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Ethanol and caffeine consumption modulates the expression of miRNAs in the cerebellum and plasma of UChB rats

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ABSTRACT

Aims: The present study aimed to verify changes in cerebellar and plasmatic expression of miRNAs after the chronic consumption of ethanol and caffeine in the UChB rat, an experimental model for alcoholism.

Material and methods: Male rats at 5 months of age, were divided into the following groups ($n = 10$/group): 1. Ethanol (UChB rats receiving 10% ethanol solution and water ad libitum); 2. Ethanol + caffeine (UChB rats receiving 10% ethanol solution + 3g/l caffeine and water ad libitum); 3. Control (rats receiving water ad libitum). The cerebellum and plasma of the animals were collected and processed by RT-PCR for the *miRNAs-155-5p, -146a-5p, -126-3p, -132-3p, -339-5p*.

Key findings: Ethanol and caffeine were capable of regulating the expression of miRNAs associated with the inflammatory process in the tissue and plasma of the UChB rats. Increased expression of the analyzed *miRNAs-155-5p, -146a-5p, -126-3p, -132-3p* was observed for the cerebellar tissue in the Ethanol group and reduced expression of them in the Ethanol + caffeine group. In plasma, caffeine significantly elevated the *miR-126-3p* and *miR-132-3p* levels and decreased *miR-155-5p* levels. Ethanol consumption increased *miR-146a-5p* expression and decreased *miR-339-5p* levels. In brief, altered plasmatic levels of the miRNAs did not reflect the miRNAs levels found in cerebellar tissue.

Significance: Considering the results herein, we concluded that ethanol predisposes to an inflammatory process while caffeine has a neuroprotective effect on the cerebellar tissue.

1. Introduction

The indiscriminate consumption of ethanol is one of the main risk factors affecting health, responsible for approximately 5.9% of the total amount of deaths worldwide [1]. The ethanol is considered a neurotoxin capable of altering properties of plasmatic membranes, affecting migration and neuronal differentiation [2]. The post-natal effects of ethanol consumption are clear in the whole encephalon with the pathological enlargement of grooves and slenderizing of the gyrus through the loss of cortical cellular elements and white matter [3]. The cerebellar neurons are strictly controlled by inhibitory GABAergic inputs provided by interneurons located in the molecular and granular layers [4]. Modifications in the cellular structure of the cerebellum were observed after chronic consumption of ethanol in rats, resulting in dilation of smooth endoplasmic reticulum, a process associated with neuronal death and axonal apoptosis [5].

In addition to ultrastructural modifications, the ethanol ingestion increases the levels of molecules known to be involved with the innate immune response, pointing to the installation of a neuroinflammation process in the Central Nervous System (CNS) [6,7]. In rats submitted to the chronic ethanol consumption there was an increase in the expression of COX-2, TNF-α and IL-15 [8]. Similar events occurred to microglial cell line (BV2) treated with different ethanol concentrations (25–100 mM) [9].

Evidence suggests miRNAs as central molecules in the control of inflammation control [7]. Su et al. [10] observed that the altered expression of miRNAs was identified in pathological conditions including the neuroinflammation and neurodegeneration. The miRNAs are small

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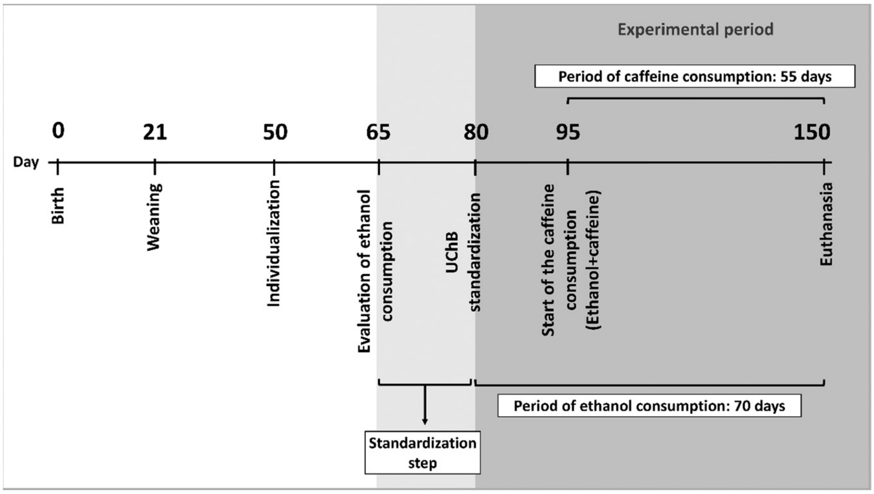

Fig. 1. Experimental design for the UChB and UChB + caffeine group from the birth to the lineage standardization. Modified from Martinez et al. (2018) [19].

non-coding RNA molecules with 21 to 23 nucleotides and widely affect the cell function regulating the translation of specific RNAs after binding to its regulators sequence. Tissue and plasma miRNAs are altered after ethanol exposure [11]. Ten Berg et al. [12] have reported changes in serum miRNAs after unrestricted social consumption of ethanol in young men. A total of 1305 miRNAs were identified in the plasma of these individuals being further sequenced. Among them, 265 miRNAs were found to be significantly elevated after ethanol consumption.

Caffeine, an alkaloid xanthine, is one of the most consumed psychoactive substances worldwide [13]. This substance has an antagonistic non-selective role of the adenosinergic receptors A1, A2a, A2b e A3, also present in the human CNS; they are recognized as modulators of the neurotransmission and neuroinflammatory response through microglial activation [14,15]. Therefore, reinforcing the hypothesis of caffeine as a neuroprotector, epidemiologic studies suggest that the caffeine consumptions decrease the occurrence of Alzheimer and Parkinson diseases, whose pathogenesis axis is related to chronic neuroinflammation [16].

Concerning the alcoholism, the UChB model was used as experimental model for chronic alcoholism study. The UChB rats present high levels of voluntary ethanol consumption (5–7 g of ethanol per kilogram of bodyweight per day). This fact was well established by the pioneering work of Mardones at University of Chile [17]. The present study aims to detect alterations in the gene expression of the *miRNAs-155-5p*, *-146a-5p*, *-126-3p*, *132-3p* and *-339-5p* in cerebellum and plasma. These miRNAs are related to the inflammatory process being possibly influenced by the interaction between ethanol and caffeine intake in the UChB model.

2. Material and methods

2.1. UChB standardization and experimental procedures

The selection of ethanol-drinking rats and standardization of the UChB variety was performed according to the protocol by Mardones and Segovia-Riquelme (1983) protocol [18]. The day of the birth was considered as day 0 and the weaning occurred after 21 days. At the 50th day, the rats were individualized and at 65th day, the evaluation of ethanol consumption started: one bottle containing 10% ethanol solution and one bottle containing water were offered during fifteen days, being positioned in different places inside the box. The animals had free choice for water or ethanol solution until 80th day. At the end of this period, rats that had a consumption of ethanol between 5 and 7 g of ethanol per kilogram of bodyweight per day were standardized as UChB strain.

The experimentation period started at 80th day, when 20 male UChB rats were housed in individual boxes, with solid floor and shavings, under controlled conditions of luminosity (12 h of darkness and 12 h of lightness) and temperature (20–25 °C). During this period, the animals received chow (Purina), water ad libitum and ethanol solution (1:10). At 95th day, 10 male rats standardized as UChB were randomly selected to the Ethanol + caffeine group. In the Ethanol + caffeine group, the bottle of ethanol solution was replaced by a bottle containing the ethanol solution (1:10) added by caffeine in a concentration of 3 g/l. For the preparation of the solutions, the ethanol used was ethanol P.A. (CAS-6417-5, Neon) and the caffeine (1,3,7-trimethylxanthine, 98.5 to 101.0%, Fisher Chemicals, Fair Lawn, USA).

The rats were divided into the following groups: Ethanol group: 10 UChB rats that consumed ethanol solution (1:10) and water ad libitum from the 80th to 150th day. This group consumed ethanol during 70 days except the days for standardization. The rats had free choice for water or ethanol, being the bottles alternately positioned inside the box each seven days; Ethanol + caffeine group: 10 UChB rats that consumed ethanol solution (1:10) and water ad libitum from the 80th to 95th day. At the 95th day, caffeine at a dose of 3 g/l was added to the ethanol solution. This group consumed ethanol solution isolated during 15 days, except the days for standardization, and the solution of ethanol and caffeine for 55 consecutive days. The rats had free choice for water or ethanol + caffeine solution, being the bottles alternately positioned inside the box each seven days (Fig. 1). As the UChB strain are derived from Wistar rats and the lineage pass through a selection process by which is necessary to consume ethanol for being standardized, one third group was used as control for the UChB model. The Wistar group was proposed as a control that did not intake alcohol during any period of experimentation. This Control group was composed of 10 male Wistar rats consuming water at *libitum* and being euthanized at 150th day.

From the end of the selection period to euthanasia (80–150 days old), the ethanol consumption was measured every week for the UChB rats (7 days). The experimental protocol followed the ethical principles according to National Council for the Control of Animal Experimentation (Brazil) and it was previously approved by Committee on Ethics in the Use of Animals (CEUA) from UNICAMP (protocol 4651-1/2017) and UFSCar (protocol 9895280815). After the experimental period, the euthanasia of the animals was performed by overdose of anesthesia (Xylazine 300 mg/kg and Ketamine 40 mg/kg, via intraperitoneal injection). After euthanasia, cerebellar tissue and 1 ml of blood of each animal (removed from ventral artery of the tail) were subjected to the molecular techniques, being stored in a freezer at −80 °C.

MiRNAs-155, -132, -126, -146a e -339-5 expressions in the cerebellar tissue.

250 μl of PBS and 750 μl of Trizol® (Invitrogen, EUA) were added to each frozen sample. The samples were homogenized using Politron®. After the permanence in room temperature during 5 min, 200 μl of chloroform was added and mixed vigorously during 15 s. The final solution was centrifuged during 15 min (4 °C and 13.200 rpm) and the aqueous phase of the flasks was transferred to new tubes. The RNA was precipitated using 500 μl of isopropyl alcohol during 12 h at −20 °C.

Then, the solution was centrifuged again (20 min at 4 °C, 13.200 rpm), and the supernatant was discarded. 1000 μl of ethanol at 75% was added and newly centrifuged by 5 min (13.200 rpm). The superior phase was despised and the dry pellet was dissolved in water treated with diethyl pyrocarbonate (DEPC) during 15 min. This material was aliquoted and stored at −80 °C.

The samples were submitted to electrophoresis in agarose gel at 1% to RNA and to the verification of the RNA integrity. For the cDNA synthesis, the reverse transcription was performed using the commercial kit (Applied Biosystems), High Capacity cDNA Reverse Transcription Kit, according to the manufacturer's instructions. For each 1 μg of RNA was added 2.5 μl of Reverse Transcriptase Buffer, followed of 1 μl of dNTP's, 2.5 μl of Random Primers and 1.25 μl of the MultiScribeTM, enzyme, completing the volume with DEPC water to 20 μl.

The differential expression of the miRNAs was quantified for the chosen miRNAs: *rno-miR-155-5p (Assay ID 002571)*, *rno-miR-132-3p (Assay ID 000457)*, *rno-miR-126-3p (Assay ID 002228)*, *rno-miR-146a-5p (Assay 000468)*, *rno-miR-339-5p (Assay ID 002257)*. The β-actin gene was used as endogenous control.

With the cDNA obtained from the samples, an amplification was performed through quantitative Polymerase Chain Reaction (PCR-RT) in real time using TaqMan Master Mix (Applied Biosystems). For the quantitative analysis of the gene expressions were used the commercially available systems TaqMan Assay-on-demand, composed of oligonucleotides and probes (Applied Biosystems). A gadget of PCR detection in real time 7500 Real Time PCR System (Applied Biosystems) was used with the software Sequence Detection System for obtaining the CT values.

The reactions of Real-Time PCR were performed in duplicates using TaqMan Master Mix (Applied Biosystems, EUA). The amplification was performed in a final volume of 10 μl, using 5 μl of the specific reagent TaqMan Master Mix, 0.5 μl of each specific probe and 4.5 μl of cDNA diluted in 1:10. The data were exported to Excel spreadsheets to calculate ΔCT. The standard conditions for the amplification were 50 °C during 2 min, 95 °C during 10 min, followed by 40 cycles of 95 °C during 15 s and 60 °C during 1 min (simultaneous annealing and extension).

2.2. Expression of the plasma miRNA-155, -132, -126, -146a and -339-5p

Blood samples were collected in a tube containing EDTA and were centrifuged (10 min, 2500 rpm). The plasma was transferred to another tube. The fraction of small RNAs was obtained through the kit miRNeasy Serum/Plasma (QIAGEN, Hilden, Germany), according to instructions. The integrity of the small RNAs was analyzed using the equipment Agilent Bioanalyzer (Agilent, California, USA). The U6 was used as endogenous control for the posterior normalization of the miRNA's expressions. For the cDNA the kit TaqMan® microRNA Reverse Transcription (Thermo-Fisher) was used, according to instructions. Around 5 ng of RNA extracted from samples were used for the synthesis in the presence of 1.5 μl of buffer 10×, 0.15 μl of dNTP mix (100 mM), 0.19 μl of RNAses inhibitor (20 U/μl), 1 μl of reverse transcriptase Multiscribe® (50 U/μl), 3 μl of specific miRNA primer and 4.16 μl of ddH20. The reaction was performed in ice and then submitted to 16 °C during 30 min, 42 °C during 30 min, followed by inactivation of transcriptase at 85 °C during 5 min, in thermocycler Mastercylcer Pro (Eppendorf, Hamburgo, Alemanha). The real time PCR method was used for the differential expression of the miRNAs as described in the previous item, for the *microRNAs-155*, *-132*, *-126*, *-146a*, *-339-5p*.

2.3. Statistical analysis

All data was exported to Excel and transferred to the GraphPad Prism 4.0 software (GraphPad Prism, Inc., San Diego, CA, EUA. The values were considered to be parametric after performing the normal distribution Shapiro-Wilk Test. The analyses of significance among the groups were performed by One-way ANOVA complemented by the Tukey's test; statistical significance was set at $p < 0.05$.

3. Results

3.1. Caffeine restored the expression of some miRNAs in the cerebellum after ethanol consumption

The miRNAs measured in the cerebellar tissue had an increased expression for the Ethanol group, after the ethanol consumption. For these miRNAs, a decrease in the expression was observed for the Ethanol + caffeine group, being compared to the levels of the Control group. Only the *miR-146a-5p* presented levels of expression lower in the Ethanol + caffeine group than the Control group. The results indicate that caffeine consumption reverted the expression of some miRNAs in the cerebellum despite the ethanol consumption (Fig. 2).

3.2. Caffeine consumption elevated the levels of circulating miRNAs

The consumption of caffeine elevated the *miR-126-3p* and *miR-132-3p* levels in the Ethanol + caffeine group. A significative augment was observed for *miR-126-3p* which was about forty-five times more elevated after the caffeine and ethanol consumption than the ethanol consumption alone. For the *miR-132-3p*, the expression in the Ethanol group was found to be eight times higher when compared to the Ethanol + caffeine group (Fig. 3).

3.3. Ethanol consumption influenced the expression of the circulating miRNAs

The *miR-146a-5p* expression was elevated in the plasma after the ethanol consumption. The caffeine intake was able to reduce this miRNA to levels comparable to the Control group. The expression of *miRNA-155-5p* was diminished for the Ethanol + caffeine group and the *miR-339-5p* had different expression levels in every analyzed groups, thus having the ethanol consumption attenuated its expression (Fig. 4).

4. Discussion

The ethanol consumption leads to neuroinflammatory modifications in the cerebral cortex, hippocampus and cerebellum [20]. During the injury period of neural tissue, signalization pathways activate microglia and astrocytes thereby contributing to create a neuroinflammatory environment [21]. The miRNAs have a remarkable role in the epigenetic regulation of the inflammatory process inside de CNS [22]. This study demonstrated an increased expression of the *miRNAs-155-5p*, *-146a-5p*, *-126-3p*, *-132-3p*, *-339-5p* in cerebellar tissue in the Ethanol group. Therefore, we conclude that chronic ethanol consumption is able to harm the neural tissue. Lewohl et al. [23] demonstrated that the levels of some miRNAs were significantly elevated in the prefrontal cortex of alcoholic human beings and, among them, the *miR-339-5p* and *miR-146a*. This study also suggested that the reduction in gene expression due to ethanol consumption in humans could have as one of its causes the increase in the miRNA expression. Fundamental processes to neural plasticity could represent the majority of the targets regulated by the analyzed miRNAs [23].

One of the miRNAs linked to microglial activation is the *miR-155-*

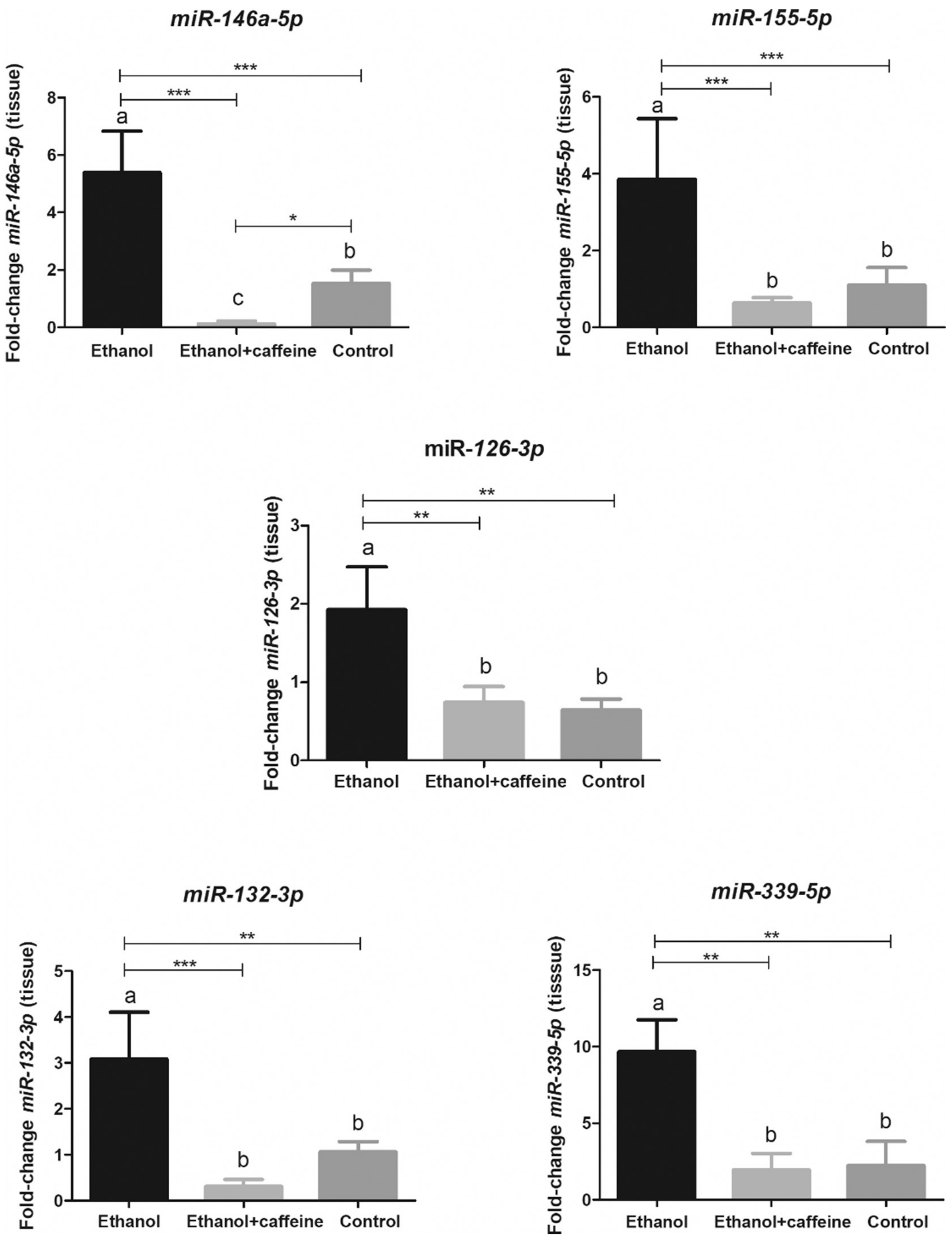

Fig. 2. Fold-change analysis of *miR-146a-5p*, *-155-5p*, *-132-3p*, *-126-3p*, *-339-5p* expressions in the cerebellum of the animals. Data are expressed as the mean ± SD. Different letters indicate statistical difference among the groups. $*p < 0,05$, $**p < 0,01$, $***p < 0.001$.

5p, which has SOCS-1 (suppressor of cytokine signaling-1) a target, protein which negatively regulates the inflammatory response in this cellular type and the elevation of this miRNA leads to an increase in secretion of proinflammatory cytokines and nitric oxide [24]. The *miR-155-5p* has proinflammatory actions and is critical for the effectiveness of some responses originated from macrophages and T cells. In the macrophages, the *miRNA-155* is stimulated by binders of receptors as the toll-like receptors (TLRs) and by cytokines as the interferon-γ (IFN-γ) [25]. Lippai and colleagues [26] showed that the deficiency in the *miRNA-155* protected against the increase of proinflammatory cytokines induced by ethanol at the cerebellum of mice, protecting from the overproduction of NFkB through TLR4-dependent pathway. The data of our study is in agreement with the literature, considering the elevated expression of the *miR-155* in the cerebellum of the Ethanol group after the ethanol intake. The ingestion of caffeine mitigated the level of this miRNA comparably to the Control group, suggesting a recovery of the inflammatory process previously established in the Ethanol group.

Some miRNAs play a resolutive role in the inflammatory process, as *miR-146a-5p*. This miRNA overexpression in microglial cells has demonstrated the ability to induce elevation in IL-10 levels, essential interleukin that prevents the activated state of inflammation [27]. In a cell line of astrocytes treated with IL-1β, the elevated *miR-146a* expression was related to the negative regulation of the inflammation by reducing factors such as IL-6 and COX-2 [28]. The targets of *miR-146a-*

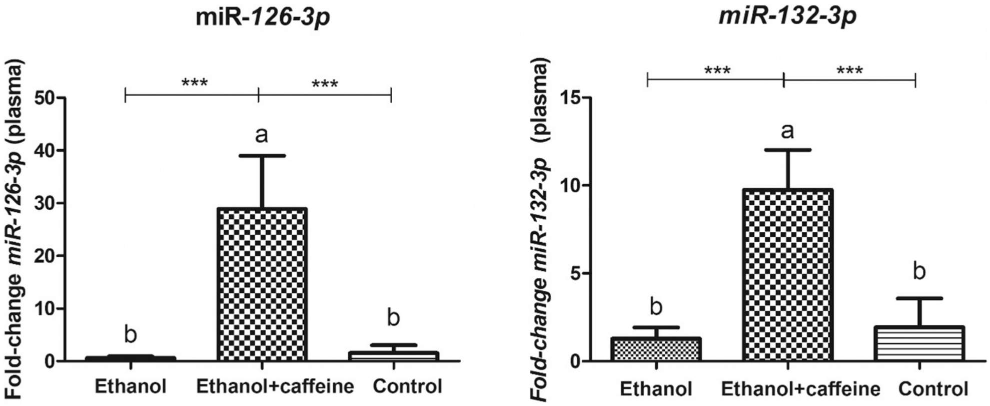

Fig. 3. Fold-change analysis of *miR-126-3p* and *-132-3p* expressions in the plasma of the animals. Data are expressed as the mean ± SD. Different letters indicate statistical difference among the groups. $*p < 0,05$, $**p < 0,01$, $***p < 0.001$.

5p are genes related to the adaptation of MyD88, IRAK-1 and TRAF-6, important components for the NFkB pathway activation through the activation of TLR's [28]. Therefore, an increased level of *miR-146a* matches with high increased expression of NFkB, implying an attempt to suppress the overproduction of this transcription factor [28,29]. After the ethanol consumption, there was an elevation in the *miR-146a* in the cerebellar tissue of the Ethanol group responding to the probable augment of inflammatory conditions, pointed by the elevated expression of *miR-155* in the same group. Moreover, the caffeine consumption reduced the expression of miR-*146a* at lower levels than the Control group. And, an increase in the *miR-155-5p* expression was reported concomitantly with the increase of *miR-146a-5p* in the inflammatory context [25].

Lippai and colleagues [26] observed that chronic ethanol consumption increased the cerebellar *miRNA-132* levels. Similarly, an elevation of this miRNA was observed for the Ethanol group. The *miR-132* is not only related to the inflammatory process but it is also found in neural synaptic terminals having broad relation with the integrity of the synaptic fields of the dendrites and neural plasticity. In the mature nervous system, dysregulation of miR-132 has been suggested to play a role in a number of neurocognitive disorders characterized by aberrant synaptogenesis [30]. Kawashima et al. [31] demonstrated that elevated *miR-132* expression induced positive augment in glutamatergic receptors GluA1, GluN2A e GluN2B in a culture of cortical neurons. These events suggested ethanol as a modulator not only of inflammatory proteins, but also of structural proteins. The *miR-132* was elevated in

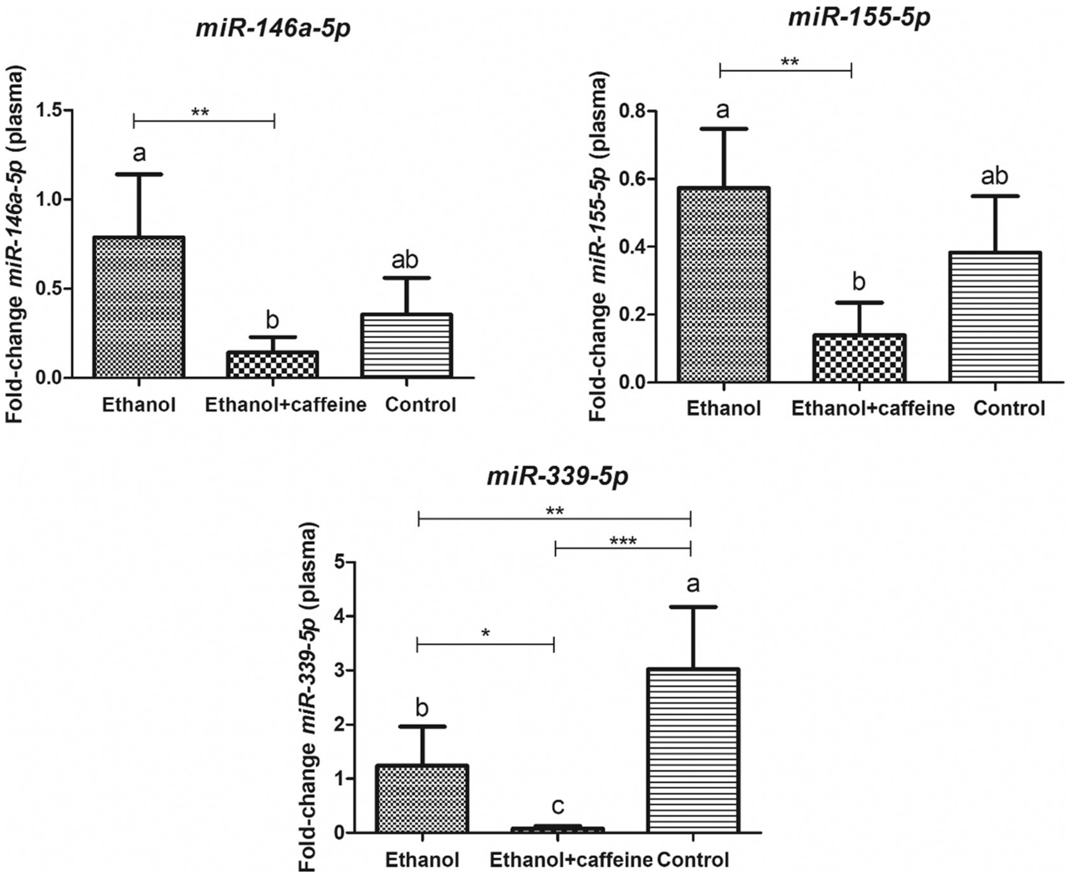

Fig. 4. Fold-change analysis of *miR-146a-5p*, *-155-5p* and *-339-5p* expressions in the plasma of the animals. Data are expressed as the mean ± SD. Different letters indicate statistical difference among the groups. $*p < 0,05$, $**p < 0,01$, $***p < 0.001$.

the Ethanol group and the targets of this miR include IRT1, AChE, PTEN, FOXO3a and p300 and it might be related to neuropsychiatric disorders [32].

In addition, Zhang et al. [33] showed *miRNA-339-5p* increase in an ethanol-induced neuroinflammation condition. They observed significative augment in proinflammatory cytokines as IL-1β, IL-6 and TNF-α in the microglial cells. This miRNA directly targets the IKK-β and IKK-ε proteins, important regulatory factors of the NFkB activation [34]. Furthermore, the miR-339-5p was found to be deregulated in neurodegenerative processes and it was found overexpressed in the cerebral cortex of murines during the period of ethanol dependence [35].

There are few studies exploring the *miRNA-126-3p* function in the human brain specifically. Despite that, it has a great expression in the neural tissue and it seems to have a preponderant role in angiogenesis process and in the maintenance of vascular epithelium, being important for the vascular homeostasis [36,37]. Also, actions have been related to the immune innate response [38]. In inflammatory process, the *miRNA-126* seems to suppress the production of the reactive oxygen species in endothelial cells through the modulation of the High Mobility Group Box 1 expression [39].

In the cerebellar tissue, the Ethanol + caffeine group had expressions of all analyzed miRNAs comparable to the Control group or even lower than the Control group. Thus, it is suggested as that, from the initial disturbance caused by the ethanol and evidenced by the alterations in Ethanol group, caffeine was able to close the expression of the miRNAs to basal levels. Varma and Kovtun [40] reported caffeine to be capable of modulating the expression of miRNAs in the lenses of rats submitted to high galactoses diet. Once the elevation of miRNAs silences the translation of the proteins, caffeine attenuated this overexpression and its beneficial effect could be attributed to the ability of suppressing the toxic miRNA elevation.

The effects of the ethanol and caffeine were not restricted to the cerebellum. When analyzed the expression of the plasmatic miRNA, systemic reverberation of these small RNAs was observed. Since its origin in the intracellular compartment, miRNAs can be extracellularly secreted after AGO2 action or even being secreted in extracellular vesicles, the exosomes, serving as specific signatures of the disease state [41].

Momem-Heravi et al. [42] reported that when cellular lineages, animal models and patients with alcoholic hepatitis were exposed to ethanol, there was an increase in the number of the circulating exosomes with alterations also in the miRNA cargo. Once it is present in the blood flow, miRNAs may have systemic actions, addressed to many organs and tissues [11,43]. Rahman and colleagues [44] concluded that alcohol can alter contents of extracellular vesicles such as miRNAs, protein and cytokines, in hepatic and extra-hepatic cells. The transfer of these alcohol modified extracellular vesicles to nearby or distant cells can play vital role in inflammatory pathways in alcohol induced pathogenesis and comorbidities. The ability of ethanol in modulating the plasmatic miRNAs can reflect one important pathway for the alterations found in many organs after the abuse of this substance.

Ten Berg et al. [12] showed alterations in the miRNAs present in serum of young adult rats after unrestricted exposure to the ethanol. Approximately, 1305 miRNAs were identified and sequenced and, among them, 265 miRNAs had the expression augmented after one single episode of ethanol consumption. Gardiner et al. [45] performed a prospective study with a group of pregnant women who consumed or not ethanol during the pregnancy. The miRNAs *125a-5p*, *-602-5p*, *-126-3p*, and *-3180-3p* were found altered in the serum of women that consumed ethanol.

The ethanol consumption elevated the *miR-146a* and *-155* levels in the plasma of the Ethanol group rats. The increased level of these miRNAs in the serum was observed in a model of alcohol-induced liver disease [46,47]. The expression of these miRNAs was also elevated for the *miR-155* and diminished for *miR-146a* in patients with Parkinson's disease and its signature might be related to a neurodegenerative state [48].

The caffeine consumption elevated the *miR-126-3p* and *-132-3p* expression at superior levels when compared to the Control and Ethanol group, showing that these miRNAs were specifically altered by the consumption of this substance. Few studies related the modulation of the caffeine about plasmatic miRNAs being the most part of them restricted to the effects of this substance in specific tissues [49]. Thus, it is interesting to consider that, despite the lower expression of *miR-126-3p* and *-132-3p* in the cerebellar tissue for the Ethanol group, there was a higher plasmatic expression of these miRNAs in the Ethanol + caffeine group. It indicates that the expression of a determined miRNA in a tissue does not always reflect its systemic effects, as reported by Sierzega et al. [50].

Also, Martinez et al. [19] analyzed the action of the simultaneous consumption of ethanol and caffeine in the cerebellum of UChB rats and showed the ability of caffeine in reverting the apoptotic process caused by the ethanol in Granule and Golgi neurons and also in the glial cells present in the white matter of the cerebellum; this is likely due to the suppression of Caspase-3 while upregulating the expression of XIAP. However, it is not clear how caffeine is able to act to attenuate neuronal loss. It is believed that caffeine causes delay in the neurodegenerative processes in addition to the decrease of apoptotic rate, leading to effect positive against neuroinflammation [51,52].

5. Conclusion

We concluded that ethanol and caffeine promoted changes in miRNA's expression in the cerebellar tissue and plasma of UChB rats, being the ethanol responsible for inducing an inflammatory process and the caffeine acting as a neuroprotector agent for the cerebellar tissue. Moreover, we concluded that the plasmatic miRNAs did not always reflect the cerebellar levels of the same. Finally, we suggest that the analysis of these markers should be evaluated as a complementary parameter and not singularly in the signaling of different biological processes.

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Declaration of Competing Interest

The authors declare that there was no conflict of interests.

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