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Agmatine Reverses Sub-Chronic Stress-induced Nod-like Receptor Protein 3 (NLRP3) Activation and Cytokine Response in Rats

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Abstract: The activation of Nod-like receptor protein 3 (NLRP3) has lately been implicated in stress and depression as an initiator mechanism required for the production of interleukin (IL)-1β and IL-18. Agmatine, an endogenous polyamine widely distributed in mammalian brain, is a novel neurotransmitter/neuromodulator, with anti-stress, anxiolytic and antidepressant-like effects. In the present study, we examined the effect of exogenously administered agmatine on NLRP3 inflammasome pathway/cytokine responses in rats exposed to restraint stress for 7 days. Rats were divided into three groups as stress, stress+agmatine (40 mg/kg; i.p.) and control groups. Agmatine, significantly down-regulated the gene expressions of all stress-induced NLRP3 inflammasome components (NLRP3, NF-κB, PYCARD, caspase-1, IL-1β and IL-18) in the hippocampus and prefrontal cortex (PFC), and reduced pro-inflammatory cytokine levels not only in both brain regions but also in serum. Stress-reduced levels of IL-4 and IL-10, two major anti-inflammatory cytokines, were restored back to normal by agmatine treatment in the PFC. Findings of the present study suggest that stress-activated NLRP3 inflammasome and cytokine responses are reversed by acute administration of agmatine. Whether antidepressant-like effect of agmatine can somehow, at least partially, be mediated by the inhibition of NLRP3 inflammasome cascade and relevant inflammatory responses requires further studies in animal models of depression.

There is strong evidence that psychological stress induces pro-inflammatory cytokines such as interleukin (IL)-1β and tumour necrosis factor (TNF)-α in the periphery and the brain [1-6]. It has been repeatedly shown that the administration of IL-1β receptor antagonists can ameliorate depressive-like behaviours of animals that are exposed to acute or chronic stress

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[2,7,8]. In fact, it is common sense that IL-1β might be a key factor in stress-related conditions and serves as a biomarker in the diagnosis of depression [9]. At this point, much attention has lately been drawn to the importance of a particular initiator mechanism of IL-1β-mediated inflammatory responses, namely as *the inflammasome*, in psychological stress and depression as a novel target in these conditions [1,2]. The nucleotide binding and oligomerization domain-like receptor protein 3 (NLRP3) inflammasome is a multiprotein complex that is formed by the activation and oligomerization of NLRP3 and responsible for initiating IL-1β- and IL-18-mediated inflammatory responses [1,10-12]. Accumulating evidence suggests that NLRP3 inflammasome is activated in animal depression models and in patients with major depressive disorder [13-16].

NLRP3, a member of the nod-like receptors family (NLRP1, NLRC4, AIM2), is a cytosolic receptor protein that is located in macrophages and microglia and responsible for recognizing a wide range of danger signals and triggers IL-1β- and IL-18-mediated inflammatory processes [17,18].

NLRP3 structurally consists of three domains: C-terminal leucin rich repeat (LRR), centrally localized nucleotide binding oligomerization (NACHT) and N-terminal pyrin (PYD) domains [19]. When macrophage/microglia cells are encountered with danger signals, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and pro-caspase-1 bind to NLRP3 which consequently forms the inflammasome structure. ASC functions as a bridge between NLRP3 and pro-caspase-1 with its pyrin and CARD domains that bind the N-terminal PYD of NLRP3 and the CARD domain of pro-caspase-1, respectively [12]. This binding results in caspase-1 activation and consequently production of the mature forms of pro-IL-1β and pro-IL-18; IL-1β and IL-18. Toll-like receptors (TLRs) (especially TLR2 &

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TLR4) are located on the cell membrane of macrophages/microglia, are the main pattern recognition receptors for activating NLRP3 inflammasome complex and subsequently allowing the production of IL-1β and IL-18. When TLRs are stimulated, the gene transcriptions of IL-6, TNF-α, pro-IL-1β, pro-IL-18 and NLRP3 are increased by nuclear factor kappa B (NF-κB) pathway while a separate second-base mechanism is required for the maturation of pro-IL-1β and pro-IL-18 which is the activation of the NLRP3 inflammasome and release of caspase-1 from pro-caspase-1 to transform pro-IL-1β and pro-IL-18 into their mature forms [1,17,18,20-23].

Agmatine, a novel neurotransmitter/neuromodulator in the mammalian brain [24], is an endogenous amine that is synthesized through decarboxylation of L-arginine by mitochondrial enzyme arginine decarboxylase and metabolized either by agmatinase into different polyamines or by diamine oxidase to guanido butanoic acid [25-27]. Agmatine is a competitive inhibitor of both neuronal nitric oxide synthase (nNOS) and inducible NOS (iNOS) [28] whereas it conversely stimulates endothelial NOS (eNOS) [29]. Agmatine binds to imidazoline-binding sites [30], and α_2-adrenoreceptors and blocks N-methyl-D-aspartate (NMDA) receptors via voltage- and concentration-dependent mechanisms and other ligand-gated cationic channels including nicotinic and 5-HT_3 receptors [26,27,30,31].

Our group and other research groups have repeatedly demonstrated that exogenous agmatine has a variety of pharmacological effects improving a wide spectrum of central nervous system conditions, like algesia including neuropathic pain [32,33], mood disorders [34], epilepsy [35,36], morphine type addiction [37-40], neuro injury [41-43] and cognition [44,45]. Perhaps one of the most well-established effects are anti-stress, anxiolytic and antidepressant-like effects produced by agmatine [34,46,47].

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Up to the present, there are less but suggestive reports regarding agmatine's anti-inflammatory effects [48-50]. In a recent study, agmatine has been shown to reverse depressive-like behaviours induced by TNF-α administration in mice [51]. However, there is still lack of understanding regarding the molecular mechanisms which may at least partially contribute to the proposed immunomodulatory effects of agmatine in stress-related conditions.

The aim of the present study was to examine the effect of acute agmatine administration on NLRP3 inflammasome activation and relevant cytokine responses in sub-chronic restraint stress model of rats. We examined NLRP3 inflammasome components in two brain regions: prefrontal cortex (PFC) and hippocampus. We further examined the expressions of IL-1β, IL-18 and some other pro-/anti-inflammatory cytokines both in the brain and the peripheral blood serum of rats.

Materials and Methods

Animals

Male adult Sprague-Dawley rats weighing 250-350 g were used (Marmara University, Experimental Animal Implementation and Research Centre) and housed in groups of 4-5 rats per cage (dimensions; 27x40x18 cm) under standard laboratory conditions (12-hr light/dark cycle; room temperature 21±2 °C). The animals were allowed to acclimate to the housing room and handled for seven days prior to the experiment. Food and water were provided *ad libitum* except the duration of stress procedure. The study was approved by the Animal Ethics and Care Committee of Marmara University.

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Experimental design and drugs

Twenty-two rats were used in total and divided into three experimental groups: stress group (n=8), stress+agmatine (n=8) group and control group (n=6). The control group was kept under standard housing conditions and only applied daily handling stress for about 5 min./day for 7 days. Stress groups underwent restraint stress for seven consecutive days, about 4 hr/day (8 a.m. – 12 noon). The stress and agmatine group was intraperitoneally administered agmatine sulfate (Sigma-Aldrich, USA; A7127), 40 mg/kg, dissolved in 0.9% NaCl while the control group received 0.9% NaCl (0.1 ml/100 g) 30 min. prior to the last stress procedure. Agmatine dose and administration route (40 mg/kg; intraperitoneally) was chosen based on our previous work demonstrating the antidepressant and anxiolytic-like effects in the forced swimming and elevated plus maze tests (34).

Acute restraint stress procedure

Metal restraint modules containing air holes were used for the stress procedure. Rats were gently replaced in the modules and immobilized by a separate reverse U-shaped metal apparatus which were attached to the appropriate holes on the module where the rat's body ends. It was taken care of that the metal apparatus was replaced in the correct hole for each rat to prevent any body harm or lack of immobilization due to the body weight differences between the test subjects. Restraint modules and metal apparatus were washed and dried after each experiment.

Stress procedure was conducted for seven consecutive days, 4 hr/day between 8 a.m. and 12 noon on each day. Rats in the stress groups were individually brought to the experiment room just before the procedure and brought back to the housing room after the end of the

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stress session while the control group subjects were kept in the housing room and only exposed to daily handling stress (5 min.) for 7 days. Rats were killed by a rapid decapitation on the seventh day, immediately after the last stress session. The brains were dissected out and peripheral blood samples were collected for gene and protein analysis of NLRP3 inflammasome and relevant cytokines.

Collection of tissue samples

The subjects from each group were killed immediately after the last stress procedure and peripheral blood samples were collected using dry centrifuge tubes, then blood serum were isolated for protein expression analysis of pro-/anti-inflammatory cytokines (7-Plex Panel & IL-1β; Antigenix America). PFC and hippocampus tissues were separated following the brain dissection for the protein and gene analysis of cytokines and NLRP3 inflammasome cascades. In order to do this, both tissues were divided into two and stored separately (4 tissue samples per rat; two PFC samples, two hippocampus samples). Each samples of PFC and hippocampus were treated with RNAlater for gene expression analysis or with tissue homogenization lysis buffer (pH 7.4; 0.5% Triton X-100, 150 mM NaCl, 15 mM Tris, 1 mM $CaCl_2$, 1 mM $MgCl_2$) for protein expression analysis. All samples were stored at -80°C.

Measurement of cytokines

Pro-inflammatory (IL-1β, IL-2, IL-6, IL-17, TNF-α, IFN-γ) and anti-inflammatory (IL-4, IL-10) cytokine protein levels were measured by bead-based assay, using Super X-Plex Th1-2 Cytokine Assay Kit (Antigenix America, USA) in flow cytometry (FC500, Beckman Coulter, USA). This kit utilizes multiple bead populations differentiated by size and levels of fluorescence intensity. This enables the resolution of distinct bead populations on the flow

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cytometer. Briefly, after daily quality control and standardization of fluorescence measurement (Flow Check beads for laser alignment, Flow Set beads for fluorescence calibration, both obtained from Beckman Coulter, USA) and check for colour compensation (CytoComp, Beckman Coulter, USA), standard reagents were run and adjustments were finalized. Cytokine concentrations were based on the measured R-Phycoerythrin emission. Concentrations of the sample were determined by comparison to known concentrations of a standard curve obtained by running standard reagents. IL-18 was measured by ELISA (Shanghai Sunred Biological Technology Co., Ltd, PRC).

qPCR Measurements

All samples were collected into RNAlater stabilization solution (Life Technologies, USA) and kept at -20^{o}C. After thawing, tissue homogenates were obtained by using ultrasonic homogenizator (The Omni Sonic Ruptor Ultrasonic Homogenizer, Omni International, USA). RNA isolations from hippocampus and PFC tissue homogenates were conducted with Trizol-Plus RNA isolation kit (Life Technologies, USA). All samples were examined for their RNA quality by spectrophotometer (Epoch, BioTek Instruments, Inc., USA). A260/280 was used to determine the purity of the RNA sample. Those samples which had an OD 260/280 ratio of 1.8 to 2 were considered as well-isolated samples and isolation procedures were repeated for those with lower ratio values. All procedures were accomplished according to manufacturer instructions. More specifically, tissue samples were homogenized in 1 mL Trizol® Reagent per 50 – 100 mg tissue using a tissue homogenizer. The sample volume did not exceed 10% of the volume of Trizol® Reagent used for homogenization. Lysates were incubated with Trizol® Reagent at room temperature for 5 min. to allow complete dissociation of nucleoprotein complexes. Then, 0.2 mL chloroform per 1 mL Trizol® Reagent

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was added. The tubes were shaken vigorously by hand for 15 sec. and then incubated at room temperature for 2-3 min., centrifuged at 12,000 x *g* for 15 min. at 4^{o}C. Upper phase containing RNA was transferred to RNase-free tubes. After adding ethanol, the sample was transferred to a spin cartridge and then binding, washing and elution steps were performed. In order to perform efficient RNA isolation and to remove remaining genomic DNA, samples were treated with DNAse I. cDNA synthesis from obtained RNA samples were performed with High-Capacity cDNA Reverse Transcription kit (Applied BioSystems, USA). cDNA samples were kept at -80^{o}C. Gene expression analysis was performed for NLRP3 inflammasome components (NLRP3, caspase-1, ASC, IL-1β, IL-18, NF-κB) and pro-inflammatory cytokines (IL-6 and TNF-α). Taqman Gene Expression Master Mix (Applied BioSystems, USA) was used for all analyses and GAPDH was used as an internal control (housekeeping gene). Primers and probes were obtained as a ready-to-use kit (Life Technologies, USA). Relative quantitation was calculated with 2^$^{-}$(ddCT).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software Inc., USA). Data were presented as the means ± SEMs. The one-way analysis of variance (ANOVA) followed by Tukey's HSD test was used to determine whether there were any significant differences between the means of two or more independent groups. For all analyses, $p < 0.05$ was regarded to be statistically significant.

Results

NLRP3 inflammasome is activated in both brain regions (PFC and hippocampus) after stress exposure

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After 7 days of restraint stress exposure, gene expressions of NLRP3 inflammasome components significantly increased in the specific brain regions, PFC and hippocampus, compared to the non-stressed control group. In the stress group, the hippocampal gene expressions of all inflammasome components; NLRP3 (F=21.06, p<0.001), caspase-1 (F=13.96, p<0.001), PYCARD domain (ASC) (F=21.12, p<0.001), NF-κB (F=79.03, p<0.001), IL-1β (F=20.90, p<0.001), and IL-18 (F=24.07, p<0.001) exhibited a significant fold increase compared to the control group (fig. 1A). The PFC gene expressions were similar to those seen in hippocampus, however, with smaller but significant differences as; NLRP3 (F=25.12, p<0.001), caspase-1 (F=21.63, p<0.001), PYCARD domain (F=14.56, p<0.001), NF-κB (F=21.97, p<0.001), IL-1β (F=6.345, p<0.05) and IL-18 (F=9.155, p<0.01) (fig. 1B).

The mRNA levels of IL-6 and TNF-α were also examined and it was found that IL-6 (F=17.60, p<0.001 in hippocampus; F=12.08, p<0.001 in PFC) and TNF-α (F=15.98, p<0.001 in hippocampus; F=8.453, p<0.01 in PFC) were significantly elevated in both brain regions in the stress group compared to the control group (fig. 2).

In addition to mRNA levels, the protein expressions of IL-1β and IL-18, two pro-inflammatory cytokines of which production is dependent on NLRP3 inflammasome activation also significantly elevated after stress exposure in hippocampus (IL-1β; F=46.66, p<0.001, IL-18; F=608.6, p<0.001) and PFC (IL-1β; F=49.32, p<0.001, IL-18; F=1907, p<0.001) compared to the non-stressed group (fig. 3).

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Agmatine reverses stress-induced NLRP3 inflammasome activation in both brain regions

When administered 30 min. prior to the last stress session, agmatine (40 mg/kg; .i.p.) reversed stress-induced NLRP3 inflammasome activation in both brain regions by reducing the relative gene expression levels of all inflammasome components except for IL-18. In the hippocampus, the stress+agmatine group exhibited significant-fold decreases in mRNA levels of NLRP3 (F=21.06, p<0.001), caspase-1 (F=13.96, p<0.05), PYCARD domain (F=21.12, p<0.001), NF-κB (F=79.03, p<0.001) and IL-1β (F=20.90, p<0.01) compared to the stress-only group (fig. 1A). However, stress-induced IL-18 levels remained high after agmatine treatment unlike seen in other components (fig. 1A). In the PFC, agmatine treatment significantly decreased stress-induced gene expressions of NLRP3 (F=25.12, p<0.001), caspase-1 (F=21.63, p<0.01), PYCARD domain (F=14.56, p<0.001), NF-κB (F=21.97, p<0.001) and IL-1β (F=6.345, p<0.05) (fig. 1B). Meanwhile, agmatine slightly but not significantly reduced IL-18 gene expression in the PFC (F=9.155, p>0.05) (fig. 1B).

In addition to the gene expressions, we also examined the effect of agmatine on protein levels of IL-1β and IL-18 in both brain regions. Agmatine administration significantly reduced the protein expressions of IL-1β in both regions (F=46.66, p<0.001 in hippocampus; F=49.32, p<0.001 in PFC) (fig. 3). IL-18 protein levels remained high in the hippocampus of agmatine-treated rats (fig. 3A). Unlike hippocampus, PFC IL-18 protein levels were significantly reduced by agmatine treatment compared to the stress-only group (F=1907, p<0.001), but they were still significantly higher compared to the control group (p<0.001) (fig. 3B).

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Stress resulted in increased hippocampal protein levels of pro-inflammatory cytokines with having no effect on anti-inflammatory cytokine levels

We also analysed the protein expressions of other pro-inflammatory cytokines including TNF-α, IL-6, IL-2, IFN-γ, IL-17 together with two anti-inflammatory cytokines; IL-4 and IL-10 in flow cytometry. According to our results, the shift between pro- and anti-inflammatory cytokines was in favour of pro-inflammatory cytokines in the hippocampus of the stress group since the protein levels of all of the investigated pro-inflammatory cytokines were significantly higher (IL-6; $F= 5.615$, $p<0.05$, TNF-α; $F= 423.8$, IL-2; $F=35.22$, IFN-γ; $F= 25.76$, IL-17; $F= 39.82$, $p<0.001$) (fig. 4A) whereas there was no significant change in levels of anti-inflammatory cytokines, IL-4 ($F=0.591$, $p=0.5641$) and IL-10 ($F=0.5474$, $p=0.5878$ (fig. 4B), in the hippocampus of stressed rats compared to the control group.

Stress resulted in increased PFC protein levels of pro-inflammatory cytokines coupled with a tendency of decrease in anti-inflammatory cytokine levels

As in the hippocampus, the protein expressions of pro-inflammatory cytokines were also increased in the PFC of stressed rats (IL-6; $F=27.02$, TNF-α; $F=71.36$, IL-2; $F=41.02$, IFN-γ; $F=45.21$, IL-17; $F=25.15$, $p<0.001$) (fig. 5A). However, this time, there was a tendency of decrease in the protein levels of anti-inflammatory cytokines; IL-4 and IL-10 (fig. 5B). The PFC IL-4 protein levels were slightly but not significantly decreased ($F=9.034$, $p>0.05$) while IL-10 levels were significantly decreased in the stress group ($F=47.58$, $p<0.001$) compared to the control group (fig. 5B). Again, the balance between pro- and anti-inflammatory cytokines in the PFC was on behalf of pro-inflammatory ones in the presence of sub-chronic stress.

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The serum protein levels of pro-inflammatory cytokines are elevated in stress-exposed rats

Serum pro-inflammatory cytokines were significantly increased in the stress group compared to the control group: IL-1β (F=138.0, p<0.001), IL-18 (F=1675, p<0.001), IL-6; (F=7.823, p<0.05), TNF-α (F=121.2, p<0.001), IL-2 (F=56.92, p<0.001), IL-17 (F=74.59, p<0.001) and IFN-γ (F=34.96, p<0.01) (fig. 6A). Serum IL-4 levels were significantly decreased in the stress group compared to the control group (F=113.0, p<0.001), whereas IL-10 levels were not statistically different from the control group (F=29.63, p>0.05) (fig. 6B).

Agmatine reduces stress-induced elevated levels of pro-inflammatory cytokines both in the brain and the periphery

Acute agmatine administration reduced the stress-induced elevated protein levels of pro-inflammatory cytokines in both brain regions (fig. 4A and fig. 5A) and in the serum (fig. 6A): TNF-α (F=423.8, p<0.001; in hippocampus, F=71.36, p<0.001 in PFC, F= 121.2, p<0.001 in serum), IL-6 (F=5.615, p<0.05; in hippocampus, F=27.02, p<0.001; in PFC, F= 7,823, p<0.01 in serum), IL-2 (F=35.22, p<0.001; in hippocampus, F=41.07, p<0.001 in PFC, F=56.92, p<0.001 in serum), IFN-γ (F=25.76, p<0.001; in hippocampus, F=45.21, p<0.001 in PFC, F=34.96, p<0.001 in serum) and IL-17 (F=39.82, p<0.001; in hippocampus, F=25.15, p<0.001 in PFC, F=74.59, p<0.001 in serum) were significantly decreased in the stress+agmatine group compared to the stress group (fig. 4A and fig. 5A). In the hippocampus, acute agmatine treatment resulted in a significant decrease in relative mRNA levels of TNF-α (F= 15.98, p<0.01) and IL-6 (F= 17.60, p<0.05) compared to the stress-only group (fig. 2A). In the PFC region, there was a slight but not significant-fold decrease in TNF-α (F= 8.453, p>0.05) and significant-fold decrease in IL-6 mRNA levels (F=12.08, p<0.05) in the agmatine-treated group compared to the stress group (fig. 2B).

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Agmatine restores anti-inflammatory cytokines in the PFC and serum but not in the hippocampus

In the PFC, acute agmatine treatment significantly increased the protein levels of IL-4 (F=9.034, p<0.01) and IL-10 (F=47.58, p<0.001) compared to the relatively decreased levels seen in the stress group (fig. 5B). Moreover, the increased levels of IL-4 and IL-10 by agmatine treatment were similar to the levels observed in the control group (fig. 5B).

On the contrary, there were no significant changes in hippocampal levels of IL-4 (F=0.5911, p>0.05) and IL-10 (F=0.5474, p>0.05) in stressed rats compared to the control group. Agmatine treatment did not exhibit any significant differences in hippocampal anti-inflammatory cytkoines levels (fig. 4B).

In peripheral blood serum, the stress+agmatine group demonstrated significant increases in the protein levels of both IL-4 (F=113.0, p<0.001) and IL-10 (F=29.63, p<0.001) compared to the stress-only group (fig. 6B). Elevated IL-10 levels by agmatine treatment were significantly higher than the levels seen in the control group (p<0.001).

Discussion

In the present study, NLRP3 inflammasome is activated in hippocampus and PFC of rats exposed to restraint stress for 7 days which was accompanied with elevated serum and brain levels of certain pro-inflammatory cytokines. When administered acutely, agmatine (40 mg/kg) was able to decrease all NLRP3 inflammasome components [NLRP3, NF-κB, PYCARD (ASC), caspase-1, IL-1β and IL-18] included in this present study. Additionally, agmatine reversed relevant cytokine responses by shifting an anti-inflammatory state not only in the brain but also in the periphery.

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To date, the link between psychological stress and increased inflammatory responses has been widely reported [1,3,5,52,53]. Especially, IL-1β seems to be a valuable predictive factor for this linkage among other pro-inflammatory cytokines in the pathology of depression and stress-related conditions [9,52]. Considering this strong association, the attention has been lately drawn to the NLRP3 inflammasome pathway as an initiator mechanism responsible for the release of IL-1β and IL-18 [1]. Presently, a limited number of encouraging studies have recently reported the activation of NLRP3 inflammasome in animal models of depression and patients with major depressive disorder [13-16,54-59]. NLRP3 inflammasome activation and subsequent increase in PFC but not serum IL-1β levels of rats were reported in chronic unpredictable mild stress (CUMS) by a recent study [14]. In contrast to this report, we found that not only PFC levels but also serum protein levels of IL-1β and IL-18 were increased in response to stress condition. CUMS procedure in mice also induced hippocampal NLRP3 inflammasome activation and elevated IL-1β levels both in the hippocampus and serum, which is consistent with our findings [15].

NLRP3 inflammasome activation was also examined in patients with major depressive disorder [13]. It was shown that NLRP3 inflammasome was activated in peripheral blood mononuclear cells of depressed patients and this was coupled with elevated serum protein levels of IL-1β and IL-18. Moreover, amitriptyline treatment reduced NLRP3 and caspase-1 gene expression, and IL-1β and IL-18 serum levels [13].

Similar with the previous studies, one of the latest findings from another study reported that NLRP3 inflammasome was activated in the hippocampus of CUMS-exposed rats [54]. Furthermore, this effect was along with increased hippocampal oxidative-nitrosative markers, inflammatory cytokines and iNOS which supports the notion of agmatine as an

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antidepressant molecule that competitively inhibits iNOS and have anti-inflammatory and antioxidant properties due to the fact that it could suppress NLRP3 inflammasome activation and inflammatory processes in response to stress [24,28,34,46,51]. Thus, in the present study, we found that acute agmatine treatment was able to inhibit NLRP3 inflammasome activation and reduce subsequent pro-inflammatory cytokine levels not only in both brain regions but also in serum of stressed rats.

The balance between pro- and anti-inflammatory cytokines has been shown to be shifted in direction of pro-inflammatory cytokines in animal models of depression and depressed patients [60-63]. In our study, we also measured protein levels of certain pro-/anti-inflammatory cytokines (TNF-α, IL-6, IL-2, IFN-γ, IL-17, IL-4 and IL-10) in addition to NLRP3 inflammasome-mediated cytokines (IL-1β and IL-18) in the brain and serum of rats. We found that all examined pro-inflammatory cytokine levels were increased in PFC, hippocampus and serum of stressed rats, whereas analysis of anti-inflammatory cytokines, IL-4 and IL-10, exhibited inconsistent results. IL-4 levels were reduced significantly in serum and exhibited a tendency to decrease in PFC but not in hippocampus of the stressed rats. On the other hand, the reduction in IL-10 levels was observed only in PFC of the stressed rats.

Interestingly, agmatine treatment reduced pro-inflammatory cytokines induced by stress in the hippocampus, PFC and serum while causing a considerable elevation in anti-inflammatory cytokine levels in the PFC and serum. However, our findings regarding hippocampal anti-inflammatory cytokine levels not showing any differences between control and stress groups remains yet to be elucidated.

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To date, a growing number of studies have been exploring the neuroprotective and/or anti-inflammatory effects of agmatine which served the basis for our study [48,49,51,64-67]. It has been shown that astroglial and macrophage iNOS cell expression induced by LPS/cytokine incubation was dose-dependently decreased by agmatine treatment [49]. In an another study, agmatine was shown to attenuate LPS-induced anorexia, sickness behaviour, and IL-6 and TNF-α serum levels in rats [66]. In an acute lung injury model, agmatine treatment reduced inflammation by inhibiting NF-κB, and certain pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 [68]. Restraint stress-induced depressive-like behaviour and hippocampal pro-/anti-oxidative homeostasis imbalance in mice were shown to be abolished by agmatine treatment [64]. In a very recent study, agmatine reversed depressive-like behaviours induced by TNF-α in mice [51]. Moreover, Freitas *et al.* have recently reported that agmatine is protective against the detrimental effects of corticosterone in the hippocampal neuronal cell line by at least partially inducing the activation of nuclear factor (erythroid 2-derived)-like 2 (Nrf2), one of the key transcription factors of the cellular antioxidant defences, which has been lately implicated in the depression [69]. The possible link between Nrf2 activation and agmatine's antidepressant-like effects was further examined in corticosterone-induced depression model of mice. Agmatine treatment for 21 days was not able to demonstrate antidepressant like effect in Nrf2-knockout mice [70]. However, in wild-type mice, agmatine treatment not only abolished corticosterone-induced depressive-like behaviour but also induced neuroplasticity, ameliorated microglia and astrocytes morphology by a mechanism that seems to be at least partially dependent on the activation of Nrf2 which could also be related with agmatine's proposed anti-inflammatory effects, an issue to be addressed in the future studies.

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This present study has certain limitations. Firstly, we did not include behavioural experiments for assessing depressive-like behaviours in sub-chronic restraint stress model and therefore we are unable to draw a conclusion on whether the inhibition of NLRP3 inflammasome cascade and relevant cytokine responses by agmatine could be associated with anti-depressive/anxiolytic-like properties. We have previously shown that agmatine, when administered acutely at a dose of 40 mg/kg as in the present study, elicited antidepressant and anxiolytic-like effects in the forced swimming and elevated plus maze tests [34]. Further, Lavinsky *et al.* reported that agmatine (40 mg/kg) induces anxiolytic-like behaviour in the elevated plus maze task [71]. Based on these previous works, we rather focused on the effect of agmatine on stress-induced NLRP3 inflammasome activation and subsequent cytokine responses by conducting a wide spectrum of analyses.

In conclusion, despite the mentioned limitations, our study clearly demonstrates that even when administered acutely, agmatine reverses stress-induced NLRP3 inflammasome activation and relevant cytokine responses. The present findings therefore could provide novel insights for future translational studies addressing the role of agmatine's suggested immuno-modulatory actions for improving the behavioural alterations seen in depression and stress-related disorders.

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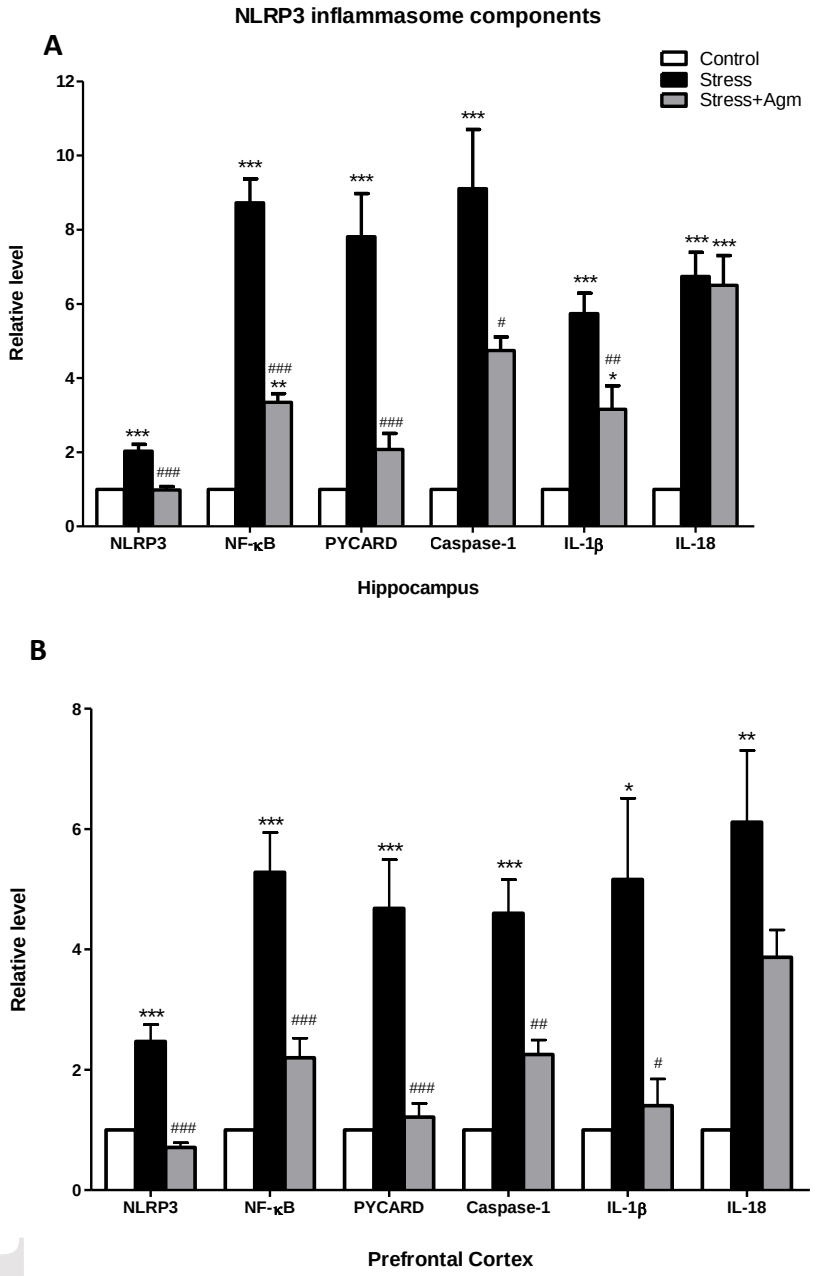

Fig. 1. The effect of acute agmatine treatment on relative mRNA levels of NLRP3 inflammasome components in hippocampus (A) and prefrontal cortex (B) of stressed rats. Agmatine (Agm) (40 mg/kg; i.p.) was administered 30 min. prior to the last stress session. All data are expressed as means ± SEM (n=6-8) and represented as fold change relative to control group. *p<0.05, **p<0.01, ***p<0.001 *versus* control group; $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 *versus* stress group.

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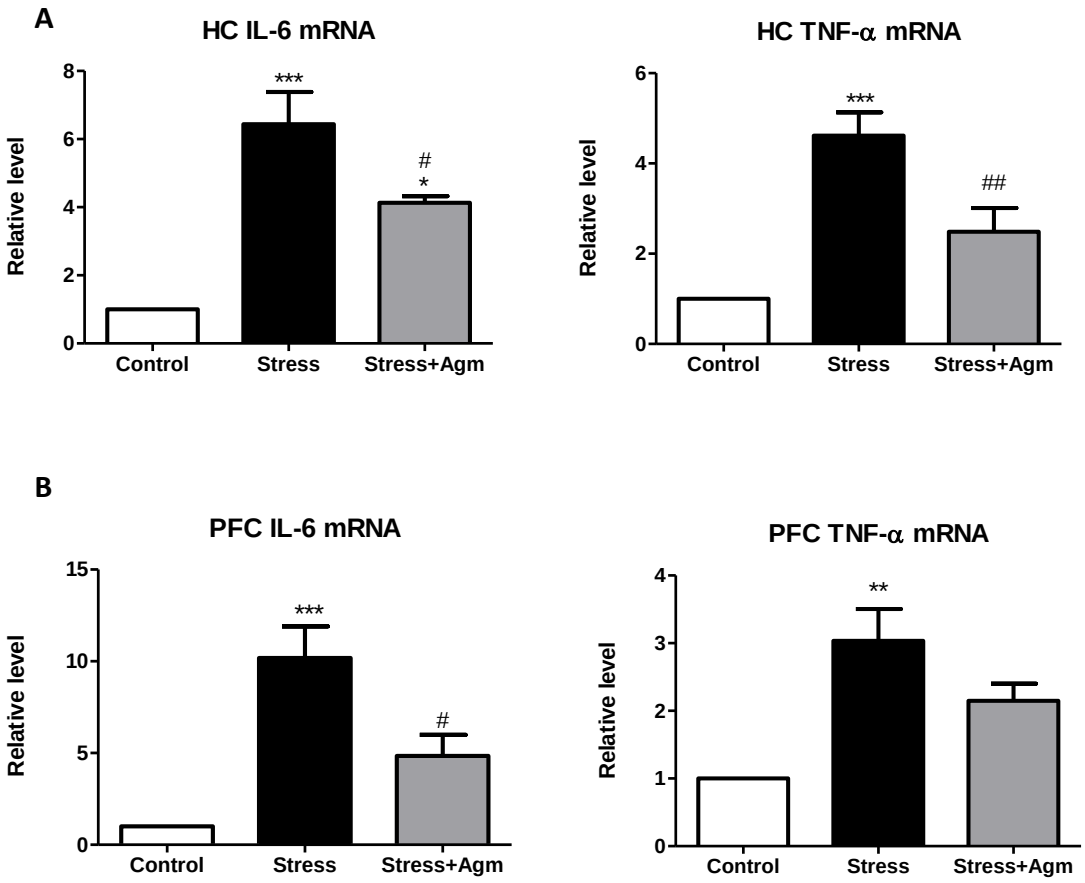

Fig. 2. The effect of acute agmatine treatment on gene expressions of IL-6 and TNF- α in hippocampus (HC) (A) and prefrontal cortex (PFC) (B) of stressed rats. Agmatine (Agm) (40 mg/kg; i.p.) was administered 30 min. prior to the last stress session. All data are expressed as means ± SEM (n=6-8). *p<0.05, **p<0.01, ***p<0.001 *versus* control group; $^{\#}$p<0.05, $^{\#\#}$p<0.01 *versus* stress group.

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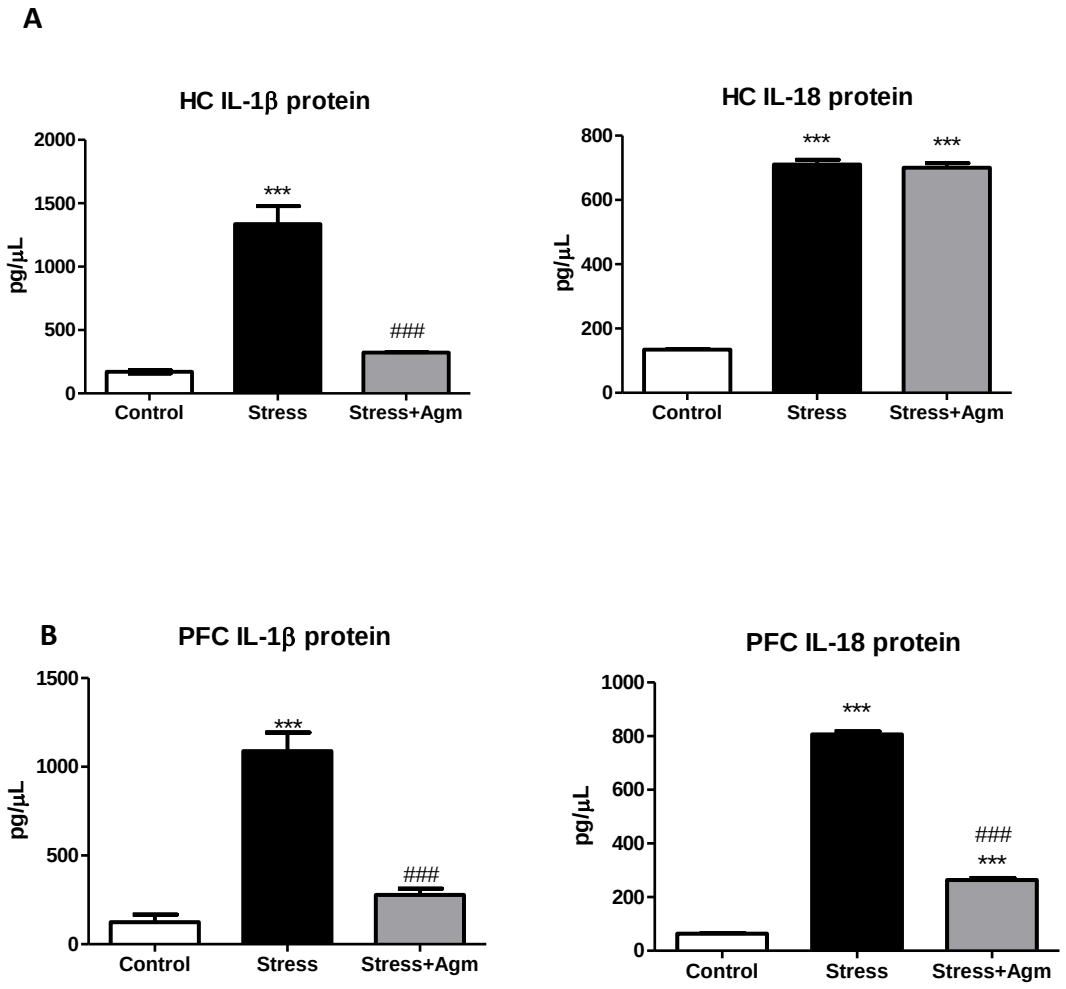

Fig. 3. The effect of acute agmatine treatment on protein expressions of IL-1β and IL-18 in hippocampus (HC) (A) and prefonral cortex (PFC) (B) of stressed rats. Agmatine (Agm) (40 mg/kg; i.p.) was administered 30 min. prior to the last stress session. All data are expressed as means ± SEM (n=6-8). ***p<0.001 *versus* control group; ### p<0.001 *versus* stress group.

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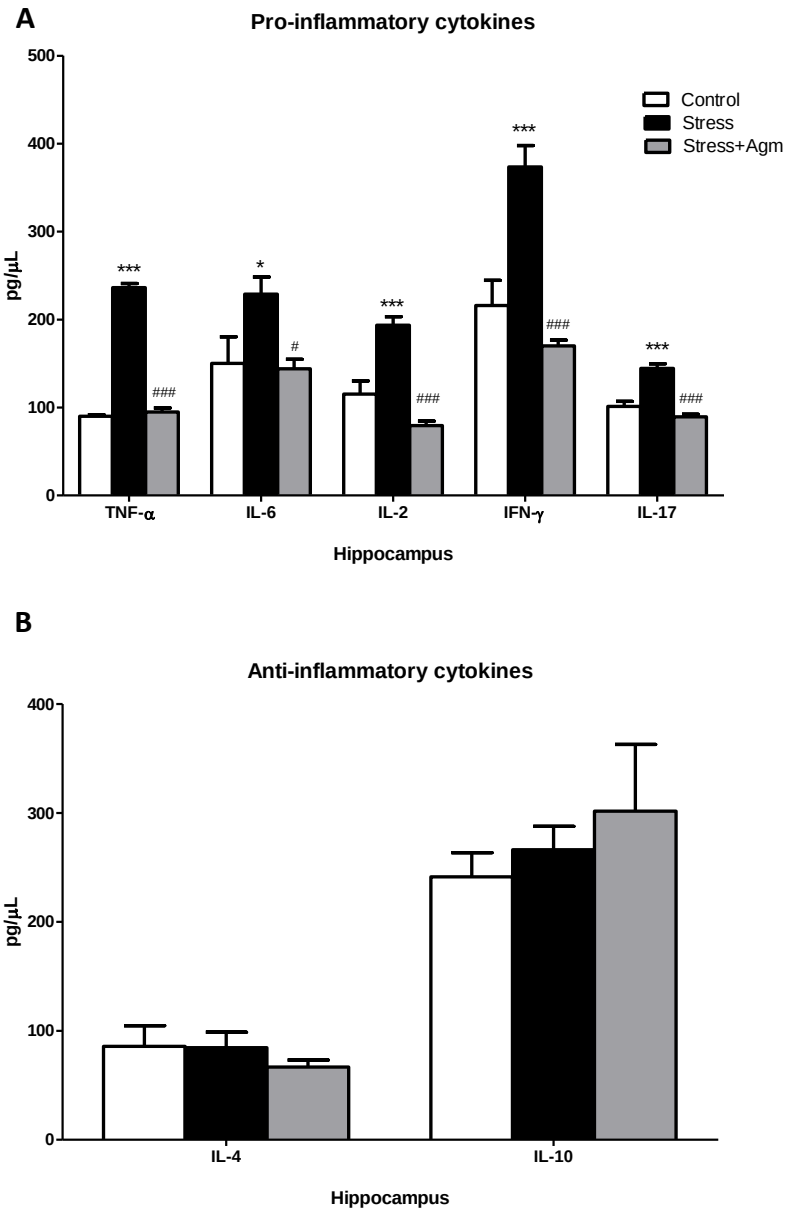

Fig. 4. The effect of acute agmatine treatment on protein expressions of certain pro- (A) and anti- (B) inflammatory cytokines in hippocampus of stressed rats. Agmatine (Agm) (40 mg/kg; i.p.) was administered 30 min. prior to the last stress session. All data are expressed as means ± SEM (n=6-8). *p<0.05, ***p<0.001 *versus* control group; $^{\#}$p<0.05, $^{\#\#\#}$ p<0.001 *versus* stress group.

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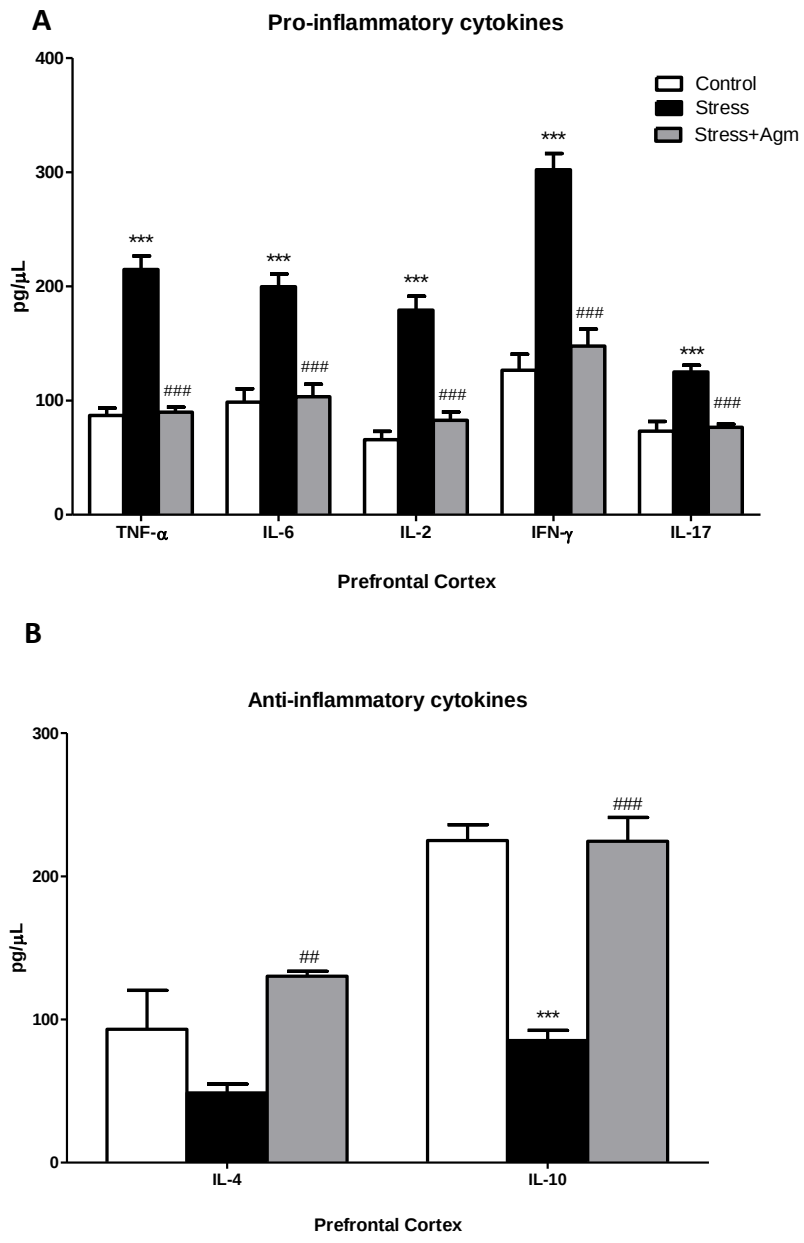

Fig. 5. The effect of acute agmatine treatment on protein expressions of certain pro- (A) and anti- (B) inflammatory cytokines in prefrontal cortex of stressed rats. Agmatine (Agm) (40 mg/kg; i.p.) was administered 30 min. prior to the last stress session. All data are expressed as means ± SEM (n=6-8). ***p<0.001 *versus* control group; $^{\#\#}$p<0.01, $^{\#\#\#}$ p<0.001 *versus* stress group.

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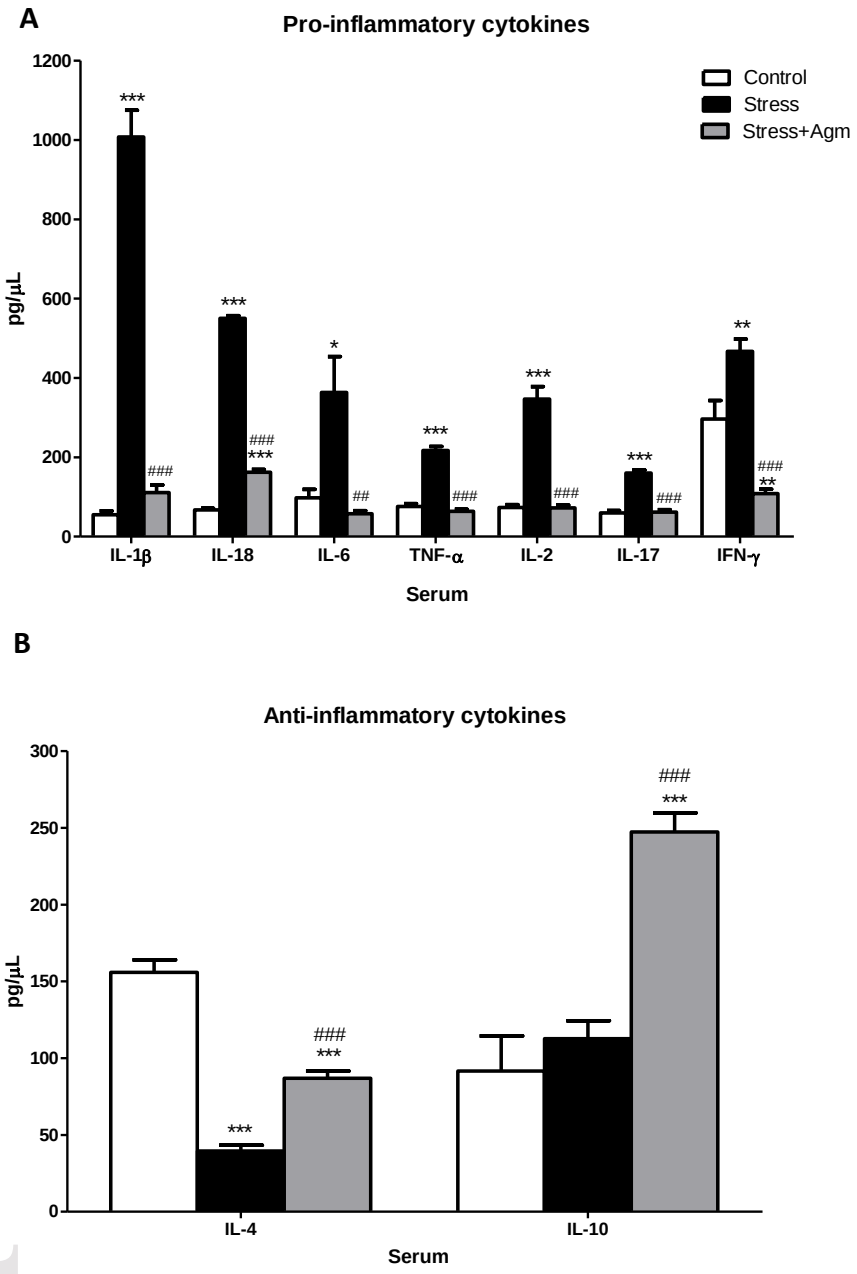

Fig. 6. The effect of acute agmatine treatment on protein expressions of certain pro- (A) and anti- (B) inflammatory cytokines in serum of stressed rats. Agmatine (Agm) (40 mg/kg; i.p.) was administered 30 min. prior to the last stress session. All data are expressed as means ± SEM (n=6-8). *p<0.05, **p<0.01, ***p<0.001 *versus* control group; $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 *versus* stress group.

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