

Effects of alkylating agents on hippocampal neuronal injury and miR-204-5p expression

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ABSTRACT

Introduction: The molecular mechanisms underlying chemotherapy-associated hippocampal neurodegeneration, particularly following exposure to alkylating agents, remain poorly understood. This study investigated the association between melphalan- and chlorambucil-induced hippocampal injury and alterations in the expression of apoptosis- and autophagy-related genes and miR-204-5p.

Methods: Adult male Wistar rats were treated daily for 30 days with saline (control), melphalan (Mel), or chlorambucil (Chl). Hippocampal tissue was analyzed using histological assessment of dark neurons and RT-qPCR analysis of apoptosis-related genes (Bax, Bcl-2, Caspase-3), autophagy-related genes (LC3 β and Beclin-1), and miR-204 expression.

Results: Both Mel and Chl significantly increased the density of dark neurons in the hippocampus compared with the control group. Molecular analysis revealed increased mRNA expression of the pro-apoptotic genes Bax and Caspase-3 together with reduced expression of the anti-apoptotic gene Bcl-2. In addition, the expression levels of the autophagy-related genes LC3 β and Beclin-1 were significantly decreased following treatment. Furthermore, miR-204 expression was significantly upregulated in both Mel and Chl groups and was accompanied by increased dark neuron density and coordinated alterations in the expression of apoptosis- and autophagy-related genes.

Conclusion: These findings indicate that systemic exposure to melphalan and chlorambucil is associated with hippocampal neuronal injury, accompanied by altered expression of apoptosis- and autophagy-related genes. The consistent upregulation of miR-204 suggests that this microRNA may be associated with the molecular changes observed following alkylating agent-induced hippocampal injury; however, further protein-level and functional studies are required to establish whether miR-204-5p plays a causal mechanistic role and to determine its potential therapeutic relevance in chemotherapy-associated neurotoxicity.

Keywords:

Hippocampus
Neuronal injury
Melphalan
Chlorambucil
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Received 4 May 2026; Revised from 20 July 2026; Accepted 5 August 2026

Citation: Ebadi AH, Moghimian M, eyzi S, Saadatian Z, alikhani V, Soltani M. Effects of alkylating agents on hippocampal neuronal injury and miR-204-5p expression. *Physiology and Pharmacology* 2026; 30: 364-373. <http://dx.doi.org/10.22034/phypha.30.3.364>

Introduction

Chemotherapy remains a cornerstone in cancer treatment, yet its long-term neurotoxic consequences in survivors—particularly those involving alkylating agents such as chlorambucil and melphalan—are incompletely understood (Morgillo and Marovino, 2021). These agents exert their cytotoxic effects by alkylating DNA and inducing DNA cross-links and strand breaks, ultimately leading to cell cycle arrest and cell death (Siddik 2002). Both chlorambucil and melphalan have been reported to reach the central nervous system, although their extent of brain penetration and pharmacokinetic properties may differ (Greig et al., 1988). The hippocampus, particularly the dentate gyrus, may be especially vulnerable to such insults because of its capacity for adult neurogenesis, a process critical for synaptic plasticity, learning, and memory. Damage to hippocampal structure and neuronal integrity caused by chemotherapeutic agents has been associated with cognitive deficits in cancer survivors (Sekeris et al., 2021). Emerging evidence suggests that certain alkylating chemotherapeutic agents may promote excessive production of reactive oxygen species (ROS), potentially contributing to mitochondrial dysfunction, lipid peroxidation, neuroinflammation, and activation of apoptotic pathways within the hippocampus (Alhowail and Aldubayan, 2021). Oxidative stress is considered one of the major mechanisms contributing to chemotherapy-induced cognitive impairment and hippocampal neuronal injury (Keeney et al., 2018).

Our prior work demonstrated that melphalan and chlorambucil were associated with oxidative stress, altered apoptotic markers, and reduced proliferation in gonadal tissues, suggesting a broader capacity for systemic oxidative damage in highly proliferative, non-malignant tissues (Ebadi et al., 2025a). Extending this paradigm to the brain, we hypothesized that hippocampal neurotoxicity induced by these alkylators may be mediated through dysregulation of microRNAs (miRNAs)—small non-coding RNAs (18–26 nt) that post-transcriptionally silence target mRNAs by binding to their 3' untranslated regions, thereby modulating key cellular processes including apoptosis, autophagy, and neuronal homeostasis (Ghafouri-Fard et al., 2022; Soutschek and Schratt, 2023; Tüncel et al., 2022).

Among these, miR-204-5p has been implicated in the regulation of neuronal integrity, axonal stability, and ox-

idative stress responses (Gao et al., 2014; Wang et al., 2018). Elevated miR-204-5p expression has been documented in models of cognitive impairment and neurodegenerative disorders, where it promotes apoptosis (Liu et al., 2021; Zhu et al., 2022) and inhibits autophagy (Zhang et al., 2021). Mechanistically, miR-204 has been implicated in the regulation of multiple apoptosis- and autophagy-related genes. Increased miR-204 expression has been reported to be associated with suppression of the anti-apoptotic protein BCL2 (Lin et al., 2017), enhanced apoptotic signaling, and reduced expression of autophagy markers such as LC3 and Beclin-1 (Feng et al., 2020). However, these regulatory relationships have been established in specific experimental models and have not been directly investigated in the hippocampus following exposure to melphalan or chlorambucil.

Although several miRNAs, including miR-21 and miR-34a, have been implicated in neuronal injury and neurodegenerative disorders (Hussein and Magdy 2021), miR-204 was selected because accumulating evidence suggests that it is involved in the regulation of oxidative stress responses, apoptosis, and autophagy, three interconnected processes implicated in chemotherapy-induced hippocampal injury. Furthermore, previous studies have consistently reported altered miR-204 expression in models of cognitive dysfunction and neurodegeneration, making it a biologically relevant candidate for investigating molecular alterations associated with alkylating agent-induced neurotoxicity (Ebadi et al., 2025b).

In this study, we investigated whether systemic administration of melphalan and chlorambucil is associated with hippocampal neuronal injury, increased dark neuron density, altered miR-204-5p expression, and changes in the expression of apoptosis- and autophagy-related genes.

Materials and Methods

Study Design

This experimental research was performed on 24 adult male outbred Norway Wistar rats aged 8 weeks and weighing 200–250 g. The animals were maintained at the animal house of Gonabad University of Medical Sciences under controlled environmental conditions, including a 12-hour light/dark cycle, temperature of $22 \pm 2^\circ\text{C}$, proper ventilation, and unrestricted access to standard laboratory chow and drinking water.

Ethical Considerations

All experimental procedures were approved by the Ethics Committee of the Ministry of Health and Medical Education of the Islamic Republic of Iran (Approval No: IR.GMU.AEC1404.005; Date: 3 November 2025). The experimental protocol complied with international guidelines for animal care and was conducted according to the 3Rs principles (Replacement, Reduction, Refinement). Reporting of the study followed ARRIVE recommendations to ensure transparency and ethical compliance.

Group Allocation and Treatment Protocol

After an adaptation period, animals were randomly assigned to three groups ($n = 8$ per group) and treated for 30 consecutive days:

- Control group: received daily intraperitoneal injections of normal saline.
- Chlorambucil group (Chl): administered chlorambucil at 0.2 mg/kg/day via the intraperitoneal route.
- Melphalan group (Mel): received melphalan at 1.5 mg/kg/day intraperitoneally.

Twenty-four hours after the final injection, anaesthesia was induced using ketamine (50 mg/kg) and xylazine (10 mg/kg) intraperitoneally (Soltani et al., 2022). The brains were rapidly excised, divided along the midline, and processed as follows: the right hippocampus was fixed in 10% neutral buffered formalin for microscopic examination, whereas the left hippocampus was immediately frozen and homogenized for subsequent molecular assays, including mRNA and miRNA analysis. In this study, melphalan was administered as an intraperitoneal injection at a dose of 1.5 mg/kg daily and chlorambucil at a dose of 0.2 mg/kg daily. The selected doses were based on previously published experimental studies demonstrating reproducible systemic toxicity without excessive mortality and were considered suitable for evaluating chemotherapy-induced tissue injury in rats (Hafström and Naredi 1998; Olayinka and Ore 2014). Drug doses were individually calculated according to each animal's body weight, and the concentration of each solution was adjusted so that all animals received a constant injection volume of 1 mL.

Quantitative Real-Time PCR (RT-qPCR)

Total RNA was extracted from the left hippocampal tissue using a commercial RNA isolation kit (Yektatajiz,

Iran) in accordance with the manufacturer's protocol. RNA integrity was verified by agarose gel electrophoresis, whereas RNA purity was assessed spectrophotometrically using the A260/A280 ratio (1.8–2.0). Complementary DNA (cDNA) synthesis was performed using 1 µg of total RNA and a commercial reverse transcription kit.

Quantitative real-time PCR was carried out using a StepOne Real-Time PCR System (Applied Biosystems, USA). Each 10 µL reaction mixture consisted of cDNA template, specific forward and reverse primers (0.2 µM each), SYBR Green Master Mix (Ampliqon, Denmark), and nuclease-free water. The amplification program included an initial denaturation at 95°C for 10 minutes, followed by 40 amplification cycles (95°C for 15 seconds, 58°C for 30 seconds, 72°C for 30 seconds). A melt curve analysis was conducted to verify amplification specificity. Primer specificity was confirmed by melt curve analysis, and all primer pairs showed amplification efficiencies of approximately 100% ($E \approx 2.0$).

Expression levels of apoptosis-related genes (*Caspase-3*, *Bax*, *Bcl-2*) and autophagy-associated genes (*LC3β*, *Beclin-1*) were normalized against *GAPDH*. Relative expression was determined using the $2^{-\Delta\Delta CT}$ calculation method (Roshanaee et al., 2022).

Quantification of miR-204-5p by Stem-Loop RT-qPCR

The expression of miR-204 was evaluated using stem-loop reverse transcription followed by quantitative PCR. After RNA extraction, cDNA was synthesized using miRNA-specific stem-loop primers. The reverse transcription reaction (10 µL total volume) contained reverse transcriptase enzyme, RT buffer, dNTPs, stem-loop primer, RNA template (~500 ng), and nuclease-free water. The thermal protocol consisted of incubation at 16°C for 30 minutes, 42°C for 30 minutes, and final enzyme inactivation at 82°C for 5 minutes.

Real-time PCR amplification was performed using a miR-204-specific forward primer and a universal reverse primer. U6 small nuclear RNA served as the internal reference gene. (Saadatian et al., 2023.) The primer sequences used in this study are listed below:

Bax: F: TTG CTA CAG GGT TTC ATC CA, R: GAG TAC CTG AAC CGG CAT CT

Bcl-2: F: GCTACCGTCGTGACTTCGC, R: CCCCACCGAACTCAAAGAAGG

Caspase-3: F: TGTGCTCCAGGCTTCCTTAATC, R:

AGGCTTATGGGAAATGCTGGAC

Beclin-1: F: CGAAAGGTGGTGGCAGAAAAC, R: ACTATATTCTCGCTGGTACTGAGC

LC3β: F: TCAGTGAGAGCTGCCTCTGTC, R: AG-CAGTGGGGATTACACAGTG

GAPDH: F: GGTCTACATGTTCCAGTATGACTC, R: CATTGATGTTAGCGGGATCTCG

miR-204-5p: RT: GTCGTATCCAGTGCAGGGTC-CGAGGTATTCGCACTGGATACGACGGCAT, F: CCCCTTCCCTTTGTATCCT

U6: RT: GTCGTATCCAGTGCAGGGTCCGAGG-TATTCGCACTGGATACGACAAAAT, F: GCTTCGG-CAGCACATATACTAAAAT

Histological Assessment and Toluidine Blue Staining

Formalin-fixed right hippocampal samples were dehydrated through graded alcohols, cleared in xylene, and embedded in paraffin. Sections of 4 μm thickness were prepared using a rotary microtome and mounted on glass slides. After deparaffinization and rehydration, slides were stained with hematoxylin and eosin for general histological evaluation.

To identify dark neurons, sections were additionally stained with 0.03% toluidine blue solution (pH 4.5) for 1–2 minutes. After rinsing and differentiation in 75% ethanol, sections were dehydrated, cleared, and cover-slipped.

Quantification of Dark Neurons

Dark neurons were quantified using a systematic random sampling approach. From each hippocampal region (CA1, CA3, dentate gyrus), 10 non-overlapping microscopic fields were randomly selected, totaling 30 fields per animal. Observations were conducted at ×400 magnification, with each field covering an area of

0.032 mm². Dark neurons were identified according to previously established morphological criteria, including intensely basophilic cytoplasm, triangular or angular shrunken cell bodies, condensed nuclei, and loss of normal neuronal architecture (Asiaei et al., 2017).

Cell density was calculated using the following equation:

Dark neuron density (cells/mm²) = Number of counted dark neurons / (Number of examined fields × Area per field)

To minimize observational bias and improve reproducibility, dark neuron counting was independently performed by two blinded observers who were unaware of the experimental group allocation. Any discrepancies between the observers were resolved by joint re-evaluation and consensus.

Statistical Analysis

Results are presented as mean ± SEM. The Kolmogorov–Smirnov test confirmed normal distribution ($p > 0.05$), and homogeneity of variances was verified using Levene's test ($p > 0.05$). Statistical comparisons among groups were performed using one-way ANOVA followed by Tukey's post hoc analysis in SPSS version 20. A significance level of $p < 0.05$ was considered statistically meaningful.

Results

Histological Finding

Histological examination of hippocampal sections stained with toluidine blue revealed normal neuronal morphology and a low density of dark neurons (DNs) in the control group. In contrast, rats treated with either melphalan or chlorambucil exhibited a marked increase

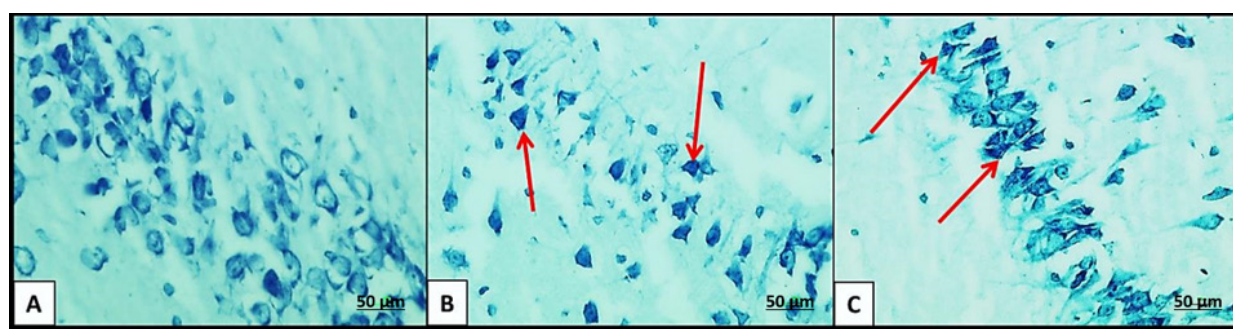


FIGURE 1. Representative photomicrographs of rat hippocampal sections stained with toluidine blue. Panel A: Control group; Panel B: Melphalan-treated group; Panel C: Chlorambucil-treated group. Arrows indicate representative dark neurons, characterized by shrunken cell bodies and hyperchromatic cytoplasm. Original magnification: ×400. Scale bar = 50 μm.

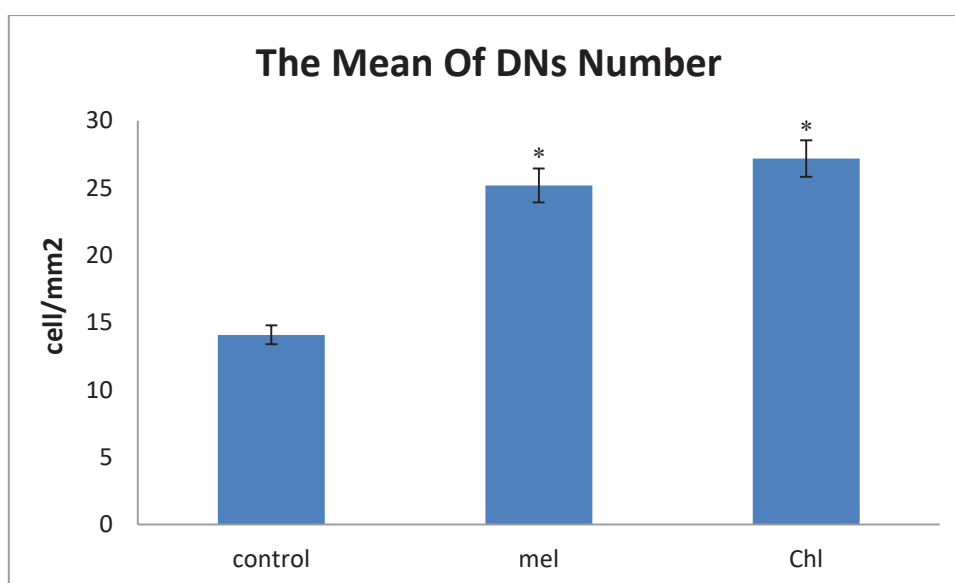


FIGURE 2. Representative photomicrographs of rat hippocampal sections stained with toluidine blue. Panel A: Control group; Panel B: Melphalan-treated group; Panel C: Chlorambucil-treated group. Arrows indicate representative dark neurons, characterized by shrunken cell bodies and hyperchromatic cytoplasm. Original magnification: $\times 400$. Scale bar = 50 μm .

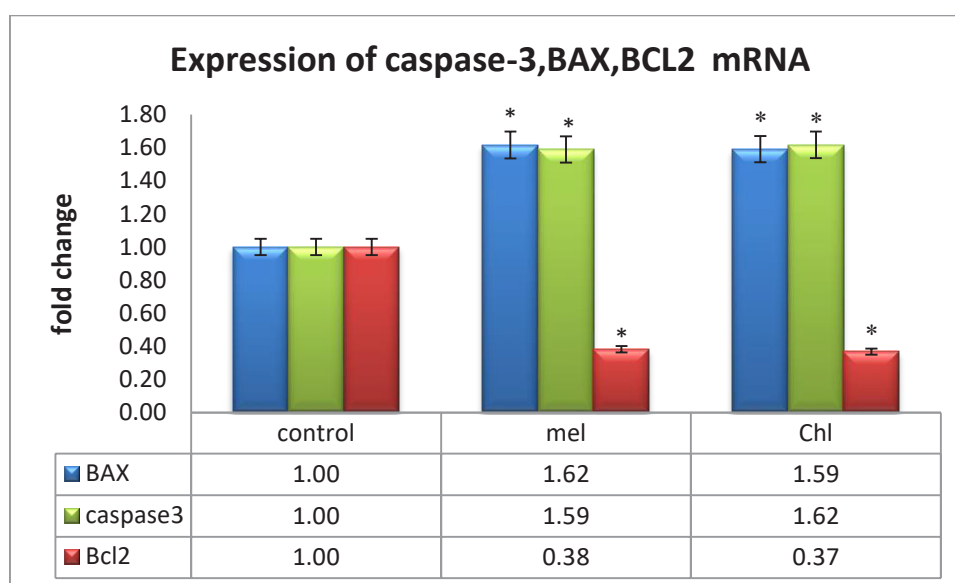


FIGURE 3. Relative mRNA expression of apoptosis-related genes in the rat hippocampus. Relative expression of Caspase-3, BAX, and BCL2 was determined by qRT-PCR following melphalan (Mel) and chlorambucil (Chl) treatment. Gene expression levels were normalized to GAPDH and calculated using the $2^{-\Delta\Delta C_t}$ method. Data are presented as mean $\pm$ SEM. * $P < 0.05$ versus the Control group.

in the number of dark neurons, characterized by intensely stained, shrunken neuronal cell bodies with condensed nuclei (Figure 1). Quantitative analysis demonstrated a significant increase in dark neuron density in both treatment groups compared with the control group ($P = 0.011$). Although the chlorambucil-treated rats showed a slightly higher mean density of dark neurons than the melphalan-treated rats, this difference was not

statistically significant (Figure 2).

Expression of Apoptosis-Related Genes

Real-time PCR analysis demonstrated significant alterations in the expression of apoptosis-related genes following exposure to both alkylating agents (Figure 3). Compared with the control group, Bax expression increased to 1.62-fold in the melphalan group and

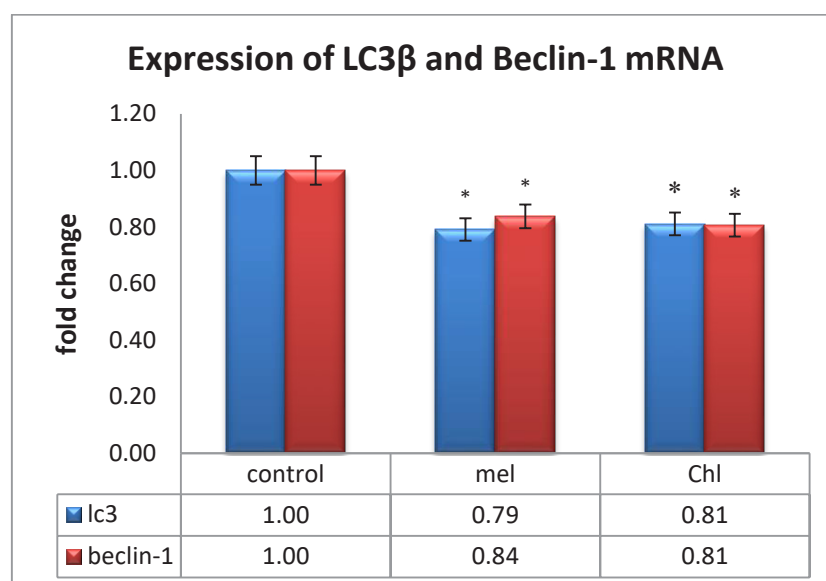


FIGURE 4. Relative mRNA expression of autophagy-related genes in the rat hippocampus. Relative expression of LC3 β and Beclin-1 was determined by qRT-PCR following melphalan (Mel) and chlorambucil (Chl) treatment. Gene expression levels were normalized to GAPDH and calculated using the $2^{-\Delta\Delta C_t}$ method. Data are presented as mean $\pm$ SEM. * $P < 0.05$ versus the Control group.

1.59-fold in the chlorambucil group ($P = 0.021$). Similarly, Caspase-3 expression increased to 1.59-fold and 1.62-fold in the melphalan and chlorambucil groups, respectively ($P = 0.001$). In contrast, the expression of the anti-apoptotic gene Bcl-2 was markedly reduced to 0.38-fold in the melphalan group and 0.37-fold in the chlorambucil group compared with controls ($P = 0.012$). No statistically significant differences were detected between the melphalan- and chlorambucil-treated groups for any of the apoptosis-related genes.

Expression of Autophagy-Related Genes

As shown in Figure 4, administration of both alkylating agents significantly reduced the expression of autophagy-related genes in hippocampal tissue. LC3 β expression decreased to 0.79-fold in the melphalan group and 0.81-fold in the chlorambucil group Relative to controls ($P = 0.011$). Likewise, Beclin-1 expression declined to 0.84-fold and 0.81-fold following melphalan and chlorambucil treatment, respectively ($P = 0.031$). No significant differences in the expression of these genes were observed between the two chemotherapy-treated groups.

miR-204 Expression

Quantitative stem-loop RT-qPCR analysis demonstrated a significant increase in hippocampal miR-204 ex-

pression following treatment with both alkylating agents (Figure 5). Relative to the control group, miR-204 expression increased to 1.57-fold in melphalan-treated rats and 1.68-fold in chlorambucil-treated rats ($P = 0.001$). Although the chlorambucil group exhibited slightly higher miR-204 expression than the melphalan group, the difference did not reach statistical significance.

Discussion

Despite their well-established efficacy in improving cancer survival outcomes, the systemic administration of chemotherapeutic agents—particularly alkylating drugs such as melphalan and chlorambucil—raises significant concerns regarding their long-term neurotoxic consequences, especially within the hippocampus, a region critical for neuroplasticity and cognitive function (Morgillo and Marovino 2021).

In this study, we investigated the hypothesis that these agents are associated with hippocampal neuronal injury and alterations in cell death-related pathways, with a particular focus on the potential involvement of miR-204-5p in these processes. Histopathological analysis revealed a marked increase in dark neurons within the hippocampal formation following treatment, corroborating our previous observations of alkylating agent-induced tissue injury (Ebadi et al., 2026). This finding was accompanied by transcriptional alterations con-

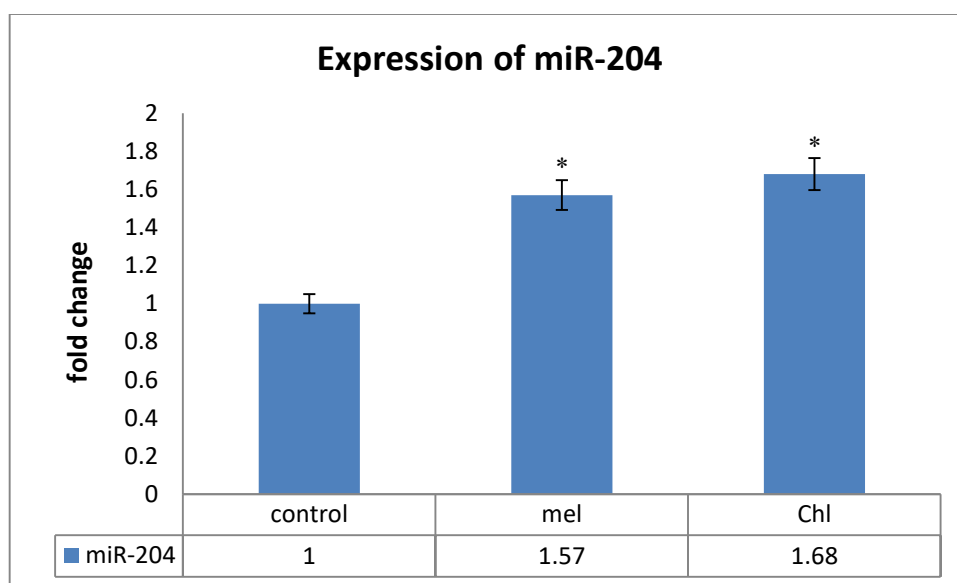


FIGURE 5. Relative expression of miR-204-5p in the rat hippocampus. Relative expression of miR-204-5p was quantified by stem-loop RT-qPCR following melphalan (Mel) and chlorambucil (Chl) treatment. Expression levels were normalized to U6 small nuclear RNA and calculated using the $2^{-\Delta\Delta C_t}$ method. Data are presented as mean $\pm$ SEM. * $P < 0.05$ versus the Control group.

sistent with enhanced pro-apoptotic signaling, including upregulation of the pro-apoptotic genes Bax and Caspase-3, downregulation of the anti-apoptotic gene Bcl2, and reduced expression of the autophagy-related genes LC3 β and Beclin-1. Collectively, these findings suggest an association between increased expression of apoptosis-related genes and reduced expression of autophagy-related genes following exposure to alkylating agents. However, because autophagic flux and protein expression were not evaluated, the present data should not be interpreted as definitive evidence of impaired autophagy, and further protein-based analyses are required to confirm these molecular changes (Martinet et al., 2009). Notably, shares similarities with mechanisms previously documented in cancer cells, where inhibition of autophagy by alkylating agents enhances chemotherapeutic efficacy by promoting apoptosis (Bordin et al., 2013); however, in the healthy hippocampus, this same mechanism results in irreversible neuronal loss.

In our previous studies, systemic administration of alkylating agents significantly increased serum oxidative stress markers while promoting apoptosis in peripheral tissues, indicating that these agents can induce systemic oxidative imbalance (Ebadi et al., 2026; Ebadi et al., 2025b). Together with previous reports describing chemotherapy-associated oxidative damage in the central nervous system, these findings raise the possibility that

oxidative stress may also contribute to the hippocampal alterations observed in the present study. However, because oxidative stress biomarkers were not directly measured in hippocampal tissue, this proposed mechanism remains hypothetical and requires further experimental confirmation.

Notably, we identified a significant and consistent upregulation of miR-204-5p in hippocampal tissue following both melphalan and chlorambucil exposure, which was accompanied by increased dark neuron density and altered expression of apoptosis- and autophagy-related genes. miR-204-5p has been implicated in the regulation of neuronal development, axonal integrity, and stress-response pathways (Gao et al., 2014). Its overexpression has been linked to increased susceptibility to neuronal injury and neurodegenerative processes in experimental models (Tao et al., 2021; Zhou et al., 2020), where it promotes apoptosis by directly targeting BCL2 and inhibiting autophagic flux via suppression of LC3 and Beclin-1.

In cervical cancer models, miR-204-5p overexpression was shown to reduce BCL2 and LC3I/II protein levels while elevating BAX and cleaved caspase-3 (Li et al., 2019), although direct comparisons with the present study should be interpreted cautiously because our study assessed mRNA expression rather than protein levels and was conducted in hippocampal tissue rather

than cancer cells (Liu et al., 2021).

Consistent with these observations, our findings demonstrated a significant upregulation of miR-204-5p accompanied by increased expression of apoptosis-related genes and reduced expression of autophagy-related genes following exposure to melphalan and chlorambucil. Although this coordinated pattern is compatible with a potential role for miR-204-5p, the present study establishes an association rather than a causal relationship. Therefore, functional studies employing miR-204-5p gain- and loss-of-function approaches are required to determine whether this microRNA directly regulates apoptosis- and autophagy-related pathways in response to alkylating agents.

Experimental inhibition of miR-204-5p has been reported to alleviate neuroinflammation, axonal degeneration, and cognitive impairment in experimental models of neurodegeneration (Liu et al., 2021; Zhu et al., 2022), supporting its potential relevance in neuronal injury. Nevertheless, whether therapeutic modulation of miR-204-5p can safely attenuate chemotherapy-induced neurotoxicity without compromising the antitumor efficacy of alkylating agents remains unknown and requires further preclinical investigation.

This study has several limitations. First, oxidative stress biomarkers were not evaluated; therefore, the contribution of oxidative stress to the observed hippocampal alterations remains hypothetical. Second, only mRNA expression of apoptosis- and autophagy-related genes was assessed. Protein-level validation, including cleaved Caspase-3 and autophagic flux markers (e.g., LC3-II/LC3-I ratio and p62), was not performed, limiting the functional interpretation of these molecular findings. Third, no functional manipulation of miR-204-5p (e.g., using mimics or inhibitors) was conducted; therefore, the observed association between miR-204-5p upregulation and hippocampal neuronal injury should not be interpreted as evidence of causality. Fourth, behavioral assessments of hippocampus-dependent cognitive function, such as the Morris water maze or novel object recognition test, were not performed; therefore, the functional consequences of the observed molecular and histological alterations remain to be established. Finally, only young adult male rats were included in this study to minimize variability associated with sex hormones and the estrous cycle. Although this approach reduced biological variability, it limits the generalizability of the

findings, and future studies should include both female and aged animals.

Conclusion

Our findings demonstrate that systemic administration of melphalan and chlorambucil is associated with hippocampal neuronal injury, accompanied by upregulation of miR-204-5p, increased expression of apoptosis-related genes, and reduced expression of autophagy-related genes. These findings suggest that altered miR-204-5p expression is associated with the molecular responses observed following alkylating agent-induced hippocampal injury. However, because the present study evaluated only gene expression and did not include functional manipulation of miR-204-5p or assessment of oxidative stress and protein-level changes, further mechanistic studies are required to establish causality and determine the therapeutic potential of targeting miR-204-5p in chemotherapy-induced neurotoxicity.

Acknowledgments

The authors are grateful to Gonabad University of Medical Sciences for cooperation in all aspects of the present study.

Conflict of interest

The authors have no relevant financial or non-financial interests to disclose.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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