

Vitamin B12 Regulates the Expression of Methotrexate-Induced Increased Markers of Autophagy: An Immunohistochemical Study



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Submission: November 17, 2020; **Published:** January 19, 2021

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Abstract

In this study, the immunohistochemical evaluation of autophagy in brain tissue in the presence and absence of folate was aimed. Therefore, Vitamin B12, a water-soluble folic acid, and Methotrexate (MTX), a folate antagonist, were used in the study. 28 rats were used in the study. Control group (n=7), Vitamin B12 group (n=7) 3µg/kg/ip B12 (during 15 days), MTX group (n=7) injected with 20mg/kg/ip MTX on 8th day of experiment, MTX+Vitamin B12 group (n=7) injected with a single dose of 20mg/kg ip MTX on 8th day of experiment + 3µg/kg/ip Vit B12 (15 days) per. After the histological procedure, microtubule-associated protein 1 Light Chain 3 (LC3B), microtubule-associated protein 1 Light Chain 3 (LC3A) and glucose-regulated protein 78 (GRP78) immunohistochemistry staining were performed on the brain tissue. According to our results, all three protein expressions were observed in the cerebral cortex. Expressions of LC3A, LC3B and GRP78 were statistically significantly increased in the MTX group compared to the control. Vitamin B12 administration showed a statistically significant improvement in the expression results of all proteins. Vitamin B12 to be used before MTX treatment may prevent abnormal autophagy in brain tissue.

Keywords: Autophagy; Endoplasmic reticulum stress; Methotrexate; Vitamin B12

Abbreviations: SAM: Adenosylmethionine; BBB: Blood-Brain Barrier; CNS: Central Nervous System; ER-Stress: Endoplasmic Reticulum-Stress; GRP78: Glucose-Regulated Protein 78; MXT: Methotrexate LC3: Microtubule-Associated Protein 1 Light Chain 3

Introduction

Vitamin B12 is a water-soluble vitamin produced by microorganisms in nature, which we take into our bodies through diets [1]. It is the cofactor of methionine synthase, which is involved in the synthesis of methionine, which is the precursor of S-Adenosylmethionine (SAM) [2]. Therefore, it plays an important role in DNA synthesis, erythropoiesis and healthy neurological functions [3,4]. Vitamin B12 deficiency is a common cause of macrocytic anemia, and also causes clinical disorders including peripheral and central neurological manifestations [3,5]. Therefore, bone marrow and nerve tissue are considered to be the organs most susceptible to vitamin deficiency [6]. Methotrexate

(MTX) is a folate antagonist used for Central Nervous System (CNS) cancers [7]. However, the presence of the Blood-Brain Barrier (BBB) in the CNS causes the drug to be prevented from entering the brain [8]. Various doses are administered intravenously or intrathecally in order to obtain effective treatment [7]. MTX exerts its effect by limiting one-carbon fragments required for the synthesis of purines in DNA synthesis and cell proliferation and inhibiting dihydrofolate reductase [9]. It has been reported that MTX used for the CNS causes neuron degeneration in different parts of the brain [10]. MTX has been reported to induce apoptosis, which is programmed cell death in brain tissue [11,9]. There is limited information about the

effects of MTX on autophagy, another programmed cell death in brain tissue. Autophagy is a highly complex physiological process that recycles cellular components. This process is important in maintaining cellular homeostasis in certain tissues such as the brain and liver muscle [12]. Autophagy can cause cell survival, as well as irregular autophagy can cause cell death. Autophagy occurs under the influence of the Autophagy-Related gene (Atg) family [13]. The yeast Atg8 homologue is rat microtubule-associated protein 1 Light Chain 3 (LC3) [12]. Three subfamilies of LC3 (LC3A, LC3B and LC3C) have been identified in humans [14]. While autophagy occurs in the cell, first cytosolic LC3-I is produced in the cytoplasm, then transformed into LC3-II, which can localize to autophagosomal membranes [15]. Incorporation of LC3-II into the membrane is associated with Atg activation and the process ends with the formation of mature autophagosomes [12]. Thus, the amount of LC3-II correlates with the number of autophagosomes [16]. Therefore, LC3s are important in showing autophagy in tissue. Information about the relationship of MTX used in CNS cancers with autophagy is quite limited. In this study, we aimed to demonstrate immunohistochemically the role of autophagy in the changes that MTX will cause in brain tissue. In order to eliminate the inhibition of MTX on folate, we aimed to show immunohistochemically whether autophagy is affected by using Vitamin B12. In addition, we evaluated glucose-regulated protein 78 (GRP78) immunohistochemically in the tissue in order to evaluate the possible Endoplasmic Reticulum-Stress (ER-stress) that will occur in the tissue if autophagy is affected.

Material And Methods

Animals

Twenty-eight adult male Wistar albino rats (2-3 months/ 8-12 weeks, weighing 200-300 g) was used from Erciyes University Experimental and Clinical Research Center (DEKAM), Kayseri, Turkey. Rats were housed in plastic cages in a well-ventilated rat house and allowed ad libitum access to food and water and kept at a 12-h light: dark cycle. All the animals received humane care according to the standard guidelines. Ethical approval for the study was obtained from Erciyes University Animal Research Local Ethics Committee and the ethic at regulations were followed in accordance with national and institutional guidelines (date 2018, decision no 18/116).

Chemicals

Methotrexate (MTX) (500mg/20ml, Koçak Farma, Turkey) and Vitamin B12 (Vit B12) (Vitamin B12 Rubranova, 5,000 µg; Bristol-Myers Squibb) was used as medicine.

Experimental design

In the study, 28 Wistar albino rats were divided into 4 equal groups. Control group (n=7) injected was applied Intraperitoneally (ip) saline during the experiment to this group. Vitamin B12 group

(n=7) 3µg/kg/ip B12 (15 days) per day throughout the experiment. Methotrexate group (MTX) (n=7) injected with a single dose of 20 mg/kg/ip MTX on 8th day of experiment. Methotrexate+Vitamin B12 group (MTX+Vitamin B12) (n=7) injected with a single dose of 20mg/kg ip methotrexate on 8th day of experiment + 3µg/kg/ip Vit B12 (15 days) per day throughout the experiment.

Preparation of brain tissues

At the end of the experimental, the animals were killed by decapitation under ip ketamine (75mg/kg) + xylazine (10mg/kg) anesthesia. The brain tissues were quickly removed and were fixed in 4% formaldehyde fixative for histological examination. Following dehydration (50%, 70, 80, 96, 3 times absolute alcohol) and clearing (xylene) embedded in parafin. Sections were stained with immunohistochemistry. Photographs were taken with an light microscope (Olympus BX51, Center Valley, PA, USA).

Immunohistochemistry procedure

Immunohistochemistry was applied to the brain tissue sections. The kit (TA-125-HDX, Thermo Fisher Scientific, Waltham, MA, USA) was used to determine the expression intensities of the antibodies. Antibodies are as follows: microtubule-associated protein 1 light chain 3 (LC3AB) (D3U4C, Cell Signaling, USA), microtubule-associated protein 1 Light Chain 3 (LC3B) (NB100-2220, Novus, USA) and glucose-regulated protein 78 (GRP78) (bs1219R, Bioss, USA). A citrate buffer was used for antigen recovery. Phosphate buffered saline (PBS) was preferred for washings. The sections were visualized using 3,30-p-diaminobenzidine tetrahydrochloride (TA-060-HDX, Thermo Fisher Scientific, Waltham, MA, USA) lightly counter-stained with Gill hematoxylin. Under the light microscope (Olympus BX51, Center Valley, PA, USA) and images were obtained. Antibody's immunoreactivities were measured with the Image j program [17].

Statistical analysis

All statistical analyses were carried out by using GraphPad Prism version 7.00 for Mac, GraphPad Software, La Jolla, California, USA. D'Agostino Pearson omnibus test was used to identify the normal distribution of the data. In the case of normal distribution, quantitative variables were compared using one-way analysis of variance and Tukey's posthoc test. The data were expressed as the mean of normalized data±standard deviation of the mean. p<0.05 was considered as statistically significant.

Results

Evaluation of LC3AB and LC3B expressions

LC3AB and LC3B expressions were observed in the brain tissue of all groups. Expression intensities were mostly in neuron cytoplasm located in the midline of the cerebral cortex. LC3AB expression showed a statistically significant increase in both the

Vitamin B12 group and the MTX group compared to the control. LC3B expression was similar to control and Vitamin B12. It increased statistically significantly in the MTX group. In the MTX + Vitamin B12 group, it decreased, showing a result similar to the control. There was also a statistically significant difference

between the vitamin B12 group and the MTX group. LC3AB expression decreased in the MTX + Vitamin B12 group, showing a similar result to the control. Between-group differences in LC3AB and LC3B expressions are shown in Table 1, Figure 1.

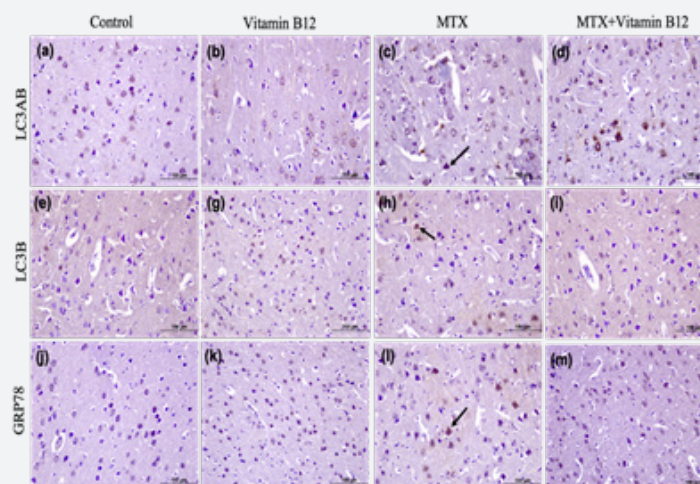


Figure 1: Immunohistochemistry stain of brain sections. LC3AB (a-d), (a) Control group, (b) Vitamin B12 group, (c) MTX group, (d) MTX+Vitamin B12 group. LC3B (e-h), (e) Control group, (f) Vit B12 group, (g) MTX group; (h) MTX+Vitamin B12 group. RIP3 (i-l) (i) Control group, (j) Vitamin B12 group, (k) MTX group; (l) MTX+Vitamin B12 group. Black arrows show the expressions of the proteins. Scala bar 100µm. Abbreviations; MTX: methotrexate, LC3, microtubule-associated protein light chain 3; GRP78, glucose-regulated protein 78.

Table 1: LC3AB, LC3B and GRP78 immunoreactivity measurements of experimental groups. All data are expressed as the mean ± SD. $p < 0.05$ was considered as significant.

Groups	Control	Vitamin B12	MTX	MTX+Vitamin B12	p
LC3AB	77.15±2.68a	78.10±2.35ab	80.26±3.16c	78.51±2.59b	0.001
LC3B	77.24±3.61a	76.76±3.34a	81.26±4.52b	77.04±3.16a	0.001
GRP78	78.50±1.70a	77.36±2.67a	80.45±2.61b	78.53±2.16a	0.001

Note: There is no significant difference among groups with same letters (a, b, c). Abbreviations: MTX, methotrexate; LC3, microtubule-associated protein 1A/1B-light chain 3; GRP78, glucose-regulated protein 78 (GRP78).

Evaluation of GRP78 expression

GRP78 expression was observed especially in the neuron cytoplasm with a pyramidal shaped cell body. There was no statistical difference between the control group and the Vitamin B12 group. There was a statistically significant increase in the MTX group compared to the control. The MTX + Vitamin B12 group was also similar to the control. GRP78 expression is shown in Table 1, Figure 1.

Discussion

MTX shows its effect on cells by inhibiting dihydrofolate reductase, indirectly inhibiting purine synthesis, by inhibiting both DNA synthesis and cell proliferation [18]. Because MTX is a folate antagonist. Intracellular folate deficiency indirectly causes anemia and nerve damage as vitamin B12 deficiency

causes inhibition of both purine and pyrimidine synthesis [19]. In our study, we preferred MTX because of its effects on folate and evaluated autophagy in nerve tissue and related ER-stress. In order for the normal physiological process to continue, the cell performs many activities together and regularly. However, under adverse conditions, cell death mechanisms can be triggered. Apoptosis and autophagy are processes that play an important role in maintaining homeostasis. Autophagy, the mechanism of programmed cell death, is essential for normal development and cellular activity, and LC3 (also known as Atg8) is a marker for autophagosomes that occur in autophagy formation [20]. As long as the physiological process continues normally, autophagy is a process that only removes old and degraded cellular components. However, increased abnormal autophagy can also cause cell death. The main difficulty in using MTX in the treatment of brain tumors is the presence of a blood-brain barrier that does not allow tissue

passage. For this reason, high doses, various drugs and methods have been developed to obtain the effective concentration of the drug in the tissue [8,21,22]. In our study, we evaluated the autophagy marker LC3 and ER-stress marker GRP78 in brain tissue by applying 20mg/kg MTX. Both LC3AB and LC3B expressions in brain tissue increased in MTX group. LC3 expressions were in the cell cytoplasm of neurons. Neurons are the cells of the nervous system that hear, interact, and transmit. Pyramidal neurons of the cerebral cortex are large cell bodied neurons with the same morphological prototype [23]. LC3 expressions were stained specifically in the cytoplasm of these neurons, not Betz cells. Cisplatin, a chemotherapeutic agent such as MTX, has been reported to up-regulate autophagy-related genes in the breast cancer cell line [20]. In a study in which neoadjuvant was used to increase the effect of MTX on Ehrlich acid carcinoma, it was reported that the autophagic gene beclin1, up-regulated by MTX, decreased after neoadjuvant drug use [24]. It has been reported that MTX increases LC3 expression in the spermatocyte cell line and is induced in apoptosis due to this increase [25]. In this study, we can say that the decreased folate concentration after MTX administration causes an increase in both LC3 expressions in the brain tissue. Because we obtained similar results to the control in both LC3 expressions in MTX + Vitamin B12 group, which we used to complement the reduced folate. In parallel with the increase in LC3 expressions in the MTX group, we also found an increase in GRP78 expression. GRP78, as one of the important chaperones on ER, participates in the ER protein folding and assembly process [26]. In response to stress conditions, GRP78 overexpression is a harbinger of the presence of large amounts of unfolded protein [27]. It has been reported that substances such as lead impair GRP78 function in the nervous system, increasing the unfolded protein response [28]. It has been reported that low doses of MTX cause a decrease in the number of apoptotic cells by reducing ER-stress in vitro and in vivo [29]. On the contrary, it is also suggested that even its topical application promotes ER-stress mediated apoptosis [30]. We found that MTX increased GRP78 expression in brain tissue after 20mg/kg dose administration. This increase was parallel to LC3 expressions. We think that the MTX dose likely increases ER-stress in neurons, causing an increase in the amount of misfolded/unfolded protein, thereby increasing LC3 expressions in response to the cell in order to eliminate these accumulated proteins. These results are not only dependent on them, but also related to more genes and proteins.

The effect of the folate antagonist MTX on LC3 and GRP78 expressions was statistically significantly improved when a folate derivative Vitamin B12 was used. In this case, it can be concluded that regular methionine synthesis has an effect on both autophagy and ER-stress. Because unlike a folic acid antagonist MTX, Vitamin B12 is a water-soluble folic acid [31]. Exogenous completion of depleted endogenous Vitamin B12 indicates that it is necessary for the normal physiological process to continue in the tissue. There is very limited literature information on vitamin B12 and

GRP78. Only in the diabetic retinopathy model, Vitamin B12 supplementation has been reported to reduce the various ER-stress markers contained in GRP78 [32]. In addition, Vitamin B12 is very important for the nervous system, and neuronal degeneration occurs in its deficiency [33]. Vitamin B12 has also been shown to reverse impaired ER-stress and cellular autophagy after stroke [34]. In our study, we found improvement in autophagic marker LC3 expression and ER-stress marker GRP78 expression with Vitamin B12 administration. This result should not be ignored in order to prevent possible abnormal autophagy.

Conclusion

In study, we demonstrated the autophagy in the brain tissue immunohistochemically using MTX and Vitamin B12. In addition, increased GRP78 expression showed us that increased autophagy can also trigger ER-stress. We think that autophagy and ER-stress should not necessarily be ignored in studies on improving the effect of MTX on CNS cancers. In addition, we believe that Vitamin B12 should be essential supplementary food for all drug-administered diseases that may cause folate deficiency.

Acknowledgement

This work was supported by Erciyes University the Scientific Research Projects Unit, TSA-2019-8673 project code.

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DOI: [10.19080/OAJT.2021.04.555648](https://doi.org/10.19080/OAJT.2021.04.555648)

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