



Cerebellar Apoptotic Parameters and Circulating MiRNAs in Senile UChB Rats (Voluntary Ethanol Consumers)

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Abstract

Despite the lack of scientific data on the real impact of alcohol consumption in the elderly, excessive drinking is relatively common in this population. Aging is accompanied by reduced water content, diminished hepatic metabolic capacity, and altered brain responsiveness, making older individuals more vulnerable to alcohol-induced neurotoxicity. The cerebellum is particularly sensitive to ethanol, with documented impairments in motor coordination, balance, and cognitive functions. However, the effects of chronic ethanol exposure during senescence remain poorly understood. This study investigated apoptotic and anti-apoptotic signaling in the aged cerebellum of UChB rats, a strain characterized by voluntary ethanol consumption, and evaluated the expression of circulating microRNAs associated with apoptotic pathways. Immunohistochemistry and gene expression analyses revealed no

significant changes in cerebellar caspase-3, XIAP, or IGFR1 expression between ethanol-consuming and control aged rats, although minor differences in protein immunolocalization were observed. Conversely, serum levels of miR-9-3p, miR-15b-5p, miR-16-5p, miR-21, miR-200a, and miR-222-3p were significantly upregulated in the ethanol group. Taken together, the findings suggest that senile UChB rats develop a tolerance to chronic ethanol intake, preventing further activation of apoptotic cascades in the cerebellum. Additionally, the modulation of specific circulating miRNAs indicates a potential regulatory or compensatory role in this adaptive response. These miRNAs may serve as molecular indicators of ethanol exposure in aging and contribute to understanding mechanisms of neurobiological tolerance.

Keywords: Ethanol; Aging; Cerebellum; Tolerance; Apoptosis; MicroRNAs; UChB rats

Introduction

Aging is associated with profound alterations in brain structure and function, including cognitive decline and increased vulnerability to neurotoxic insults [1]. Elderly individuals are particularly susceptible to the harmful effects of alcohol consumption due to age-related physiological changes, such as reduced total body water, diminished hepatic metabolic efficiency, and altered neuropharmacological responses [2]. Alcoholism is a multifactorial disease with a broad genetic impairment background. Alcohol-related disorders are defined by several criteria which can be dissected further at the genetic level [3]. Genetic factors contribute significantly to alcohol-

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seeking behavior and the abuse of its consumption, with the risk for diagnosis of alcoholism being approximately 50% from the genetic origin and 50% from the environmental conditions [4]. Ethanol metabolism is intimately linked with the physiological and behavioral aspects of ethanol consumption [5]. The cerebellum is one of the brain regions most consistently affected by ethanol exposure. Chronic or acute ethanol insults impair motor coordination, disrupt cerebellar circuitry, and contribute to neurodegeneration [6]. Ethanol-induced cerebellar damage has been documented in chronic alcoholics and in cases of prenatal exposure [7,8]. However, its specific impact during senescence remains poorly characterized. Genetic predisposition plays a fundamental role in alcohol-seeking behavior and susceptibility to ethanol-induced damage. UChB rats, selectively bred for high voluntary alcohol intake, possess metabolic and genetic features that influence their behavioral and neurobiological responses to ethanol [9,4,10,11]. ALDH2 allelic variants relevant to ethanol metabolism have been described in these strains, contributing to differential vulnerability [12,13]. MicroRNAs (miRNAs) are key post-transcriptional regulators involved in apoptosis, neuroplasticity, and responses to neurotoxic stimuli. Ethanol-sensitive miRNAs have been implicated in addiction, neuroinflammation, and neuronal survival [14,15,16]. Their evaluation in aging models may reveal biomarkers and regulatory mechanisms underlying chronic ethanol exposure. This study aimed to investigate apoptotic signaling and miRNA expression in the cerebellum and serum of senile UChB rats subjected to chronic ethanol intake. By integrating molecular, histological, and circulating biomarkers, we sought to clarify whether aging modifies ethanol-induced neurotoxicity or promotes tolerance mechanisms.

Materials and Methods

Animals

30 senile male UChB rats, with 13 months of age, were obtained from the Department of Anatomy, Institute of Biosciences, UNESP - Campus of Botucatu, SP. Two experimental groups were formed (n = 15 animals): Ethanol group (EtOH-UChB): composed of UChB rats with voluntary consumption of 10 % ethanol solution (the average of ethanol consumption in this group was 7 g ethanol per kg of body weight / day) and Control group (Ctrl-UChB): composed of UChB rats deprived of 10 % ethanol solution, with a voluntary consumption of water only.

Selection of ethanol drinking animals and experimental procedures

The rats were initially subjected to the selection for ethanol intake followed by standardization of the UChB variety in accordance with the protocol by Mardones & Segovia-Riquelme (1983) outlined by Martinez *et al.* (2018).

The experimental period started at 80th day, when 15 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 food, water *ad libitum* and ethanol solution (1:10 v/v). At the end of the selection period for ethanol consumption until euthanasia (80 to 390 days-old), ethanol consumption was measured every 7 days. The animals of the EtOH-UChB group remained 325 consecutive days with a voluntary intake of 10 % ethanol solution. The experimental protocol followed the ethical principles of the National Council for the Control of Animal Experimentation (Brazil) and the Committee on Ethics in the Use of Animals (CEUA) from UFSCar. The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies (Tveden-Nyborg *et al.* 2023).

Gene expression in aged cerebellum

250 µL of PBS and 750 µL of Trizol® (Invitrogen, EUA) were added to each frozen sample (cerebellum samples from five animals per group were used). The samples were homogenized using Politron®. After the permanence at room temperature for 5 min, 200 µL of chloroform was added and mixed vigorously for 15 seconds. The final solution was centrifuged for 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 for 5 min (13.200 rpm). The superior phase was discarded, and the dry pellet was dissolved in water treated with diethylpyrocarbonate (DEPC) for 15 minutes. This material was aliquoted and stored at - 80 °C. The samples were submitted to electrophoresis in agarose gel at 1 % to RNA and 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 1µg of RNA was added 2.5 µL of Reverse Transcriptase Buffer, followed by 1 µL of dNTPs, 2.5 µL of Random Primers, and 1.25 µL of the MultiScribe™ enzyme, completing the volume with DEPC water to 20 µL. The differential expression of caspase-3 (Assay ID Rn00563902_m1), XIAP (Assay Rn01457299_m1), and IGF1R (Rn00583837_m1) were quantified. The β-actin gene was used as endogenous control. The cDNA was obtained from the samples, and amplification was performed through quantitative Polymerase Chain Reaction (PCR-RT) in real-time using TaqMan Master Mix (Applied Biosystems). For the quantitative analysis of gene expressions, we used the commercially available system TaqMan Assay-on-

demand, composed of oligonucleotides and probes (Applied Biosystems). A gadget of PCR detection in real-time 7500 PCR System (Applied Biosystems) was used with the software Sequence Detection System for obtaining the CT values. The reactions were performed in duplicates using TaqMan Master Mix (Applied Biosystems, EUA). The amplification was performed in a final volume of 10 μ L, using 5 μ L of the specific reagent TaqMan Master Mix, 0.5 μ L of each specific probe, and 4.5 μ L of cDNA diluted in 1:10. The data were exported to Excel spreadsheets to calculate Δ CT. The standard conditions for the amplification were 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 seconds and 60 °C during 1 min (simultaneous annealing and extension).

Real-time PCR for miRNA expression

Animals were anesthetized via intraperitoneal injection of 90 mg/kg ketamine chloride (Ketalar/ laboratory: Parke-Davis) and 10 mg/kg of xylazine (Rompum/ laboratory: Bayer). Later, 1 mL of blood from rats' lateral tail veins was collected and processed for RNA extraction. The expression profiles of the miRNAs -9-3p (Assay ID: 002231), -15b-5p (Assay ID: 000390), -16-5p (Assay ID: 000391), -21-5p (Assay ID: 000397), -200a-3p (Assay ID: 000502) and -222-3p (Assay ID: 002276) were analyzed using the whole blood of each animal. Total cellular RNA was extracted using Trizol Reagent (Invitrogen, USA) and RNA was reverse transcribed to single-stranded cDNA, using a High-Capacity Kit (Applied Biosystems, USA) with specific primers provided by the TaqMan microRNAs assays according to the manufacturer's protocol. For quantitative analysis of the miRNAs -9-3p, -15b-5p, -16-5p, -21-5p, -200a-3p, and -222-3p, we used the commercially available system TaqMan Assay-on demand (Applied Biosystems). Reverse transcription was performed using 5 ng of RNA for each sample in 7.5 μ L of the total reaction mixture. The cDNA obtained was diluted 1:4 and 4.5 μ L was used for each 10 μ L of the quantitative real-time PCR reaction mixture using the TaqMan Master Mix (Applied Biosystems). All reactions were carried out duplicate and analyzed with the 7500 Sequence Detection System apparatus (Applied Biosystems). Data was analyzed using the ABI-7500 SDS software. The total RNA absorbed was normalized based on the Ct value for U6 (000391). The variation in expression levels was calculated by the $2^{-\Delta\Delta C_t}$ method, with the mean ΔC_t value for a group of miRNAs -9-3p, -15b-5p, -16-5p, -21-5p, -200a-3p and -222-3p. Samples from control animals were used as a calibrator. For these miRNAs, the expression evaluation and statistical analysis were performed using the Shapiro-Wilk normality test and One-way ANOVA. Statistical significance was set at $P < 0.05$. Graphical analysis was performed using GraphPad Prism version 4.0 go Windows, (GraphPad Software, San Diego - California USA).

Immunostaining for caspase-3, XIAP and IGFR1

5 μ m thick sections were obtained using the microtome (Biocut - Model 1130) and collected on silanized slides. Antigen retrieval was performed by incubating the tissue sections in citrate buffer (pH 6.0) at 100 °C in microwave or treatment with proteinase K, depending on the antibody. Endogenous peroxidase blockade was performed with H₂O₂ (0.3 % diluted in methanol) and posterior incubation in BSA solution (3 %) diluted in TBS-T buffer for 1 h. The membranes were immunoreacted with antibodies for caspase-3, XIAP and IGFR1 diluted in 1 % BSA (1:35-200) and stored overnight at 4 °C. The Envision HRP kit (Dako Cytomation Inc., EUA) was used for antigen detection, according to the manufacturer's instructions. The staining was revealed using diaminobenzidine (DAB), followed by counter-staining with Harris' Hematoxylin.

Morphometric parameters of immunohistochemical reactions

For immunohistochemical analysis of caspase-3, XIAP, and IGFR1, ten fields were randomly selected in each slide based on the magnification of 40 x animal /group in the cerebellar cortex. Purkinje cells, Bergmann glia, and granule neurons were particularly observed. The white matter cells were counted as a whole, excluding just the neurons of the cerebellar nuclei. The number of reactive cells was measured, and the relative frequency of staining was calculated for each cellular type. For XIAP and IGFR1, the analysis qualitatively considers difference in intensity of staining, location, or distribution between the groups).

Statistical analysis

All data were exported to Excel and transferred to GraphPad Prism 7.0 (GraphPad Prism, Inc, San Diego, CA, USA). The results obtained for each parameter were considered parametric within the group and analyzed using the Shapiro-Wilk normal distribution test. Student's t-test was used for comparison. Statistical significance was set at $P < 0.05$.

Results

Caspase-3, XIAP, and IGFR1 are differentially modulated in the aged cerebellum following chronic ethanol consumption.

Chronic ethanol intake increased the relative frequency of caspase-3-positive Purkinje cells and Bergmann glia in aged rats (Figure 1 and 2 A, B). In contrast, granule neurons and white matter cells showed comparable caspase-3 levels between the Control and Ethanol groups (Figure 1 and 2 C, D). XIAP immunostaining was reduced in Purkinje cells of the Ethanol group, while Bergmann glia and granule neurons exhibited diffuse labeling, particularly within the glomerular layer (Figure 3 E, F). Similarly, IGFR1 immunolocalization

decreased in the Ethanol group, with scattered labeling in Bergmann glia and granule neurons, especially in the glomerular zone (Figure 3 G, H).

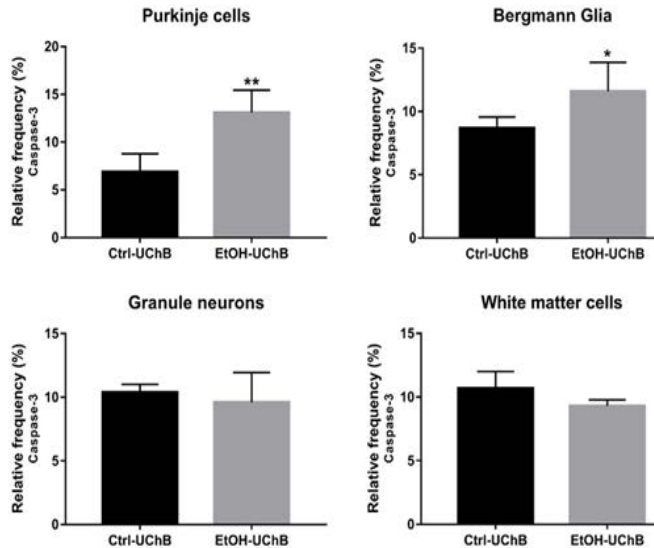


Figure 1: Mean (\pm standard deviation) of immunoreactivity for Caspase-3 in Purkinje cells, Bergmann Glia, Granule neurons, and White matter cells of the cerebellum. * $p < 0.05$, ** $p < 0.01$.

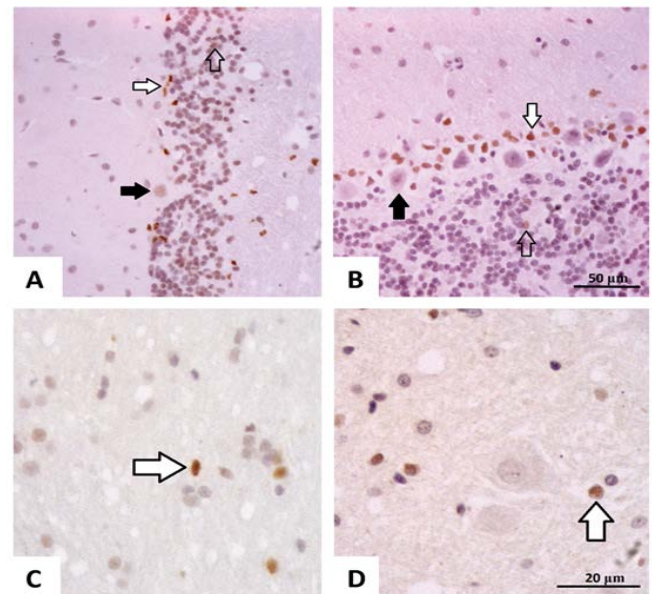


Figure 2: Photomicrographs of the cerebellum from Ctrl-UChB (A, C) and EtOH-UChB (B, D) groups. Representative images show positive labeling for Caspase-3 in the Purkinje cell layer (black arrow), for Bergmann Glia (white arrow), and the granular layer (unfilled arrow) (A, B). 400X. Positive reactivity for Caspase-3 in the White matter cells. (C, D) 1000X.

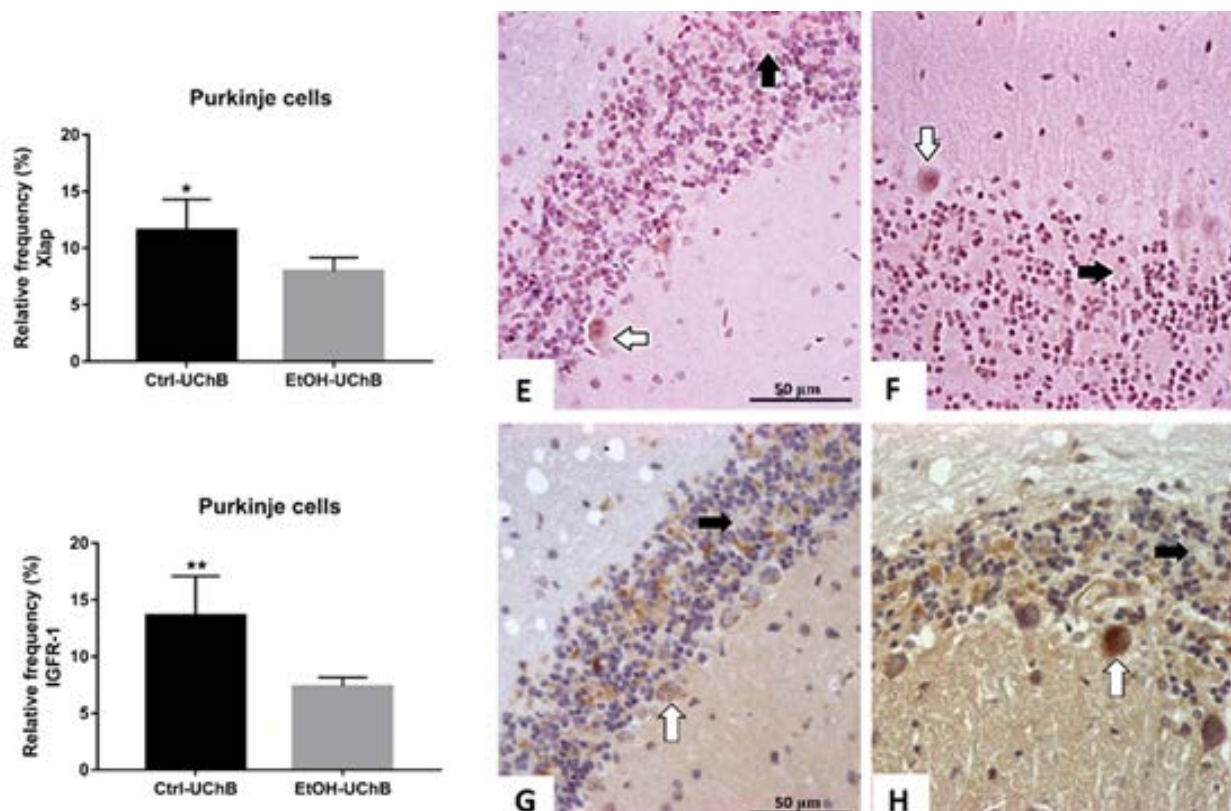


Figure 3: Mean (\pm standard deviation) of immunoreactivity for XIAP and IGFR1 in Purkinje cells of the cerebellum. * $p < 0.05$, ** $p < 0.01$. Representative images show positive immunoreactivity for XIAP (E, F) and IGFR1 (G, H) in Purkinje cells (white arrows) and scattered staining in the glomerular zone (black arrows). E, G: Ctrl-UChB group; F, H: EtOH-UChB group 400X.

Gene expression remains unchanged despite chronic ethanol exposure

Despite differences in protein localization, no statistically significant changes were detected in mRNA expression levels of caspase-3, XIAP, or IGFR1 between the groups (Figure 4).

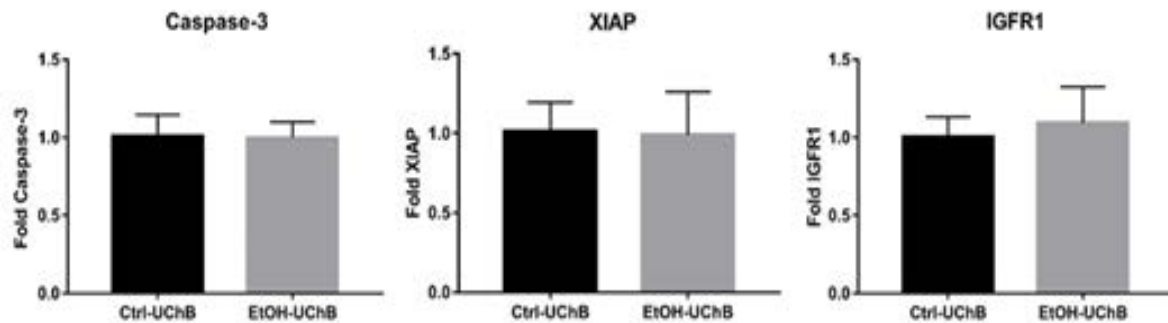


Figure 4: Gene expression analyses for Caspase-3, XIAP, and IGFR1 in cerebellar tissue. Data are expressed as the mean \pm standard deviation.

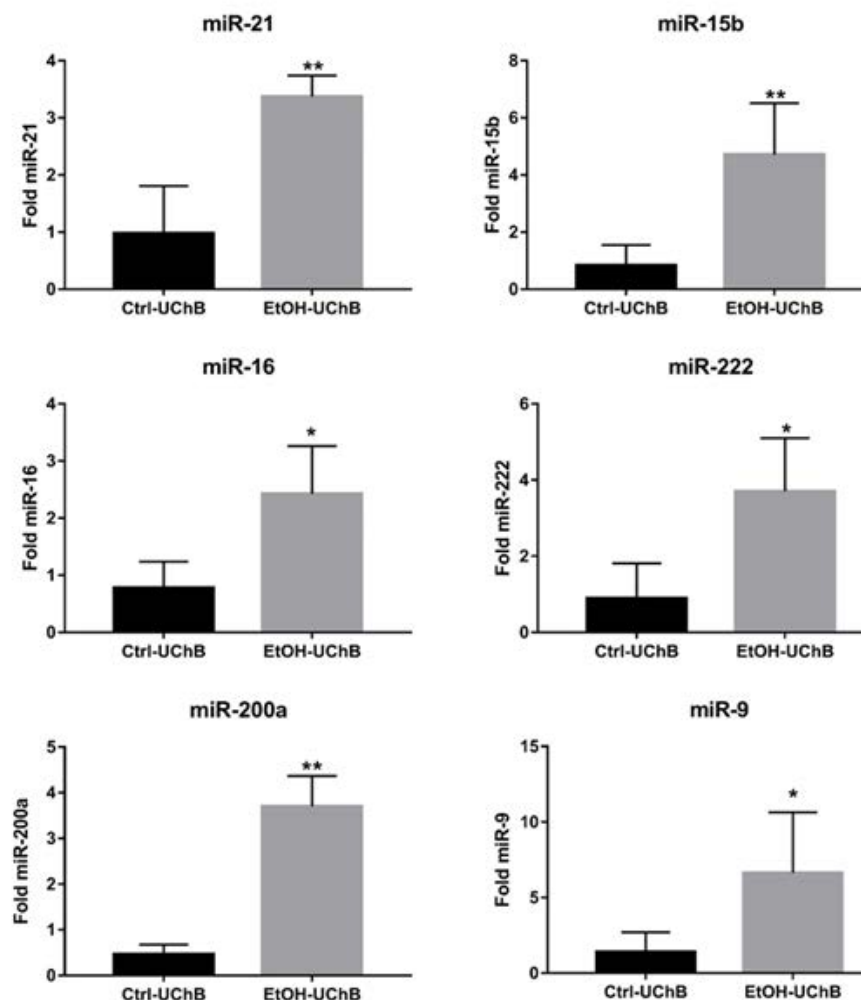


Figure 5: Fold-change analysis of miR-9-3p, -15b-5p, -16-5p, -21-5p, -200a-3p and -222-3p expressions in the serum of animals. Data are expressed as the mean \pm standard deviation, * $p < 0.05$, ** $p < 0.01$.

Discussion

Chronic ethanol consumption did not induce significant morphological or molecular alterations in the cerebellum of aged UChB rats, suggesting a state of functional tolerance that blunted the expected neurodegenerative effects. Although ethanol neurotoxicity is well documented [17,18,]. The absence of major alterations in apoptotic markers supports the idea that long-term ethanol exposure interacts with the genetic and metabolic background of UChB rats [12] shaping their neural response to chronic intake. Genetic predisposition plays a central role in alcohol dependence and the neurobiological consequences of ethanol exposure [19]. UChB rats carry ALDH2 alleles with high affinity for NAD⁺ [20,12], facilitating efficient acetaldehyde metabolism and contributing to higher voluntary alcohol intake. This metabolic advantage is also associated with the rapid development of acute tolerance, particularly to ethanol-induced motor impairment [21]. The molecular and cellular mechanisms underlying tolerance including adaptations in ion channels, intracellular signaling, and neuron glia communication are known to shape behavioral responses to ethanol [22, 23,24]. In line with these mechanisms, the present study found no significant changes in caspase-3, XIAP, or IGFR1 expression, reinforcing the notion of neuroadaptation rather than neurodegeneration in aged UChB rats. Comparisons with previous studies further support this interpretation. Both Oliveira et al [25] and Martinez et al [11] reported robust apoptotic activation in adult UChA and UChB rats exposed to ethanol. However, the current aged animals showed remarkably lower levels of caspase-3 and XIAP, suggesting that senescence-related cerebellar decline may overshadow or mask additional ethanol-induced damage. Aging alone is associated with reduced cerebellar volume, neuronal loss, and apoptosis [26,27] and when combined with ethanol exposure, may shift the brain's response from degeneration toward adaptive molecular restraint, possibly mediated by reduced apoptotic competence. Regarding IGFR1, no significant differences were detected, contrasting with studies showing ethanol inhibition of IGF-mediated survival pathways in developing or adolescent brains [28-31]. Ethanol has been shown to reduce IGF production and impair PI3-kinase signaling, leading to neuronal loss and cerebellar hypoplasia during development. However, these effects are less pronounced in senile animals, who may already exhibit blunted growth-factor signaling as part of physiological aging. Thus, chronic ethanol consumption in aged UChB rats likely fails to further suppress IGF signaling beyond the age-related baseline. The role of miRNA-mediated neuroadaptation is an important consideration when interpreting the mild cerebellar response. Ethanol-sensitive miRNAs act as molecular switches regulating plasticity, tolerance, and addiction and early-life environmental exposures can produce lasting changes in neurobehavior [32,33]. Chronic ethanol exposure

and withdrawal are known triggers of long-lasting molecular adaptations in mesocorticolimbic circuits [34-36]. These adaptations include adjustments in apoptotic pathways such as the reduction of ethanol-induced toxicity observed by Johansson et al. [37] which may contribute to the attenuated apoptotic profile found in this study. Moreover, shifts in miRNA expression during chronic alcohol exposure may confer compensatory protection against neurodegeneration and support behavioral tolerance [38,39]. Evidence from adolescent models indicates that ethanol-induced changes in mGlu2 receptor signaling alter striatal circuitry and influence tolerance development [40,41], further emphasizing the complexity of these adaptive responses. Taken together, the lack of apoptotic activation in the cerebellum of aged UChB rats aligns with a broader framework in which genetic predisposition, metabolic efficiency, age-related decline, and miRNA-regulated neuroadaptations interact to shape the neural impact of chronic ethanol exposure. Although this study used only male rats, potentially limiting generalizability, the findings highlight the resilience and compensatory capacity of the UChB strain. They also underscore the importance of considering age and genetic background when evaluating ethanol's neurobiological effects. Finally, the involvement of miRNAs and other regulatory molecules points to promising avenues for future research, particularly in identifying biomarkers or therapeutic targets that differentiate susceptibility to ethanol-induced cerebellar damage across genetically distinct populations [42].

Conclusions

Our findings reveal that aged UChB rats maintain cerebellar apoptotic stability despite lifelong voluntary ethanol intake, supporting the notion that senescence combined with genetic predisposition shapes a state of functional tolerance to ethanol neurotoxicity in apoptotic parameters. While apoptotic markers remain unchanged at the transcriptional level, the pronounced elevation of circulating miRNAs involved in apoptotic regulation suggests a systemic compensatory mechanism rather than local tissue damage. These results underscore the importance of considering age, genetic background, and metabolic characteristics when evaluating ethanol-induced neurobiological alterations and identify specific miRNAs as promising candidates for biomarkers of chronic alcohol exposure in aging populations.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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