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Research article

Crocin nano-chitosan-coated compound mitigates hippocampal blood-brain barrier disruption, anxiety, and cognitive deficits in chronic immobilization stress-induced rats

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ABSTRACT

Stressful conditions can disrupt the central nervous system's normal homeostasis and physiological functions, resulting in blood-brain barrier malfunction, memory and learning impairment, anxiety, etc. Crocin is a long-investigated natural compound that has been documented to have anti-inflammation and neuroprotective effects, albeit it comes with some limitations such as low stability and bioavailability. Therefore, we aimed to overcome crocin's limitations by coating crocin with a nano-carrier (chitosan) in the chronic immobilization stress-induced rat model. Crocin was encapsulated into chitosan nanoparticles by a modified method. A total of 35 male Wistar rats were selected as our study subjects (220–250 g) which were randomly divided into 5 groups (control, stress, nanoparticle, crocin, and chitosan). Chronic immobilization stress was induced by placing rats for 2 h into a plastic bottle with specific measurements (for 14 consecutive days) to prevent animals from moving. To evaluate the memory and learning changes, we used the Barnes maze test and the Passive avoidance test followed by the evaluation of the N-methyl-D-aspartate |(NMDA) receptor subunits genes (GRIN1 and GRIN2A) expression. Anxiety levels were evaluated by elevated plus maze test. Furthermore, the changes in the expression of genes responsible for encoding the tight junction proteins of BBB including ZO1, CLDN5, and OCLN were assessed by RT-PCR. Compared to intact crocin, the administration of crocin nano-chitosan-coated compound resulted in significant improvement of specific memory and learning indicators as well as a significant reduction of anxiety levels in chronic immobilization stress-induced rats. Finally, we observed that treatment with the crocin nano-chitosan-coated compound can elevate the expression levels of the genes responsible for encoding NMDA receptor subunits, and the genes responsible for encoding the tight junction proteins of blood-brain barriers in the hippocampus.

Abbreviations: Blood-brain barrier, BBB; Crocin nano-chitosan-coated, CNCC; Central nervous system, CNS; N-methyl-D-aspartate, NMDA.

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1. Introduction

Stress is a common phenomenon in everyday life that leads to coordinated responses from the nervous and hormonal systems. These responses are necessary for appropriate physiological and behavioral changes, such as instinctive survival responses [1]. Chronic Immobilization is a renowned method of inducing chronic stress that is widely used in rodents such as rats. During the immobilization stress body movements are restricted, which can lead to diverse pathological conditions [2].

Many in vivo studies have suggested that chronic stressful stimulants can cause impairment in neuron's synaptic plasticity in different regions of the brain including the hippocampus, which can lead to loss of memory, learning malfunction, and the development of neurological disorders [3–5]. Chronic stress usually leads to anxiety, a fear-based psychological or mental state associated with environmental stimuli perceived as threats [6].

One of the most important excitatory neurotransmitters in the central nervous system (CNS) is glutamate, and N-methyl-D-aspartate (NMDA) is considered to be the main ionotropic receptor of this abundant neurotransmitter, directly affecting the process of synaptic plasticity, memory, and learning in the hippocampus [7]. The structure of NMDA receptors consists of different subunits, two of which are GluN1 and GluN2A, encoded by Grin1 (encoding subunit 1 = GluN1) and Grin2A (encoding subunit 2A = GluN2A) genes [8,9].

The vessels that mediate blood circulation in the CNS have specific multicellular structures called the blood-brain barrier (BBB). The normal function of neuronal cells in the CNS depends on precise control over their homeostasis, which is accomplished by the flawless work of the BBB [10–12]. Alteration of BBB characteristics in CNS usually occurs in diverse stressful conditions and neurodegenerative diseases which leads to BBB malfunction or breakdown, this phenomenon is usually followed by ion dysregulation, edema, and the entrance of the peripheral immune cells into the brain, which can result in neuronal malfunction, neuroinflammation, increased intracranial pressure, and eventually neuronal degeneration [13–16].

Tight junction (TJ) proteins are one of the major components of the BBB, helping endothelial cell-to-cell adhesion in this complex and regulating the selective diffusion of molecules within the BBB [11]. Some of the most important proteins that contribute to forming the BBB's tight junctions are encoded by zonula occludens-1 (ZO-1), occludin (OCLN), and claudin-5 (CLDN5) genes [17].

A variety of disorders are associated with immobility under acute and chronic stress conditions that can disrupt multiple systems within the CNS, resulting in memory, learning, and cognitive impairment, anxiety, neuronal degeneration, abnormal blood circulation, and BBB dysfunction [18–20]. Nowadays, many synthetic medications aim to attenuate the adverse effects of stress; however, they come with many side effects and limitations, creating a dilemma for individuals. Therefore, developing medicines with fewer side effects that can reduce stress and its adverse effects on the body, especially on the BBB, is of great importance. Herbal remedies have always been the focus of many studies as they have fewer side effects compared to synthetic drugs, making them an excellent choice in the treatment of neurological disorders, including acute and chronic stress disorders, depression, Alzheimer's disease (AD), Parkinson's disease, etc [18,21–23]. Out of all these natural compounds, crocin, a carotenoid compound of saffron that can easily dissolve in water, has received special attention [24]. Previous studies have investigated crocin's properties on the adverse effects of stress as well as its neuroprotective properties against many neurological disorders [25], yet it has some limitations such as low stability in different environments and low bioavailability [26].

Chitosan is one of the most frequently used polysaccharides in the research and drug industry that is derived from chitin. Due to chitosan's excellent properties such as non-toxicity, biodegradability, and biocompatibility, it has been widely used as a drug carrier in a lot of studies focusing on different neurological disorders and stress-induced conditions [27,28]. The properties of chitosan, including flexibility in modifying the surface of nanoparticles, the capacity to attach a diversity of ligand molecules, and the ability to form stable nanoparticles under various physiological conditions, make it an excellent choice as a drug carrier, especially in nanoparticle form [28,29]. Furthermore, this polysaccharide's surface is flexible enough to be modified, making it easy to use in various drug preparation methods, resulting in the successful delivery of designated drugs through the BBB [30,31].

Therefore, in this study, we aimed to enhance crocin's stability and bioavailability by coating it with chitosan (as a nanocarrier) via a modified nanotechnology method. We then investigated the crocin nano-chitosan-coated (CNCC) compound's probable effects on the expression of genes responsible for encoding BBB's tight junction proteins and NMDA subunits in the hippocampus, as well as memory, learning, and anxiety indicators compared to the intact form of crocin in a chronic immobilization stress-induced rat model.

2. Material and methods

2.1. Selected animals

Thirty-five male Wistar rats (220–250 g) were selected as study subjects and randomly divided into five groups of seven rats each (n = 7). Rats in each group were housed in pairs of two cages, with three rats per cage, under a 12-h light/dark cycle, with no restrictions on food and water. The room temperature was maintained at 25 °C (25 ± 2 °C) to minimize environmental stress factors. Our study complied with animal welfare regulations and was conducted according to guidelines approved by international and local ethical committees. We took all necessary measures to reduce the number of rats used and to minimize their pain.

2.2. Experimental groups

Rats were placed into 5 groups of 6 rats in each group. The first group was the control group that did not undergo to immobilization

stress-inducing process and did not receive any experimental compound. The second group was the stress group that underwent to immobilization stress-inducing process but did not receive any experimental compound. The third group was the nanoparticle group that underwent to immobilization stress-inducing process and received our experimental compound (CNCC compound) with a certain dosage of 180 mg/kg, 30 min before inducing stress for 14 consecutive days. The fourth group was the crocin group which also underwent our immobilization stress-inducing process and received intact crocin with a certain dosage of 6 mg/kg, 30 min before inducing immobilization stress for 14 consecutive days. The fifth group was the chitosan group which also underwent the immobilization stress-inducing process and received intact chitosan with a certain dosage of 160 mg/kg, 30 min prior to the immobilization stress induction for 14 consecutive days.

2.3. The procedure of making nanoparticles

We used a modified method to obtain the crocin nano-chitosan-coated compound, first of all, we prepared a 5 ml solution of 0.2 % chitosan solution in 1 % acetic acid, and then 6 mg of crocin was dissolved in 1 ml of distilled water and then we added the dissolved crocin to the prepared chitosan solution under extreme delicacy.

Then 1 ml of 0.1 % TPP solution was assembled and then added to the prepared chitosan solution drop by drop while stirring it and then the final solution was stirred for an additional 2 h.

Eventually, the above-mentioned solution was centrifuged for 1 h (10000–14000 RPM), and then the supernatant was removed, after that, the desired sediment was again dispersed in MiliQ water and stored at −20 °C for use or freeze-drying [32]. Hydrodynamic particle size was investigated using DLS (SOS I, KONE (South Korea)).

To determine the exact amount of crocin content in the designed nanoparticle, the difference between the total amount of crocin used in the preparation process of nanoparticles and the amount of crocin not trapped in the supernatant was calculated. Crocin content was analyzed using a UV–Vis spectrophotometer (Infinite 200 PRO, TECAN, Switzerland) at 440 nm [33]. EE was calculated with the following equation:

$$\text{EE}\ (\%) = \frac{TC - FC}{TC} \times 100$$

(TC = Total amount of Crocin, FC = Free amount of Crocin)

Also, the loading capacity was estimated by the following equation:

$$\text{LC}\ (\%) = \frac{TC - FC}{wt\ of\ the\ nanoparticles\ retrieved} \times 100$$

2.4. Chronic immobilization stress-inducing process procedure

In this method, rats were placed in a plastic bottle measuring 25 × 7 cm, so the animal would no longer be able to move. In order to make space for breathing, at one end of the bottle we made a 1-cm hole. Each animal (except the control group's rats) is stressed for 2 h a day for 14 consecutive days. The process of immobilizing the rats was carried out every day between eight to ten in the morning [34].

2.5. Real-Time PCR

Total RNA extraction from the hippocampus was obtained by RiboEx™ total RNA isolation kit (GeneAll®, Korea, Cat No. 301-001) and the concentration was measured by NanoDrop device. Primers were designed by Primer-BLAST of NCBI for the following genes B2M, ZO-1, OCLN, CLDN-5, GRIN1, GRIN2A (Table 1.), and afterward were purchased from Sina Clone company. A reverse transcriptase kit (BioFact, Korea, Cat No. BR631-096) was used to synthesize cDNA. The Real-time PCR amplification was performed using Master Mix Syber Green (BioFact, Korea, Cat No. DQ383-40H). The RT-PCR cycling setup was as follows: 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 25 s at 60 °C, and 30 s at 72 °C. and finishes at second hold with 72 °C for 7 min at the end of each 60 °C step,

Table 1
Forward and Reverse sequence of RT-PCR primers.

Target gene	Sequence length (bp)	Primer	Reference
ZO1	142	F: GAGTCGCAATGGTTAACGGAG R: GGGTCTGGGTGACTTACAGG	**This study**
OCLN	156	F: GCTGGAGAAGTGAGAGAGAGG R: TGGAGACAGGAAACGGATGG	**This study**
CLDN5	152	F: GGTGTCTCAGAAGTACGAGCTG R: TTGGTGCTGAGTACTTGACTGG	**This study**
GRIN1	183	F: CTACTCCCAACGACCACTTCAC R: CTCGCATCATCTCAAACCAGAC	**This study**
GRIN2A	204	F: AATGTGACTTGGGATGGCAAGG R: GCTGAGGTGGTTGTCATCTGG	**This study**
B2M	151	F: CTTTCTACATCCTGGCTCACAC R: GTCCAGATGATTCAGAGCTCC	**This study**

analyzation of the fluorescence level was conducted. The 2−11CT method was used for the quantitative assessment of gene expression and for evaluating the amplification of each gene, the melting curves and the visualization on gel electrophoresis were done [35].

2.6. Barnes maze

After 10 days of administrating our experimental nano compound, rats underwent the Barnes maze test to evaluate learning and memory impairment. Barnes maze consists of 3 phases, the first one is the Adapting phase which was on the day before the first training day (day 9) when the rats of each group were placed in the testing room for 30 min to adapt to the environment, the second phase is the training phase (day 10) which consists of 4 days of training in which rats underwent the trial four times for 1 min each in which the rats learn how to estimate the spatial location and find the way to reach the destination chamber by using distal visual signs, and the third one is the testing phase on day 14 which consist of a one-time trial for 1 min for the final results. At the beginning of each trial, a dark Plexiglas cylinder was placed in the center of the plate and then rats were placed in it, and after removing the cylinder, rats could search the plate freely until they reached the destination in 1 min and if rats could not reach the target hole in the designated time, we would have helped them to reach it until they could find it on their own. The parameters that were measured and analyzed were the time latency of finding the target hole after removing the cylinder, and the number of errors while finding the goal hole. After each trial, 70 % ethanol was used for cleaning the surface of the plate and scape chamber so that we could increase the accuracy by avoiding the intra-maze odor [21].

2.7. Passive avoidance

We have used this test after ending the elevated plus maze test to estimate the effects of the questioning nano compound on learning and memory by measuring the capability of learning and remembering where the shock stimulus is present. The advantages of this procedure are the need for minimal training for producing rapid learning and also a great deal of authority on the aversive stimulus.

The intensity that we used in this study for shocking was at the minimum amount that was needed to arouse the rats, which was about 0.2–0.5 mA electric shock for 1–2 s, and each rat was subjected to this procedure only one time. This procedure consists of two phases: the training phase (day 15) which begins with placing the rat in the bright (white) compartment facing the open door toward the dark compartment. Rats were given 5 min to enter the dark compartment and when they completely enter the dark compartment the door will close and they receive an electric shock for 1–2 s subsequently, rats stay in the dark compartment for an additional 10 s, and then they are replaced into their cage boxes. We used 70 % ethanol for cleaning the apparatus between each trial. Two days after the training phase (day 17) rats undergo the testing phase for 5 min with no aversive stimulus. The rats are once again placed in the white compartment toward the open door to the dark compartment, and the time latency of re-entering the dark compartment is recorded for each rat.

2.8. Elevated plus maze

To evaluate the anti-anxiety and anti-stress effects of our experimental nanoparticle compound in chronic immobilization stress-induced rats, the elevated plus maze (EPM) was performed.

After 14 consecutive days of administrating the experimental nano compound and one day after the Barnes maze test was finished (day 15), rats underwent the elevated plus-maze test. This test was a 5-min one-time trial for each rat. At the beginning of the test, we placed rats headed toward the open arm in the intersection of the arms, and then we started filming their behaviors. The detected parameters for anti-anxiety were open arm time (% OAT $= \frac{open\ arm\ time}{open+close\ arm\ time} \times 100$), open arm entry (% OAE $= \frac{open\ arm\ entry}{open+close\ arm\ entry} \times 100$), grooming, head dipping, and freezing (time latency of staying in one place without any movement). We used 70 % ethanol after each trial on all 4 arms for cleaning and getting rid of the odor of the previous rat to avoid intra-maze odor for the next trial [36].

2.9. Statistical analysis

IBM SPSS Statistics 26 was used for the statistical analysis. The results were expressed as mean ± SEM. One-way ANOVA and Tukey's test were done to indicate the mean and significance between the groups. $P < 0.05$ was regarded as statistically significant.

3. Results

3.1. Characterization, Entrapment efficiency (%), and loading capacity

After centrifuging (as mentioned in the preparation method section), 0.9 mg of the 6 mg of the designated drug was free. Also, according to Crocin's standard curve which was drawn at 440 nm (UV–VIS absorbance) and the mentioned equation, EE (%) was calculated (Table 2.) [32]. Furthermore, our nanoparticles had 175 ± 5 nm hydrodynamic size after preparation.

3.2. Real-time PCR analysis of NMDA subunit genes

The analysis of NMDA receptor subunits genes expression in the hippocampus by RT-PCR showed that inducing chronic

immobilization stress reduces the expression levels of GRIN1 by 7.7-fold and GRIN2A by 4.5-fold compared to the control group. Furthermore, our results demonstrated that the administration of CNCC compound can increase GRIN1 expression by 5-fold (Fig. 1-a) and GRIN2A expression by 3.3-fold (Fig. 1-b) compared to the stress group. Finally, we observed that the nanoparticle group increased the expression of GRIN1 and GRIN2A more significantly compared to the crocin and chitosan as shown in Fig. 1.

3.3. Real-time PCR analysis of BBB tight junction genes

The changes in expression levels of hippocampal BBB tight junction genes by RT-PC are shown in Fig. 2. Immobilization stress resulted in a dramatic decrease in the expression levels of all the target genes including ZO-1 by 3.3-fold (Fig. 2-a), OCLN by 2.7-fold (Fig. 2-b), and CLDN-5 by 7.1-fold (Fig. 2-c) compared to the control group. Moreover, three treatment groups were designed for induced-immobilization rats, respectively with chitosan, crocin, and CNCC compounds, to evaluate the above-mentioned genes. The results demonstrate that the nanoparticle group and the crocin group can significantly increase the expression of all the target genes, however, treatment with chitosan only significantly increased the gene expression of Zo1. In the immobilized stress-induced state, we also observed that treatment with the nanoparticle elevated the expression of all the target genes more significantly compared to the crocin and chitosan groups.

3.4. Analysis of memory impairment by Barnes maze test

All groups showed similar results on the Barnes maze test's first day. However, after 4 days of training on the final day of the test, different ranges of time latency and errors in finding the goal chamber were seen. As shown in Fig. 3 (a - b) the stress group's rats needed more time to find the goal chamber (42.57 ± 6.88) and had more errors (3 ± 0.43) compared to the control group's rats (Time: 7.71 ± 1.14) (Error: 0.42 ± 0.29), and by using One-way ANOVA significant difference between groups was shown (Time: $F = 6.91$, $df = 4$) (Error: $F = 9.76$, $df = 4$) followed by Tukey's test significant difference between the stress group and the control group was confirmed ($P < 0.001$). On the other hand, the group that received the questioning nano compound showed a significant decrease in both time latency (17.71 ± 7.37) and error (0.71 ± 0.28) in process of finding the goal chamber compared to the stress group ($P < 0.01$). However other treatment groups did not show significant differences in time latency and error compared to the stress group.

3.5. Analysis of memory impairment by passive avoidance test

After passing two days of the training-day rats showed different scales of time latency for re-entering in the dark compartment. As shown in Fig. 4 the time latency that took for the stress group's rats to re-enter the dark compartment (41.71 ± 7.36) was significantly lesser than the control group's rats (214.29 ± 7.11), and by using a One-way ANOVA test and Tukey's test significant difference between all groups ($F = 136.19$, df 4) as well as the stress group and control group ($P < 0.001$) were confirmed. On the other hand, the rats of the nanoparticle group did not show any significant difference in the time latency (193.71 ± 4.23) that took for them to re-enter the dark compartment compared to the rats of the control group but they did show significant differences from the stress group ($P < 0.001$). Furthermore, the crocin group (135.43 ± 6.35) and the chitosan group (106.71 ± 3.58) showed significant time differences compared to the nanoparticle group and the control group ($P < 0.001$), they also spent significantly more time compared to the stress group ($P < 0.001$) in the bright compartment before re-entering to the dark compartment.

3.6. Analysis of Stress and Anxiety levels by Elevated plus maze (EPM)

After examining the levels of stress and anxiety through the EPM test, significant differences between groups in various parameters were observed. As shown in Fig. 5-a, the OAT parameter in the stress group (5.75 ± 2.49) was significantly lower than in the control group (27.90 ± 3.41) ($P < 0.001$) and the nanoparticle group (21.11 ± 1.33) ($P < 0.01$). Furthermore, the nanoparticle group did not show any significant difference from the control group but exhibited a significant difference from the chitosan group (7.73 ± 3.52) ($P < 0.05$). As for the OAE parameter as shown in Fig. 5-b, the control group's OAE parameter (37.62 ± 2.29) is significantly more than the stress group (19.75 ± 4.72) and it was confirmed by the One-way ANOVA test ($F = 4.93$, $df = 4$) followed by Tukey's test ($P < 0.01$). As for the nanoparticle group no significant OAE difference were seen compared to the control group.

The nanoparticle group did not show any significant difference from the control group in the head dipping parameter but as it is shown in Fig. 6-a, there were significant differences between the nanoparticle group and other groups such as the stress group and it was confirmed by the One-way ANOVA test ($F = 12.31$, $df = 4$) followed by Tukey's test ($P < 0.001$). Furthermore, the control group had no significant grooming difference compared to the nanoparticle group, however, the control group did have a significant difference compared to the stress group ($P < 0.05$) which was confirmed after analyzing its data. As for grooming (Fig. 6-b) no significant differences were seen between treatment groups. Fig. 6-c shows the time latency of freezing in all of the treatment groups efficiently

Table 2
The amount of calculated Crocin nanoparticles, EE %, and LC % are shown in the table.

Nanoparticle Size (nm)	Entrapment Efficiency (%)	Loading Capacity (%)
175 ± 5 nm	85 %	25 %

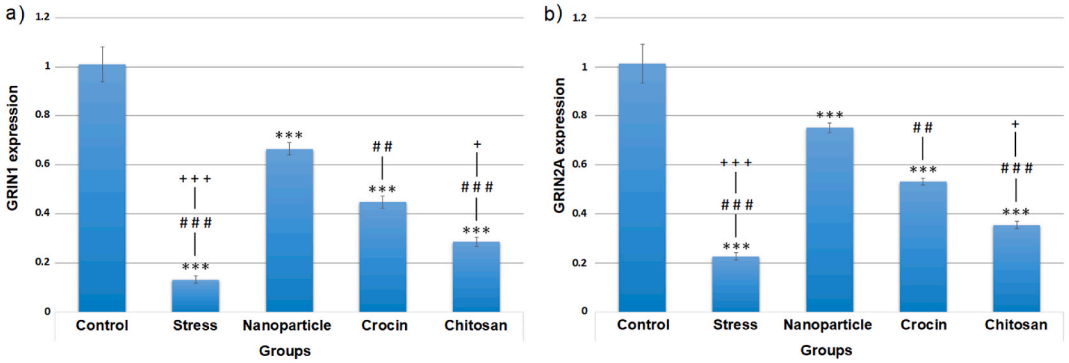

Fig. 1. Results of the GRIN1 and GRIN2A expression by RT-PCR. Mean ± SEM is demonstrated in all five experimental groups. *** = ($P < 0.001$) stands for significant differences between the experimental groups from the control group. # # # = ($P < 0.001$), and # # = ($P < 0.01$) show a significant difference between the experimental groups from the nanoparticle group. + + + = ($P < 0.001$), and + = ($P < 0.05$) shows a significant difference between the experimental groups from the crocin group.

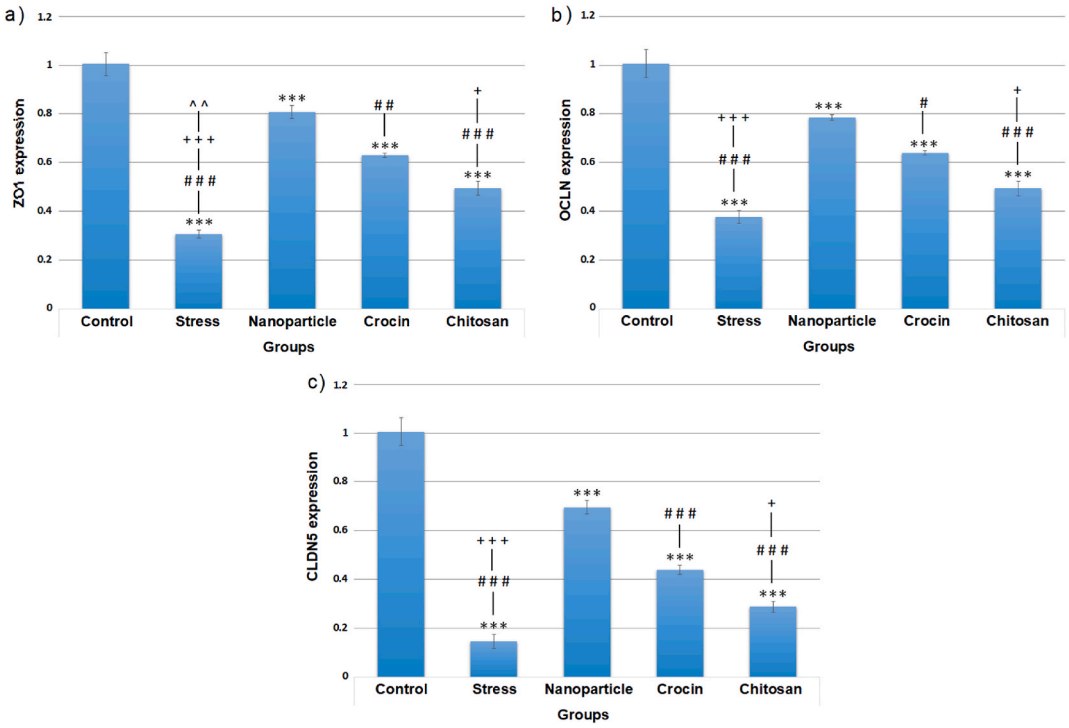

Fig. 2. Results of the gene expression by RT-PCR. Mean ± SEM ZO-1, CLDN-5, and OCLN expression are demonstrated in all five experimental groups. *** = ($P < 0.001$) stands for significant differences between the experimental groups from the control group. # # # = ($P < 0.001$), # # = ($P < 0.01$), and # = ($P < 0.05$) show a significant difference between the experimental groups from the nanoparticle group. + + + = ($P < 0.001$), and + = ($P < 0.05$) shows a significant difference between the experimental groups from the crocin group. ^^ = ($P < 0.01$) indicates a significant difference between the experimental groups from the chitosan group.

decreased compared to the stress (53.85 ± 3.18) group ($P < 0.001$), however, treatment with the CNCC compound (12.28 ± 0.91) showed significantly better results compared to the crocin (27.57 ± 2.34) and chitosan (30.83 ± 2.44) groups ($P < 0.001$).

4. Discussion

Numerous studies have explored the therapeutic potential of natural compounds such as crocin, known for their reduced side effects, as alternatives to synthetic drugs in addressing neurological disorders stemming from stressful conditions, including neurodegeneration, dementia, memory deterioration, anxiety, and depression [37–39].

In contrast to other studies that have used crocin with a high dosage between 30 and 60 mg/kg [40,41], our study focused on

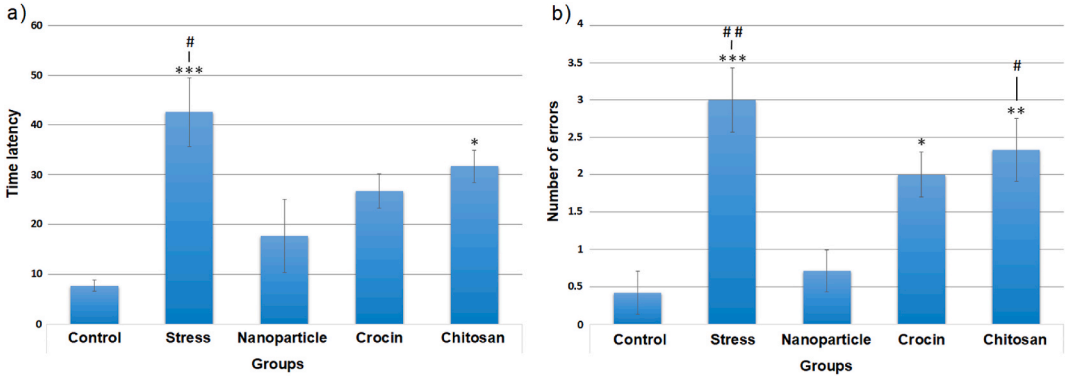

Fig. 3. Results of Barnes maze test. Mean ± SEM of time latency and errors in the process of finding the goal chamber on the testing day are demonstrated in all five experimental groups. *** = ($P < 0.001$), ** = ($P < 0.01$), and * = ($P < 0.05$) stands for significant differences between the experimental groups from the control group. # # = ($P < 0.01$), and # = ($P < 0.05$) show a significant difference between the experimental groups from the nanoparticle group.

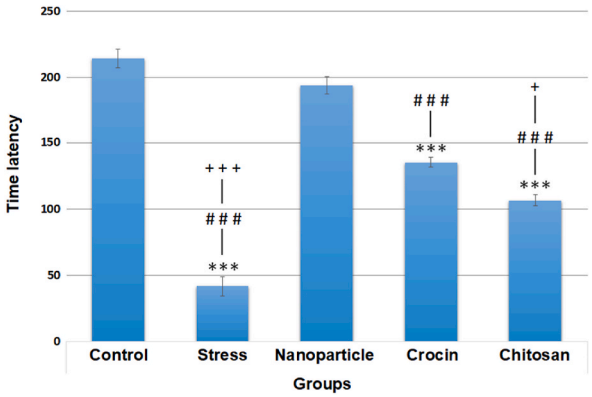

Fig. 4. Results of testing memory impairment by Passive avoidance test. Mean ± SEM of the time latency for re-entering the dark chamber on the testing day is demonstrated in all five experimental groups. *** = ($P < 0.001$) stands for significant differences between the experimental groups from the control group. # # # = ($P < 0.001$), shows a significant difference between the experimental groups from the nanoparticle group. + + + = ($P < 0.001$), and + = ($P < 0.05$) show a significant difference between the experimental groups from the crocin group.

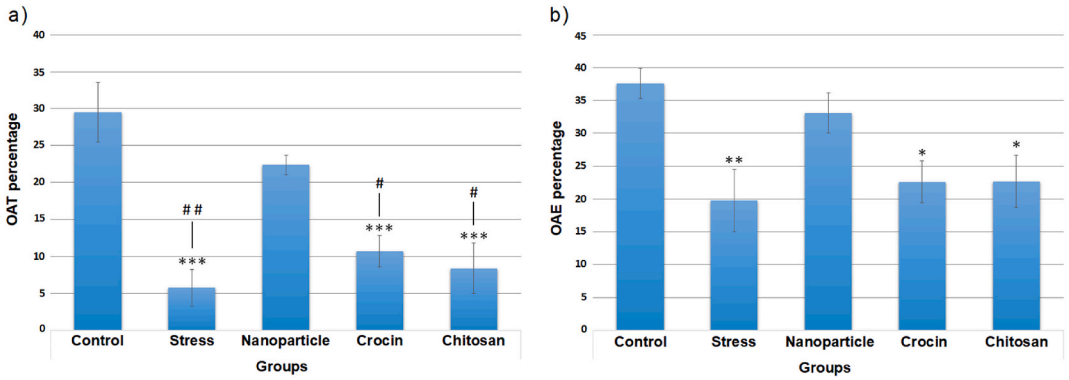

Fig. 5. Results of the anxiety level which was tested by EPM test. Mean ± SEM of OAT and OAE has been reported in all five experimental groups. *** = ($P < 0.001$), ** = ($P < 0.01$), and * = ($P < 0.05$) stands for significant differences between the experimental groups from the control group. # # = ($P < 0.01$), and # = ($P < 0.05$) show a significant difference between the experimental groups from the nanoparticle group.

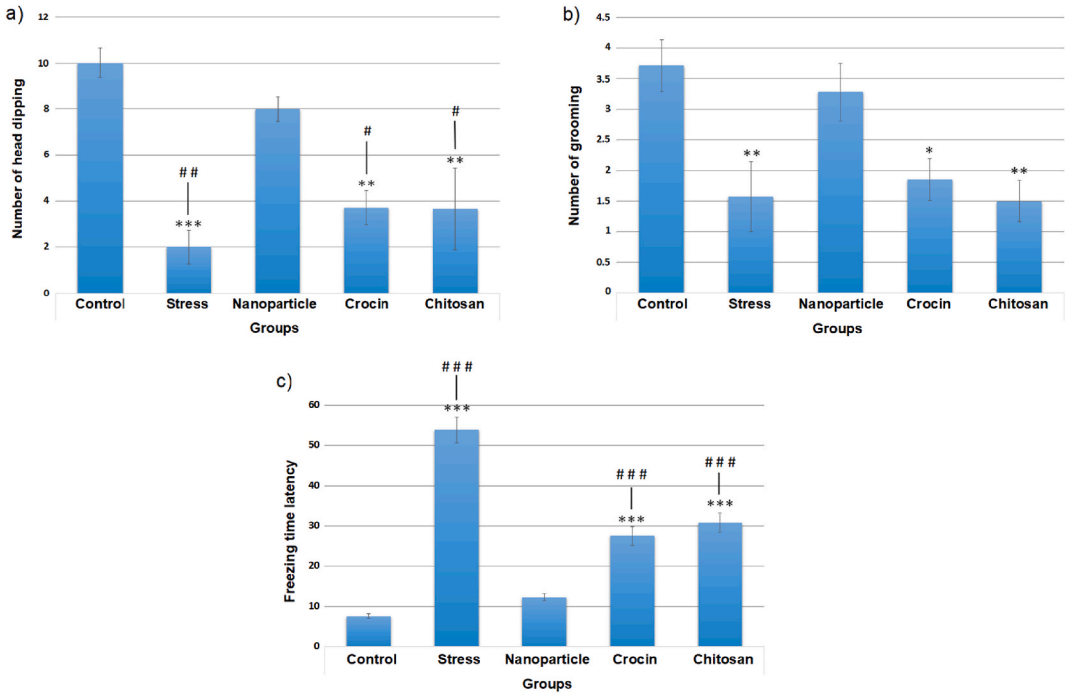

Fig. 6. Results of the anxiety level which was tested by EPM test. Mean ± SEM of head dipping, grooming, and freezing has been reported in all five experimental groups. *** = ($P < 0.001$), ** = ($P < 0.01$), and * = ($P < 0.05$) stands for significant differences between the experimental groups from the control group. # # # = ($P < 0.001$), # # = ($P < 0.01$), and # = ($P < 0.05$) show the significant difference between the experimental groups from the nanoparticle group.

enhancing the efficiency of crocin at lower doses (6 mg/kg) by applying a nanotechnology method to use chitosan as its carrier, and afterward, by comparing its effects with intact crocin, we obtained significant outcomes in both behavioral and biomolecular tests.

Stressful situations especially chronic ones, result in increased levels of corticosteroids. Additionally, excessive levels of corticosteroids have been linked to negative effects on the hippocampus in a wide range of preclinical and clinical studies Corticosteroid hormone effects in the CNS and other parts of the body are modulated by cytoplasmic steroid receptors such as glucocorticoid and mineralocorticoid receptors (GR and MR) and/or by interaction with membrane glucocorticoid receptors (mGRs) [42,43]. In 2010, Xiao Lin, and colleagues [44] reported that corticosteroids can enhance NMDA neurotoxicity in the brain by facilitating $[Ca2+]_i$ supplement and decreasing the neuroprotective NR2A-ERK1/2-mediated signaling. Moreover, It has been documented that compared to other regions of the brain, the hippocampus can have a greater number of GR and MR which makes it more susceptible to receiving a rush of corticosteroids during a chronic stress situation, this can be followed by a repressed expression response in some specific genes [43]. E. Sherwood Brown and colleagues in 2019 [45] reported that using an antagonist of NMDA receptor can attenuate the effects of corticosteroids in the DG/CA3 hippocampal subfields. Furthermore, it has been shown in various studies, in line with ours, that the chronic immobilization stress resulted in reduced expression of GRIN1 and GRIN2A genes in the hippocampus, leading to the reduction of NMDA glutamate receptor numbers, which is followed by synaptic plasticity disturbance and further memory and learning impairment [8,46]. In 2017, Asalgoo, and colleagues [35] reported that crocin can improve the adverse effects of chronic stress by affecting the hypothalamic-pituitary-adrenal (HPA) axis and modulating plasma corticosteroid levels.

A variety of studies has demonstrated that chronic immobilization stress induces BBB tight junction disintegration and reduction in ZO-1, CLDN-5, and OCLN gene expression levels which were in line we our study [20,47,48]. Moreover, recent studies have shown [49–51] that a reduction in ZO-1, CLDN-5, and OCLN gene expression levels leads to BBB malfunction and an increase in its permeability which leads to the entrance of the peripheral immune cells into the brain, triggering the glial cells activation followed by neuroinflammation and further secondary neurological disorders. In 2017, Zhang and colleagues [52] demonstrated that crocin can positively enhance the BBB tight junction genes expression of the hippocampus which was in line with our findings.

Our results of behavioral tests documented that compared to the intact form of crocin, administration of the CNCC compound can more efficiently improve memory and learning impairments caused by chronic immobilization stress. These results are probably due to the enhanced penetration of the CNCC compound from BBB and better delivery of the drug on the target site to perform its antioxidant role compared to intact crocin [31,53]. The precise mechanism of our CNCC compound in regulating corticosteroids secretion needs further investigation but we assume that these findings have resulted from the HPA axis activation and therefore reduction of the corticosteroids' adverse effects on the expression of GRIN1 and GRIN2A genes which are responsible for the accurate function of NMDA glutamate receptor in the hippocampus. Probably another reason for this better impact of CNCC compound is due to enhanced regulation of ZO-1, CLDN-5, and OCLN which are the essential elements of a physiologically stable BBB [31]. Furthermore, this

positive impact on BBB consistency leads to better cell hemostasis in CNS, resulting in the accurate function of neuronal cells in the hippocampus and suppression of secondary disorders. Our findings are consistent with the trends outlined in the comprehensive review by Esmaeil Ranjbar and colleagues (2023), which highlights the increasing importance of crocin's protective effects on endothelial and BBB disorders. This stems from its ability to prevent the decrease in the expression of tight junction proteins [54]. Additionally, compared to the intact form of crocin, it is believed that the CNCC compound can more effectively increase the amount of acetylcholine as well as decrease the amount of acetylcholinesterase in the hippocampus [55,56], which could be followed by a better performance in memory and learning process.

A recent study conducted by Pirzad-Jahromi and colleagues demonstrated that crocin can attenuate anxiety-linked behaviors, memory, and synaptic plasticity impairment in hippocampal CA1 induced by chronic immobilization-stress conditions [39].

Our data indicated that a low dosage of intact crocin (6 mg/kg) did not significantly improve anxiety-linked behaviors in the immobilization stress-induced rat model. However, the same dosage of crocin in the form of a nano-chitosan-coated compound resulted in significant improvement in anxiety-like behaviors. This effect is likely due to the restoration of BBB physiological functions, reducing disorders such as depression and neuroinflammation caused by BBB malfunction, and consequently improving cognitive impairment associated with BBB breakdown [57–59]. In addition, compared to intact crocin, probably the higher stability of chitosan-nano-coated crocin exhibited better anti-oxidant and anti-inflammation properties, which might be another reason for the better performance of rats treated with this compound regarding anxiety, memory, and learning behavioral tests. These findings are in line with our recent study on the protective effects of CNCC on anxiety and cognitive impairment induced by Alzheimer's disease [60]. Finally, we hypothesized that the greater effect of the studying compound on anxiety is probably due to better effects on the dopaminergic system and norepinephrine inhibitors [61]. Clearance by the reticuloendothelial system through opsonization is one of the main challenges in nanoparticle drug delivery and many studies implicated that the size of the nanoparticle can influence the clearance and also the distribution of the drug [62,63]. Further studies regarding the clearance of CNCC compound by the reticuloendothelial system are needed to enlighten the attainment of the subtherapeutic concentration of crocin as a therapeutic agent at the target tissues.

5. Conclusion

Taken together, our study highlights the potential of utilizing crocin in a nano-chitosan-coated form to overcome its low bioavailability. This innovative approach enabled us to achieve effective dosages of crocin at significantly lower concentrations compared to previous studies, offering promising outcomes in mitigating the adverse effects of immobilization stress, such as anxiety and memory impairment. By addressing the limitations associated with crocin's bioavailability, our research contributes to the advancement of therapeutic strategies. Through meticulous design and implementation, our study aims to provide a practical solution for enhancing the therapeutic properties of crocin, paving the way for the development of novel treatments for stress-related symptoms in rat models.

CRediT authorship contribution statement

Mohsen Khodadadi: Writing – review & editing, Writing – original draft, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Gila Pirzad Jahromi:** Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Gholam Hossein Meftahi:** Investigation, Conceptualization. **Hossein Khodadadi:** Writing – review & editing, Formal analysis. **Mohammadmehdi Hadipour:** Investigation, Data curation. **Masoud Ezami:** Resources.

Compliance with ethical standards

This study was done according to the animal welfare rules and the guidelines that were approved by the international and local ethical committees. We tried our hardest to reduce the number of rats used in this experiment and to reduce their pain and suffering during the study. This study was approved by the Baqiyatallah University of Medical Science's ethical committee, in Tehran, Iran (Ethics Code: IR.BMSU.REC.1400.151).

Data and Code availability statement

Data will be available upon request.

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Declaration of competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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