of urea is significantly higher in COVID-19 patients compared to control subjects; creatinine level is more than control also (Table 2). Glutamic oxaloacetic transaminase (GOT) enzyme activity is raised about 2-fold as a result of COVID-19 disease. Glutamic pyruvic transaminase (GPT) enzyme also showed overactivation in SARS-CoV-2 infected patients. D-Dimer level was significantly higher in COVID-19 patients compared to the control subjects ($P<0.0001$); this parameter has diagnostic value in SARS-CoV-2 infection. Unlikely, alkaline phosphatase (ALK) activity showed significant reduction in patient group, its value was estimated to be 204.67±69.74 IU/L in COVID-19 patients while ALK activity is 274.73±49.70 IU/L in healthy control.

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3.1. SARS-CoV-2 infection affects neuroactive miRNAs

Circular contents of miRNAs were evaluated by using specific primers and qRT-PCR methods (Yu et al., 2021). After normalization of data against housekeeping ncRNA (U6), the relative expression of each miRNA in COVID-19 patients showed as a column plot in comparison with control. According to the results (Fig 1A) the relative expression of miR-9 is the same in both of the COVID-19 and healthy control groups while we can see slightly reduction in miR-9 count in COVID-19 patients but this change is not significant according to the statistical analysis (P=0.057, ns). Unlikely, miR-101 count is not similar in both of the experimental groups and results manifested miR-101 expression increased in COVID-19 patients significantly (P<0.0001, ****) (Fig 1B). While circular content of miR-124 in healthy control samples is remarkably more than SARS-CoV-2 infected patients, this reduction evaluated to be more than 3-fold (Fig 1C). Cytokines storm causes significant reduction of miR-218 concentration in COVID-19 patients in comparison with control (P<0.0001, ****) (Fig 1D). miR-337 expression also decreased as a result of SARS-CoV-2 infection, the reduction was estimated about 3-fold in comparison with control samples (P<0.0001, ****) (Fig 1E). According to the results (Fig 1F), miR-802 increased significantly in the COVID-19 patients in comparison with healthy controls (P<0.0001, ****).

3.1. SARS-CoV-2 infection affects circular content of neurohormones

Dopamine is a neuromodulator catecholamine that plays role in many important body functions, including movement, memory and enjoyable reward and motivation (Bromberg-Martin et al., 2010). Changes in dopamine content are associated with numerous cognitive problems and neurological diseases (Bromberg-Martin et al., 2010). Our information is insufficient to determine how dopamine works in relation to physiological conditions and how its function

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changes during pathological conditions. By considering the neurological abnormalities in COVID-19 patients, this study aimed to evaluate the possible changes of dopamine in patient group. Our results revealed SARS-CoV-2 infection is accompanied with a significant reduction of circular dopamine content (Fig 2A). Dopamine concentration in blood serum of healthy control samples was evaluated to be 210.98±21.38 pg/ml. While this parameter in COVID-19 patients was measured as 90.30±15.69 pg/ml. Therefore, the cytokine storm that was induced by SARS-CoV-2 infection causes more than 2-fold reduction in circular dopamine content ($P<0.0001$, ****).

Oxytocin is a neurohormone that was produced by hypothalamus and released to the blood from pituitary gland (Walter et al., 2021). It participates in reproductive system function and also coordinate central and peripheral nervous systems functions (Walter et al., 2021). Fig 2B revealed oxytocin circular level reduced significantly in COVID-19 patients in comparison with healthy control. Oxytocin was evaluated to be 714.85±91.39 pg/ml in blood samples of patient group while its content was 299.18±55.39 pg/ml in SARS-CoV-2 infected patients. Comparing the obtained results revealed COVID-19 disease reduced the circular level of oxytocin near to 3-fold.

4. Discussion

New estimation of the World Health Organization (WHO) shows that more than 15 million deaths were directly or indirectly associated with the SARS-CoV-2 infection in whole of the world. Due to its breadth and scope, many studies have been conducted to clarify the ambiguous aspects and different side effects of COVID-19 disease. This viral infection consists of an

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immunomodulatory process that led to excessive amounts of interleukins and cytokines in blood, cerebrospinal fluids and other fluids of the body (Costela-Ruiz et al., 2020). These kinds of alterations include the presence of antibodies, interleukins and cytokines that may also react with the nervous system and other organs of the body (Khassawneh et al., 2020). Therefore, the most of symptoms in COVID-19 patients are related to the cytokines storm rather than the virus infection (Sarubbo et al., 2022). According to the previous study, SARS-CoV-2 infection affects neuroendocrine system and some of the patients were manifested secondary symptoms related to neurohormones dysregulation (Khassawneh et al., 2020). By considering the widespread neurological side effects in COVID-19 patients (Balcom et al., 2021), this study aimed to assess the possible changes in neurohormones circular content. Our results revealed cytokine storm make a reduction in circular level of oxytocin and dopamine hormones in comparison with healthy individuals. According to the previous report, oxytocin could interact with and inactivate the SARS-COV-2 spike protein so prevents viral entrance into the cells, it could also block the angiotensin-converting enzyme 2 and increase interferon level and number of T-lymphocytes in blood (Wang et al., 2022). In addition, oxytocin can induce secretion of body fluids through parasympathetic discharge that causes dilution and also inactivation of SARS-CoV-2 on the surface of cornea, oral cavity and gastrointestinal tract (Wang et al., 2022). Therefore, endogenous oxytocin has potential to treatment and prevention of the COVID-19 and its reduction level make human susceptible against viral infection (Imami et al., 2020). But its not clear that reduced circular content of oxytocin is as a cause or consequence of SARS-CoV-2 infection. Our results also manifested dopamine reduction as a result of COVID-19 disease. Dopamine involved in neuropsychiatric functions like thinking, memory, movement, motivation, mood, attention and more. Therefore, its reduction could be a reason for long- and short-term

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cognitive impairment in COVID-19 patients. Dopamine biosynthesis done by dopa decarboxylase enzyme (DDC) which its dysregulation was reported previously in COVID-19 patients, this enzyme has important role in serotonin and histamine biosynthesis also (Mpekoulis et al., 2021).

Our results also revealed a significant increase in liver enzymes (GOT and GPT) that can be refer to a COVID-related liver damage. The cause of hepatic injuries is not clear but direct hepatic injury, cytokine storm effects, hepatic ischemia and drug-derived damages could be considered as potential reasons (Moon and Barritt 2021). Harsh oxidative stress in severe cases of COVID-19 disease is the other potential reason. While reduced level of oxytocin as an antioxidant and anti-inflammatory hormone could aggravate hepatic harms (Hekimoglu et al., 2013).

This study also assessed some of the miRNAs involved in neurological abnormalities in COVID-19 patients including miR-9, miR-101, miR-124, miR-218, miR-337 and miR802. These miRNAs have role in different molecular pathways but their common functions are in nervous system, some of them have immunomodulatory activity also. Our results confirmed SARS-CoV-2 infection upregulated circular level of miR-101 and miR-802, whereas it caused decrease in circular concentration of the miR-124, miR-218 and miR-337. According to previous experiments miR-802 regulates cytokine storm positively. This miRNA could down-regulate the master inhibitor of NF-κB (NRF) and causes hyperactivation of the NF-κB as a transcription factor that could upregulate pro-inflammatory factors and cytokines effectively (Sun et al., 2019). Its role has been approved as an inducer of oxidative stress in liver of high-fat diet mice (Yang et al., 2019). Overexpression of miR-802 causes insulin resistance and impair glucose tolerance, while its down-regulated in obese mice improved the metabolic parameters, suggesting that increased content of miR-802 is responsible for risk of obesity-associated

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oxidative damages (Yang et al., 2019). Previous studies demonstrated that oxidative stress is a fundamental point in the pathophysiology of COVID-19 specially in severe cases (Vollbracht and Kraft 2022) possibly due to miR-802 overexpression (Fig 3). Decreased expression of miR-124 also could help to cytokine storm in COVID-19 patients (Fig 3), because it has anti-inflammatory properties through targeting P38 and P62 and inactivation of the macrophages (Han et al., 2020). Upregulation of the miR-101 also has role in increased cytokines concentration and inflammatory cascades which observed in SARS-CoV-2 infection (Zhao et al., 2021). Down-regulation of the miR-218 also could trigger inflammatory process mainly by targeting the MMP-9 expression. miR-218 could suppress the expression of the MMP-9 enzyme as a metalloprotease that help to propagation of the inflammatory agents (Guo et al., 2019, Liang et al., 2020), this process take place in COVID-19 patients due to reduced miR-218. The inflammation that induced by miRNAs network remodeling was observed in CNS during Alzheimer's and Parkinson's disease and sever cases of COVID-19 (Guo et al., 2019, Liang et al., 2020, Zhao et al., 2021). While the mentioned miRNAs play other roles in CNS function such as synaptic plasticity, cognitive function, thinking and learning ability by mediation of neurohormones (Keikha et al., 2021, Schell et al., 2022). Reduced miR-218 has negative effects on synaptic plasticity through targeting dendritogenesis and neurogenesis (Schell et al., 2022). Previous experiments also approved important role on miR-337, miR-101, miR-802 in neuroinflammation and neural damages that take place in dementia-related disease (Improta-Caria et al., 2020, Keikha et al., 2021). miR-124 is responsible for long-term plasticity of synapses in the mature nervous system and previous study reported its down-regulation in age-related dementia like Alzheimer’s disease and neurodegeneration (Sun et al., 2015). Our results also confirmed reduced circular level of miR-124 and dopamine in patient group, this

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coordinated alterations possibly play essential role in COVID-19 related neurological abnormalities.

5. Conclusion

COVID-associated short- and long-term mental health problems are challenging side effects of SARS-CoV-2 infection, this study tried to clarify some ambiguity by targeting cross talk between miRNAs and neurohormones (Fig 3). Taken together, our results revealed a positive correlation between reduced oxytocin and increased miR-802. miR-802 trigger cytokines storm as a results of virus infection through hyperactivation of NF-κB. Other modulations in miRNAs network like down-regulation of the miR-124 and miR-218 and upregulation of miR-101 are in the same line. Inflammation and oxidative stress are reciprocally reinforcing each other and trigger a systemic hyperinflammatory state which is fundamental pathological mechanisms of COVID-19. By considering crucial role of the miR-802, miR-101 and specially miR-124 in nervous system's function, it could be concluded that miRNAs network remodeling that accompanied with reduced dopamine level causes diminished synaptic plasticity, cognitive impairments and pain syndrome. Reduced level of dopamine and oxytocin are strictly associated with pathophysiological signs of COVID-19 that suggests neurohormones as therapeutic agents to improve hospitalized patients. Consequently, this study emphasizes the important role of miRNAs dysregulation in cytokines storm was observed in COVID-19 disease and introduce them as potential therapeutic targets to reduce the immunological and neurological side effects.

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Tables

Table 1: Primers sequences have been used in RT-PCR method to evaluate the expression of different miRNAs.

miRNA name	Forward and reverse primer sequence
miR-802	Forward:5′-ACGTTGTGTAGCTTATCAGACTG -3′
	Reverse: 5′- AATGGTTGTTCTCCACACTCTC -3′
miR-101	Forward: 5- TGGGCTACAGTACTGTGATA-3
	Reverse: 5- TGCGTGTCGTGGAGTC-3
miR-337	Forward: 5- CGCTTCAGCTCCTATATGA-3
	Reverse: 5- GCGAGCACAGAATTAATACGAC-3
miR-9	Forward: 5′-UCUUUGGUUAUCUAGCUGUAUGA-3′
	Reverse: 5′-AUACAGCUAGAUAACAAAGAUU-3′
miR-124	Forward: 5′-UAAGGCACGCGGUGAAUGCC-3′
	Reverse: 5′-CAUUCACCGCGUGCCUUAUU-3′
miR-218	Forward: 5′-TTGTGCTTGATCTAACCATGT-3′
	Reverse: 5′-CAGTGCGTGTCGTGGAGT-3′
U6	Forward: 5′-CTCGCTTCGGCAGCACA-3′
	Reverse: 5′-AACGCTTCACGAATTTGCGT-3′

Table 2. General characteristics of study participants. GOT is glutamic oxaloacetic transaminase, GPT is glutamic pyruvic transaminase and ALK refer to alkaline phosphatase enzyme. Significant differences were showed by steric symbol.

Parameters	Control	COVID-19 patients	P-value
Age (years)	36.77±11.95	39.80±11.19	0.56
Urea (mg /dl)	21.40 ±6.47	52.60±21.49 *	<0.0001
s.creatin(mg/dl)	0.74±0.16	1.50±0.62 *	0.007
GOT (IU/L)	21.7±6.42	42.37±15.46 *	<0.0001
GPT (IU/L)	23.70±6.42	45.53±20.92 *	<0.0001
ALK (IU/L)	274.73±49.70	204.67±69.74 *	0.0024
D-Dimer (ng/ml)	263.17±137.51	2677.90±244.2 *	<0.0001

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Figure legends

Fig 1. miRNA evaluation by using RT-PCR method. Data were normalized against U6 non-coding RNA count and represented in a column plot in each group. (A) reveals normalized count of the miR-9, (B) shows normalized level of the miR-101, (C) manifests normalized concentration of miR-124, (D) reveals normalized content of the miR-218, (E) shows normalized concentration of the miR-377 and (F) represents normalized level of the miR-802 in COVID-19 patients and healthy control. All data were expressed as mean ± SD. Significant differences indicated by star symbol above the column plots (ns, $P>0.05$; ****, $P<0.0001$).

Fig 2. The circular level of dopamine and oxytocin in COVID-19 patients and healthy individuals. (A) SARS-CoV-2 infection causes significant reduction in dopamine level of blood in comparison with control. (B) Oxytocin level also reduced in COVID-19 patients. All data were manifested as mean ± SD. Significant differences indicated by star symbol (**** $P<0.0001$)

Fig 3. Schematic illustration of studied parameters relationship. miRNAs are upstream regulators that cause oxidative stress, neurological abnormalities (by mediation of neurohormones) and cytokines storms.

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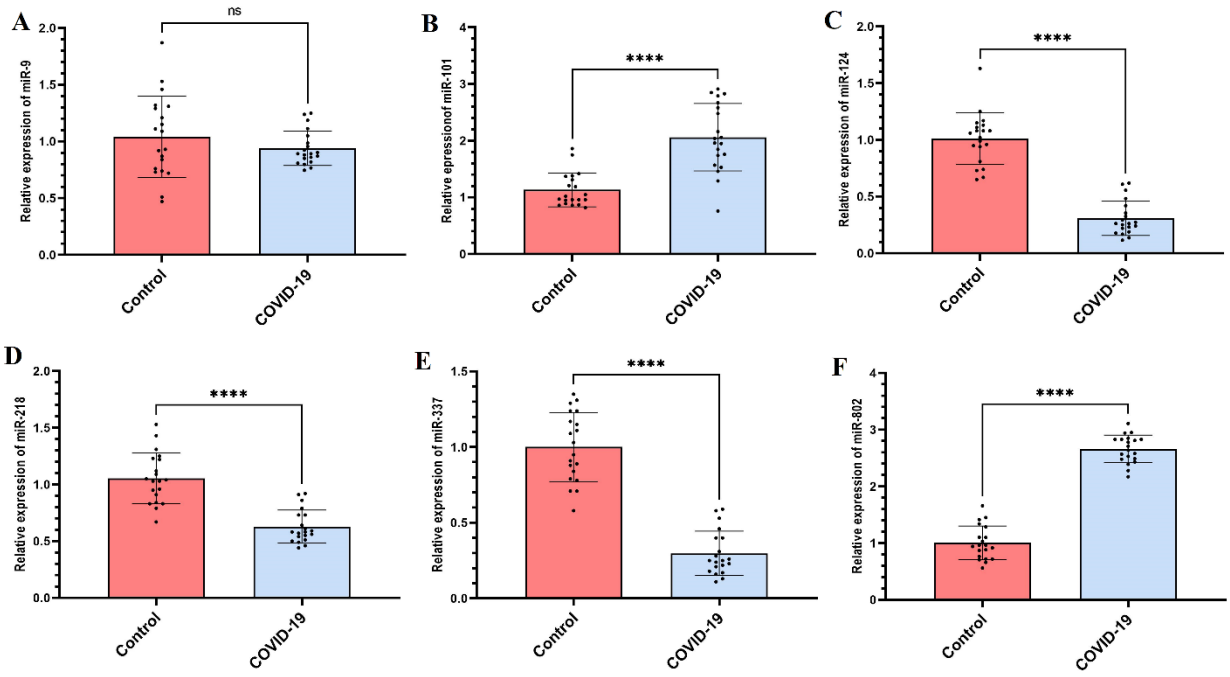

Fig. 1

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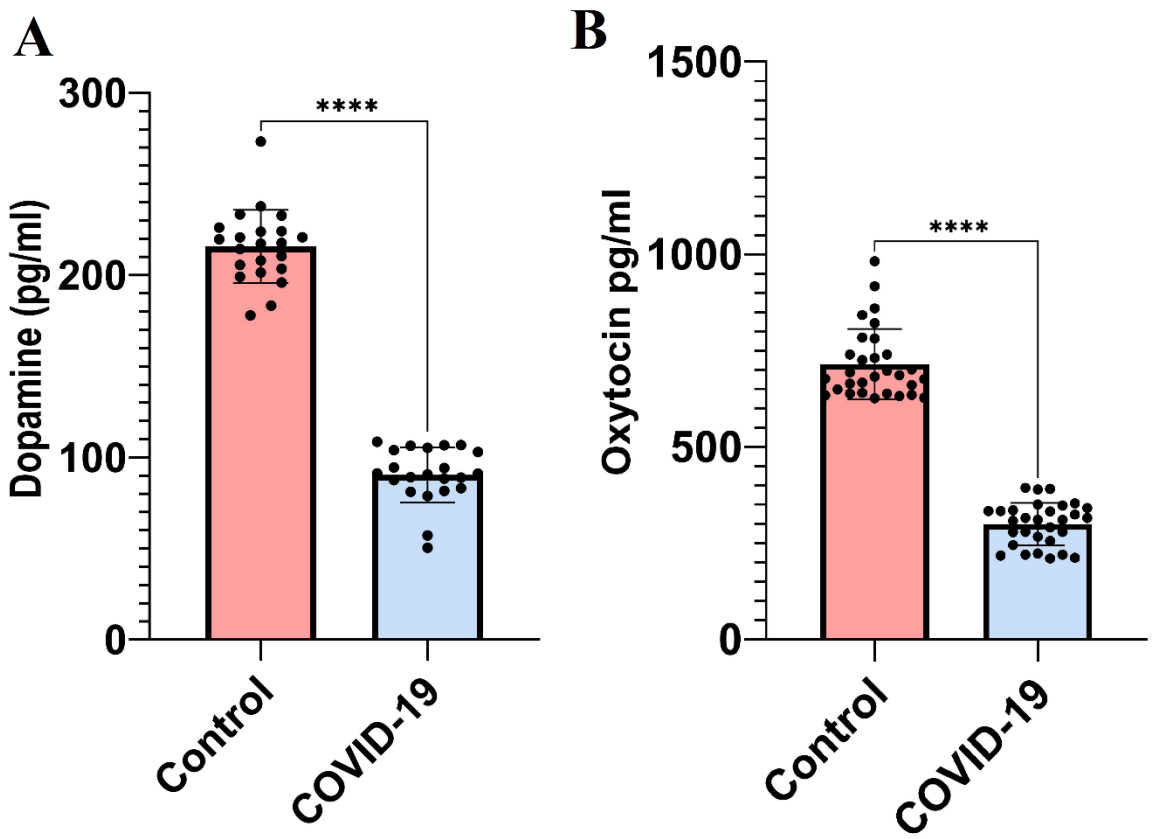

Fig. 2

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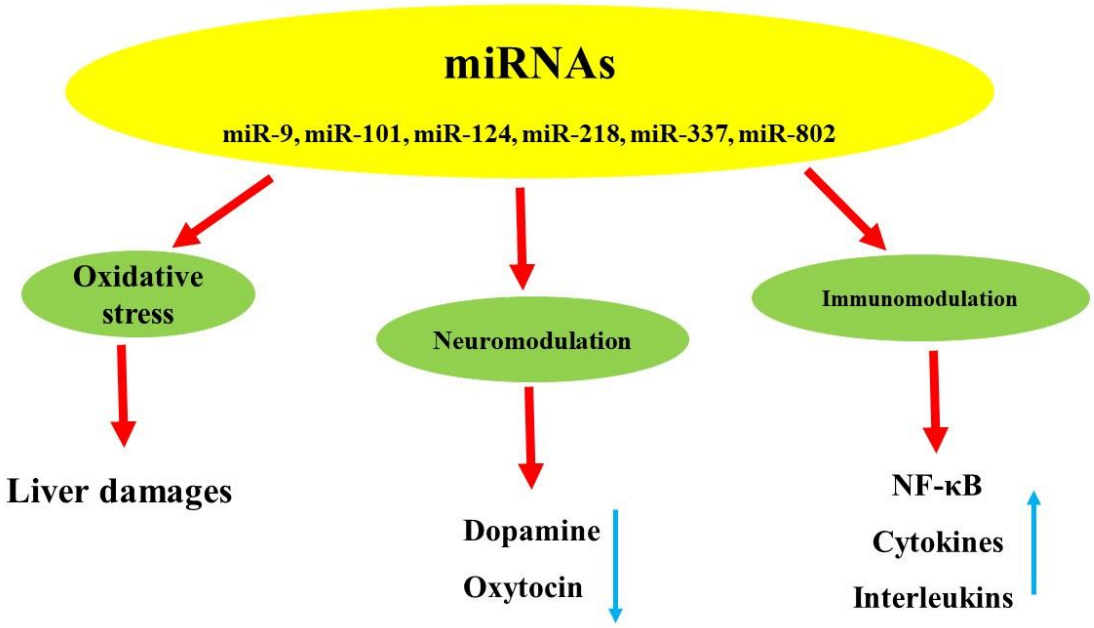

Fig. 3

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