

ORIGINAL ARTICLE

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Protective effect of metformin against lithium-induced cerebral neurotoxicity in rats**Umit Ali Malcok¹, Muserref Hilal Sehitoglu², Basak Buyuk³, Eyup Burak Sancak⁴, Halil Ibrahim Tas⁵**¹Canakkale Onsekiz Mart University, Faculty of Medicine, Department of Neurosurgery, Canakkale, Turkey²Canakkale Onsekiz Mart University, Faculty of Medicine, Department of Biochemistry, Canakkale, Turkey³Izmir Demokrasi University, School of Medicine, Department of Histopathology, Izmir, Turkey⁴Canakkale Onsekiz Mart University, Faculty of Medicine, Department of Urology, Canakkale, Turkey⁵Ordu University, School of Medicine, Department of Psychiatry, Ordu, Turkey

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**Abstract**

Neurotoxic effects are seen in patients using lithium regardless of dose. The neuroprotective activity of metformin used in the treatment of patients with type 2 diabetes mellitus is known. In this study, the protective efficacy of metformin against the neurotoxic effects of lithium was investigated. Neurotoxicity was induced by administering high dose oral lithium (40 mmol/kg) to rats for two weeks. Four groups were formed, with 6 rats in each group. OSI indices were calculated by measuring serum TAS and TOS levels. TAS, TOS, OSI, Gpx ADAM10, and ADAM17 levels were measured in the brain tissue. The neuroprotective activity of metformin was examined histopathologically by calculating the pyknosis scores, vacuolization, and interstitial edema in the hippocampus and cortex tissues. When lithium and metformin were given together, serum levels of TAS, TOS, and OSI were lower than that of the Lithium group ($p < 0.001$), whereas the TAS level in the brain tissue was higher than that of the lithium group ($p < 0.05$). With the concomitant administration of lithium and metformin, ADAM10 levels decreased ($p < 0.05$), whereas ADAM17 and Gpx levels increased ($p > 0.05$). In histopathological evaluation, pyknosis, interstitial edema, and vacuolization scores of the hippocampus and cortex regions increased with lithium alone but were low when lithium was given together with metformin. Levels of the antioxidant molecules Gpx and TAS decreased with lithium, and ADAM10 and ADAM17 proteins, which indicate the strength of intercellular bonding, increased by administering metformin. In conclusion, the toxic effect of lithium on brain tissue was reduced by metformin.

Keywords: Lithium, metformin, neurotoxicity, ADAM10, ADAM17**Introduction**

The first results about the use of lithium in bipolar disorder were published by Cade et al. in 1949 [1]. Its curative efficacy in bipolar disorder depends on the changes it has made in the transduction pathways in the central nervous system, and these pathways are known to be associated with glutamate, inositol monophosphate, and glycogen synthase kinase 3 [2,3]. The toxic side effects of lithium that can occur even in the narrow therapeutic dose range (0.6-1.2 mEq/L) cause many clinical problems in treatment and follow-up [3]. Intense biochemical changes have also been reported after acute and chronic lithium administration, including changes in inositol, choline, and phospholipid metabolisms [3]. This effect occurs when the membrane transport of choline is inhibited by lithium [4]. Lithium, which inhibits inositol monophosphatase, also promotes apoptosis [5,6]. In addition to all these effects,

lithium also inhibits AMPK, increasing apoptosis and autophagy [7]. When markers in the blood are examined, it has been shown that the serum concentration of lithium rises parallel to the TAS levels [6]. Although its clinical effects are clearly known, the details of its effect mechanisms at the molecular level still cannot be fully explained [2]. Many molecules have been investigated to reduce the toxic effects of lithium [8,9]. These molecules have in common that they are molecules that activate AMPK and inositol monophosphatase, which cause an increase in apoptosis when inhibited by lithium.

It is known that metformin exerts additional neural protective effects by activating AMPK-dependent pathways [10]. Metformin, which is widely used in the treatment of diabetes, also has antioxidant, antiapoptotic, and anti-inflammatory effects [11-13]. Besides, it reduces intracellular phosphocholine formation and corrects impaired phospholipid metabolism [14]. Another effect is to prevent cellular toxicity, and thus, neuronal damage by inhibiting cytochrome c release and caspase-3 activation caused by excess glutamate in the environment [13]. This study aims to investigate the protective effect of metformin against the toxic effects of lithium on brain tissue.

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Material and Methods

A randomized-controlled study was planned in rats. The Guide for the Care and Use of Laboratory Animals of the National Institute of Health was followed in all procedures. Twenty-four adult male Wistar albino rats weighing 250-300g were obtained from the Canakkale Onsekiz Mart University Laboratory Animal Production Unit (Turkey). One-week adaptation time was provided to the animals ($22\pm 2^{\circ}\text{C}$ temperature, $70\pm 4\%$ relative humidity, and 12-hour light/dark cycle). All subjects were fed freely with standard feed and tap water. Metformin dose was calculated according to the 800 mg/kg formula used in diabetes studies conducted with rats [15]. A lithium solution of 40 mmol/kg was prepared for each subject [16].

Subjects were randomly selected, and four groups of six rats in each group were formed: Group 1 (C): (Control group, n=6). Fed a normal diet for 14 days and placed in a metabolic cage for 24 hours on day 14. Blood and brain tissue samples were taken on the fifteenth day. Group 2 (Li): (Lithium group, n=6). Fed a lithium-rich diet (Lithium Chloride 40mmol/kg + dry food) for 14 days and placed in a metabolic cage for 24 hours on the 14th day. Blood and brain tissue samples were taken on the fifteenth day. Group 3 (Met): (Metformin group, n=6). Fed a diet rich in metformin (Metformin 800mg/kg catheter + dry food) for 14 days and placed in the metabolic cage for 24 hours on the 14th day. Blood and brain tissue samples were taken on the fifteenth day. Group 4 (Li+Met): (Lithium+Metformin group, n=6). Fed a diet rich in lithium and metformin (Lithium Chloride 40 mmol/kg + Metformin 800mg/kg catheter + dry food) for 14 days and placed in the metabolic cage for 24 hours on the 14th day. Blood and brain tissue samples were taken on the fifteenth day.

At the end of the experiment, all animals were administered deep ketamine anesthesia (50-60 mg/kg). Blood samples taken from each subject was put into a tube and centrifuged at 4000 rpm for 10 minutes. Serum samples obtained were separated and stored at -80°C for biochemical analysis. After decapitation, the whole brain was removed. Both hemispheres were separated. Hippocampus and cortex regions of the right hemisphere were separated for histopathological examination and placed into 10% formalin solution. Left hemispheres were separated for biochemical studies and kept at -80°C .

Ethical approval

The ethical approval was obtained from the Canakkale Onsekiz Mart University Animal Studies Local Ethics Committee with the decision/number 2018/05-08 and file registration number 2018/1800064463.

Preparation of samples

Homogenates were prepared for each parameter in accordance with the kit procedures before determining the levels of MDA, TAS, TOS, GPx, ADAM10, and ADAM17 with commercial kits. We suspended rat brain tissues at 50 to 100 mg/mL in Phosphate Buffer Solution (PBS pH: 7.4) and added 10 μL of BHT to 1 mL of sample volume to prevent further oxidation. All tissues were homogenized on Mixer Mill MM 400 (Retsch, Haan, Germany) then spun at 10000 rpm for 5 min to collect the supernatant for determining MDA levels in brain tissues. Rat brain tissues were weighed and suspended in an average of 100 mg/1000 mL PBS

to prepare for GPx measurement. We centrifuged the single-cell suspension at 1000 rpm for 10 minutes at 4°C . After discarding the supernatant, the remaining pellet was resuspended in ice-cold 1X Assay Buffer containing 5 pellets, containing 0.4 mM PMSF, other protease inhibitors, and 1% Triton X-100 (peroxide free).

To determine TAS, TOS, ADAM10, and ADAM17 levels, samples were prepared in an appropriate PBS and homogenized with a homogenizer. After centrifugation of the samples at 4000 rpm for 15 minutes at 4°C , the supernatant was collected and kept at -80°C until analysis.

Biochemical analysis of serum and rat brain tissues

STA-330/OxiSelect™ TBARS Assay Kit (MDA Quantitation–Cell Biolabs, Inc. San Diego, CA 92126) was used for determining MDA level in rat brains. After preparing the tissues, we added 100 μL samples and MDA standards to separate microcentrifuge tubes. Also, we added 100 μL SDS Lysis Solution to both the samples and MDA standards and mixed them thoroughly. After incubating the samples for 5 minutes at room temperature, 250 μL TBA Reagent was added to each sample and standard. We closed each tube, incubated at 95°C for 45-60 minutes, and centrifuged all sample tubes at 3000 rpm for 15 minutes. We removed the supernatant and transferred 200 μL of the MDA standards and samples to a 96-well microplate for spectrophotometric analysis. The absorbance was read at 532 nm. The results were given as $\mu\text{mole}/\text{mg}$ tissue.

We used the ADI-900-158 Glutathione Peroxidase Activity Kit (Enzolifescience Inc. Farmingdale, NY 11735) to determine the Glutathione peroxidase enzyme activity in rat brain tissues. Firstly, we prepared the reagents as defined in the kit procedure. 20 μL of the samples and standards were added into the 96-well plates, and the reaction mixture was added to all samples. We initiated the reactions by quickly adding 20 μL of Cumene Hydroperoxide to each well using a multichannel pipettor and measured absorbance at 340 nm every 30 seconds or 1 minute over a 10-15-minute period with a multi-plate reader. The results were given as $\mu\text{mole}/\text{mL.mg}$ protein.

Serum and tissue, total antioxidant status levels (TAS, Product Code: RL0017), and total oxidant status (TOS, Product Code: RL0024) were determined with spectrophotometric kits (Rel Assay Diagnostics, Gaziantep, Turkey).

After adding the reaction mixture to each sample and standards, the absorbance was read at 660 nm for TAS and 530 nm for TOS. The results for TAS and TOS were given as Trolox Equiv./L and H2O2 Equiv./L, respectively. The oxidative stress index (OSI) was obtained using the TOS/TAS ratio. The ratio percentage of TOS (μmol H2O2 equivalent/L) to TAS (μmol Trolox equivalent/L) potential was used to calculate the oxidative stress index (OSI), an indicator of the degree of oxidative stress [17].

Serum Lithium (Li) levels were measured on the Cobas c501 automatic analyzer using Roche kits (Roche Diagnostics, Indianapolis, USA). The results were given as milliequivalents per liter (mEq/L).

ADAM10 and ADAM17 levels of rat brain tissues were determined by using a commercial kit following user instructions (MBS263494- Rat A Disintegrin And Metalloprotease 10 (ADAM10) ELISA Kit, San Diego, California, United States 156308; MBS7211580- Rat Disintegrin and metalloproteinase

domain-containing protein 17 (ADAM17) ELISA Kit San Diego, California, United States 156308). The absorbance was read at 450 nm, and the results were given as (ng/mL.mg protein). The total protein levels of rat brain tissues were measured using a B6916-Bradford Reagent with Coomassie dye-binding protein assay. The results were given as ng/mL.mg protein.

Histopathological examination

Brain tissue samples were treated in 10% buffered neutral formaldehyde solution for light microscopic examination. Under suitable conditions, tissue follow-ups were completed and embedded in paraffin blocks. Sections taken at 4-micron thickness were stained with Hematoxylin & Eosin. A Leica DCM 3000 (Germany) light microscope was used for microscopic evaluation of brain tissues, and the Leica Q Vin 3 program was used as a computer-aided imaging system.

Histopathologically, changes in expansion pericellular area, karyopyknosis, and vacuolization in the cortex and hippocampus tissues of the brain were scored, and a statistical analysis of the differences between the groups was made.

Neurons in the cortex and hippocampus tissues were scored separately, and their statistical averages were obtained. Damage to the brain tissue was semiquantitatively detected and categorized at four different levels. Histopathological scoring was done as

follows: no visible change=1, minimal or little change=2, moderate change=3, and severe change=4 points.

Statistical analysis

Data were analyzed with the SPSS Package Program version 20.0 (SPSS Inc, Chicago, IL, USA). Mean and standard deviations were used in the presentation of descriptive data. The suitability of variables to normal distribution was evaluated using the Shapiro-Wilk Test. Normal distribution was observed in all groups. One-Way ANOVA was used for within-group comparison. Since the data was homogeneously distributed in the descriptive analysis, Post-hoc multiple comparisons were made with the Tukey's test. Chi-square was used to compare categorical data. A p-value of <0.05 was set as the significance threshold.

Results

Serum biochemical analysis results

Lithium, TAS, TOS, and OSI levels are given in Table 1. There was no significant difference between TAS, TOS, and OSI values of control and Met groups ($p>0.05$). TOS and OSI levels of the Li group were significantly higher than the control group ($p<0.001$). Moreover, when Li+Met were given together, the TAS, TOS, and OSI values were significantly lower than the Li group (Table 1, $p<0.001$).

Table 1. Serum biochemical analysis results

	Control	Met	Li	Li+Met	P
Lithium	0.003 ± 0.008	0.003 ± 0.005	0.045 ± 0.005 ^a	0.043 ± 0.005 ^a	<0.001
TOS	12.98 ± 2.16	11.87 ± 1.98	21.27 ± 4.35 ^a	14.39 ± 2.54 ^b	<0.001
TAS	0.86 ± 0.23	0.88 ± 0.17	0.64 ± 0.14 ^a	0.80 ± 0.12 ^b	<0.05
OSI	15.87 ± 5.03	14.11 ± 5.03	33.69 ± 7.21 ^a	18.25 ± 3.92 ^b	<0.001

Values are mean±standard deviation. Lithium (mEq/L), TAS=Total antioxidant status (μmol Trolox equivalent/L); TOS=Total oxidant status (μmol H₂O₂ equivalent/L); OSI=oxidative stress index. ^awhen compared to the control group; ^bwhen compared to the Li group

Tissue biochemical analysis results

TAS, TOS, OSI, ADAM10, ADAM17, and GPx brain tissue analysis results for the groups are given in Table 2. ADAM10, ADAM17, and GPx levels of Met and Li+Met groups are found higher than the Li group. However, when compared with the Li group, although ADAM10 enzyme activity of the Li+Met group increased significantly ($p<0.05$), there was no significant difference in ADAM17 and GPx levels ($p>0.05$). On the other hand, ADAM10, ADAM17, and GPx levels of the control group were higher than Li and Li+Met groups ($p<0.05$), and there was no statistically significant difference between the control group and Met group ($p>0.05$).

Also, there was no difference between TAS levels between the

control and Li groups ($p>0.05$). However, there was a statistically significant difference between increased TOS and OSI levels in the Li group and the control group ($p<0.05$). TAS level in the Li+Met group was significantly higher than in the Li group ($p<0.05$). The TOS level and OSI index in the Li+Met group were significantly lower than the Li group (Table 2, $p<0.05$).

According to the correlation test results to reveal the interactions between groups statistically, there was a positive relationship between ADAM17 and ADAM10 levels ($p=0.05$) and Gpx levels ($p=0.01$). Additionally, there was a positive correlation between Gpx levels and ADAM10 and ADAM17 measurements ($p=0.01$). On the other hand, the correlation between MDA and ADAM10 levels was negative ($p=0.05$). There was no correlation between TAS and TOS levels and all other groups.

Table 2. Brain tissue analysis

	Control	Met	Li	Li+Met
TAS	1.1±0.1	1.2±0.1 ^p	0.9±0.1	1.3±0.1 ^p
TOS	1.9±0.9 ^p	3.2±1.5	4.7±1.7 ^{&}	2.30±1.2 ^p
OSI	1.7±0.8 ^p	2.8±1.5 ^p	5.5±2.3 ^{&}	1.9±0.9 ^p
ADAM10	337.5±26.2 ^p	306.9±33.8 ^p	230.8±23.2 ^{&p}	280.9±31.7 ^{&p}
ADAM17	130.8±46.7 ^p	126.0±40 ^p	68.7±5.8 ^{&}	98.6±16.6 ^{&}
Gpx	118.9±25.9 ^p	107.05±10.7 ^p	69.5±12.8 ^{&}	85.7±5.1 ^{&}

Values are mean ± standard deviation. TAS=total antioxidant status (μmol Trolox equivalent/L), TOS=total oxidant status (μmol H₂O₂ equivalent/L), OSI=oxidative stress index. ADAM10=A Disintegrin and metalloproteinase 10 (ng/mL.mg protein), ADAM17=A Disintegrin and metalloproteinase 17 (ng/mL.mg protein), Gpx=glutathione peroxidase (μmole/mL.mg protein). “&” compared with the Control group p<0.05, “p” compared with the Li group p<0.05. P-value of less than 0.05 was regarded as statistically significant. In the statistical analysis of the data, One-Way ANOVA with Tukey multiple comparisons was used if variances were equal. A correlation test was conducted to reveal the interactions of the groups with each other

Histopathological evaluation

In the evaluation made concerning pyknosis, it was observed that the Met group was similar to the control group (p=0.297). However, there was a significant increase regarding pyknosis in Li+Met and Li groups compared to the control group (p<0.001). Moreover, there was a considerable difference between the Met group and Li+Met and Li groups in terms of pyknosis (p<0.001). The mean score of the Li group was 3.58±0.51, while the mean score of the Li+Met group was 2.66±0.77. When the Li+Met group was compared with the Li group, the difference was significant (p<0.01). This result shows that the development of pyknosis, one of the neurotoxic effects of lithium, is reduced by metformin (Figure 1, Figure 2, Table 3).

According to the vacuolization scores, there was no significant difference between the metformin and control groups (p=0.493). However, the rise in Li+Met and Li groups was substantial when compared with the control group (p<0.001). When compared with the Met group, the increase in Li+Met and Li groups was significant

(p<0.001). The mean score of the Li group was 3.08±0.28, while the mean score of the Li+Met group was 2.5±0.15. It was observed that the difference between the low score of the Li+Met group, which was considered as the treatment group, and the score of the Li group was significant (p<0.01). This result shows that metformin significantly reduces vacuolization, one of the neurotoxic effects of lithium (Figure 1, Figure 2, Table 3).

Results similar to statistical changes in karyopyknosis and vacuolization were obtained in the evaluation made concerning the pericellular expansion area (Table 3). In the expansion of the Pericellular area, the Li group's score was 2.16±0.57, while the mean score of the Li+Met group was 1.58±0.66. It was observed that the score was lower in the Li+Met group, the group to which the treatment was aimed, and the difference was statistically significant when compared with the Li group (p<0.05). As a result, as in karyopyknosis and vacuolization scores, the enlargement in the pericellular area increased in the lithium group also decreased with metformin administration (Figure 1, Figure 2, Table 3).

Table 3. Histopathological scoring of the hippocampus and cortex regions of the brain

		Control	Met	Li	Li+Met	*p
Cortex	Pericellular expansion area	1.16±0.40	1.66±0.81	3±0.63	1.83±0.75	<0.05
	Karyopyknosis	1.33±0.51	1.83±0.75	3.50±0.54	2.50±0.54	<0.05
	Vacuolization	1.33±0.51	1.50±0.54	3.17±0.40	2.33±0.51	<0.05
Hippocampus	Pericellular expansion area	1.33±0.51	1.33±0.51	2.33±0.51	1.66±0.70	<0.05
	Karyopyknosis	1.33±0.51	1.50±0.55	3.50±0.55	2.50±0.55	<0.05
	Vacuolization	1.33±0.51	1.33±0.51	3.17±0.41	2.33±0.51	<0.05

Values are mean±standard deviation of the histopathological scoring of the hippocampus and cortex regions of the brain. In histopathological scoring: no visible change=1; minimal or little change=2; moderate change=3, serious change=4 points. *Comparison of Li and Li+Met groups. p<0.05 was considered significant

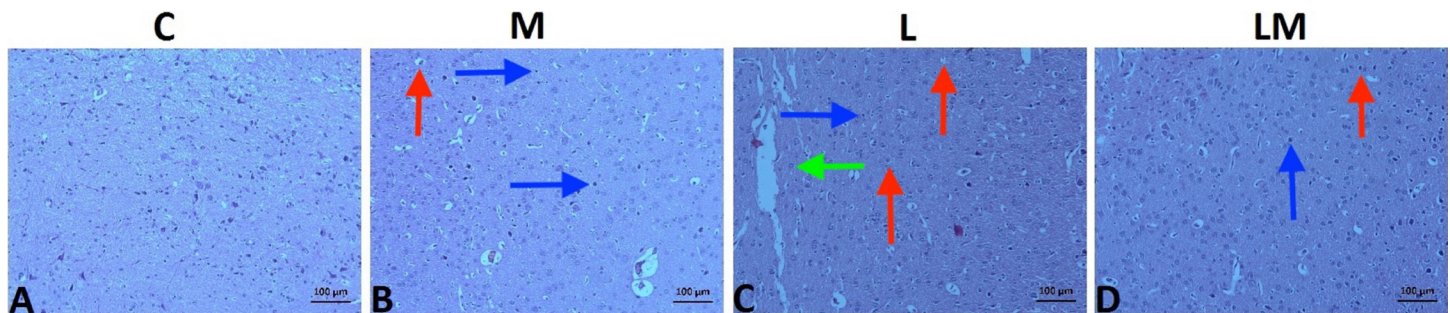


Figure 1. Representative hippocampus histopathological images (x100, scale bar=100 μ m) of H&E stained by experimental groups. A: Control (C), B: Metformin (M), C: Lithium (L), D: Lithium+Metformin (LM). The figure shows areas of enlargement (red arrows), pyknosis (blue arrows), and vacuolization (green arrows) in the pericellular area

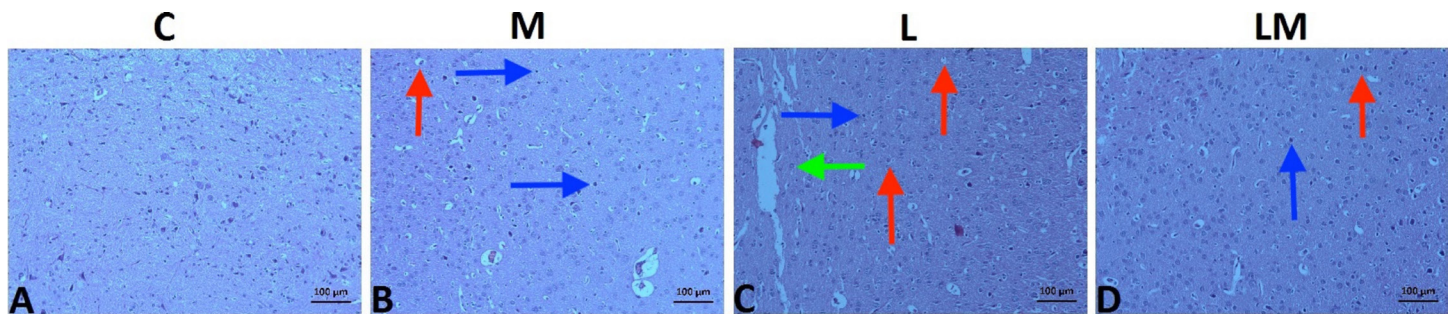


Figure 2. Representative frontal cortex histopathological images (x100, scale bar=100 μ m) of H&E stained by experimental groups. A: Control (C), B: Metformin (M), C: Lithium (L), D: Lithium+Metformin (LM). The figure shows areas of enlargement (red arrows), pyknosis (blue arrows), and vacuolization (green arrows) in the pericellular area

Discussion

Lithium is among the commonly used options in treating bipolar disorder and depression [18]. In addition to its use in treatment, it is known to cause neurotoxicity in high doses for suicidal purposes or regardless of the dose [19]. It is known that lithium increases oxidative stress in mitochondria, and thus, causes neurotoxic damage in the neurons and glial cells [6]. These pathways affected by lithium are also associated with glutamate, inositol monophosphate, and glycogen synthase kinase 3, and excessive use of these pathways depletes inositol in the environment [2,3]. Additionally, in addition to inositol consumption, it also triggers cellular damage by causing an increase in oxidants in the environment [20]. In another study, it was reported that lithium induces apoptosis, however, with clear mechanisms [21]. Although many action mechanisms of lithium have been revealed, the details of the processes that produce toxic effects are still not fully understood.

The effect of metformin on reducing neuronal damage is explained by its inhibition of glutamate-induced cytochrome c release and caspase-3 activation. In the same study, it was shown that by inhibiting these pathways, cellular toxicity and neuron damage were also prevented [13]. A different research has proven that metformin reduces cell damage during oxidative stress [11,12]. In our study, the decrease in TAS level despite the increase in TOS and OSI values obtained from serum (Table 1) and brain (Table 2) tissues indicates that lithium has an increasing effect on oxidation. Additionally, it was observed that these antioxidants increased in the Met and Li+Met groups, while oxidants decreased (Table 2). These results show that the oxidative processes caused by lithium are inhibited by the administration of metformin.

ADAM17 and ADAM10 have a structurally similar molecular sequence (Dusterhoft, S 2015). ADAM17 is a type-1 transmembrane protein involved in regenerative, immunological responses associated with uncontrolled inflammation [22]. Our study observed that the levels of ADAM10 and ADAM17 decreased besides the increase in oxidants in the group given lithium. A decrease of ADAM10 and ADAM17 proteins, which play a role in surface tension in intercellular contact, impairs tissue integrity due to intercellular bonding. On the other hand, when metformin was given alone or with lithium, it was observed that it increased ADAM10 and ADAM17 levels, thus increasing tissue integrity.

Furthermore, as it increased TAS levels, it slowed down the oxidative process in the environment. Therefore, tissue damage caused by lithium, which led to an increase in oxidant levels and a decrease in ADAM10 and ADAM17 levels, was reduced by the increasing effect of metformin on TAS and ADAM10 and ADAM17 (Table 2). In addition to these results, when lithium was given, it caused expansion in the pericellular area, karyopyknosis, and vacuolization in the hippocampus (Figure 1) and cortex (Figure 2) regions (Table 3). In our study, the decrease in brain tissue damage by administering metformin with lithium showed that metformin is a neuroprotective molecule.

It has been demonstrated that decreasing Gpx levels increase when metformin is administered to prevent renal cell damage in diabetic nephropathy following streptozotocin toxicity [23], and MDA levels decrease with the administration of metformin in the injury of granular neurons in the cerebellum due to glutamate [13]. Lithium, which causes autophagy by inhibiting inositol monophosphate [5], also induces the apoptotic process by activating the Fas death domain-containing receptor [6]. It is

known that lithium increases the oxidative process by reducing GPx levels in liver and kidney tissues, decreases the activity of the antioxidant enzyme glutathione peroxidase, and increases lipid peroxidation by increasing oxidative stress [24]. Moreover, increased autophagy in ischemic brain injury decreases with the regulating effect of metformin on AMPK [25]. Additionally, it has been shown that the antioxidant activity of metformin is related to its activation of Gpx, and it decreases the neurodegeneration caused by methamphetamine by activating the Akt/GSK3 pathway [26]. In our study, despite the significant decrease in Gpx levels in the groups given lithium, it was observed that Gpx levels increased when metformin was given, and it was observed that the oxidative process triggered by lithium was slowed by metformin.

Conclusion

The narrow treatment dose range and its potential toxic effect even at low doses make lithium difficult to use in the treatment. Analysis of biochemical and histopathological evaluations showed that lithium-induced oxidative processes were slowed by metformin. When metformin is given simultaneously, the reduction of interstitial edema, indicates cellular damage, pyknosis, and vacuolization, supporting these histopathologically. Our study is a pioneering work showing that the toxic effects of lithium decreased when used in combination with metformin. If similar results are presented in future studies, it will be possible to develop preparations containing the combination of lithium and metformin. In this way, patients who have to take lithium treatment may experience fewer side effects.

Conflict of interests

The authors declare that they have no competing interests.

Financial Disclosure

All authors declare no financial support.

Ethical approval

The ethical approval was obtained from the Çanakkale Onsekiz Mart University Animal Studies Local Ethics Committee with the decision/number 2018/05-08 and file registration number 2018/1800064463.

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