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The Role of Glycogen Synthase Kinase-3β in Zinc Mediated Neuroprotective Effect of Metformin in Rats With Glutamate Neurotoxicity

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Abstract

Metformin has been suggested to have protective effects on the central nervous system with an unclarified mechanism. The similarity between the effects of metformin and the inhibition of glycogen synthase kinase (GSK)-3β suggests that metformin may inhibit GSK-3β. In addition, zinc is an important element that inhibits GSK-3β by phosphorylation. In this study, it was investigated whether the effects of metformin on neuroprotection and neuronal survival were mediated by zinc-dependent inhibition of GSK-3β in rats with glutamate-induced neurotoxicity. Forty adult male rats were divided into 5 groups; Control, Glutamate, Metformin + Glutamate, Zinc Deficiency + Glutamate, Zinc Deficiency + Metformin + Glutamate. Zinc deficiency was induced with a zinc-poor pellet. Metformin was given orally for 35 days. D-glutamic acid was administered intraperitonally on the 35th day. On the 38th day, neurodegeneration was examined histopathologically and effects on neuronal protection and survival were evaluated via intracellular S-100β immunohistochemical staining. The findings were examined in relation to non-phosphorylated (active) GSK-3β levels and oxidative stress parameters observed in brain tissue and blood. Neurodegeneration was higher ($p < 0.05$) in rats fed with an inadequate zinc diet. Active GSK-3β levels were increased in groups with neurodegeneration ($p < 0.01$). Decreased neurodegeneration and increased neuronal survival ($p < 0.01$); decreased active GSK-3β ($p < 0.01$) levels and oxidative stress parameters, and increased antioxidant parameters were observed in groups treated with metformin ($p < 0.01$). Metformin had less protective effects in rats fed with an inadequate zinc diet. Metformin may demonstrate its neuroprotective effects and may increase S-100β mediated neuronal survival by a zinc-dependent inhibition of GSK-3β in glutamate neurotoxicity.

1. Introduction

Metformin is an agent in the group of biguanide drugs used in the first-line treatment of prediabetes and Type 2 diabetes. The one of the main effects of metformin is to increase insulin receptor sensitivity [1] and to regulate cellular energy homeostasis by Adenosine monophosphate kinase (AMPK) activation [2]. Due to positive effects of metformin on intracellular energy metabolism, it is suggested that it also has protective effects on the central nervous system (CNS) such as reducing the occurrence of post-ischemic damage [3], providing a neuroprotective effect during ischemia [4], accelerating the recovery process after ischemic injury [5]. The basic mechanisms of these effects have not been clarified yet.

One of the intracellular mechanisms of action of insulin is that it leads to phosphorylation of insulin receptor substrate-1 (IRS-1) after insulin receptor interaction. Phosphorylated IRS-1 (p-IRS-1) activates the phosphoinositide 3-kinase (PI3K)/Akt/mTOR pathway and causes cell proliferation with the inactivation of glycogen synthase kinase-3 beta (GSK-3β) by phosphorylating it over the serine 9 residue [6, 7]. On the other hand, activation of GSK-3β stops cell proliferation by many mechanisms, creates insulin resistance, and initiates apoptosis with the triggering oxidative stress and activation of many pro-apoptotic genes, especially p53. The balance mechanisms between GSK-3β activation and inhibition are very important mechanisms for cell survival [8]. Recent studies have also shown that GSK-3β activity contributes to the pathogenesis of ischemic neuronal death [9], and GSK-3β inhibition is protective against excitotoxicity

[10]. Due to the dominant role of GSK-3β in neuronal apoptosis, it has been stated that modulation of GSK-3β activity may be an important strategy to prevent ischemic neurodegeneration [9].

Similar effects of GSK-3β inhibition and metformin on the CNS suggest that metformin may exert its neuroprotective effects by inhibiting GSK-3β. However, there are no adequate studies investigating the relationship between metformin and GSK-3β in the CNS.

Zinc is a vital ion that undertakes many important functions in the body when it is at physiological levels such as reducing insulin resistance by activating IRS-1 [11], ensuring neuronal survival [12], and protection from possible toxic effects of glutamate by creating voltage-dependent and voltage-independent inhibition on N-methyl-D-aspartate (NMDA) receptors in the brain [13]. Similarly, it has been suggested that serum zinc levels are decreased in Alzheimer's disease compared to control groups [14]. On the other hand, the studies have shown that the increase in zinc ions in the cell inhibits GSK-3β [15].

S-100 Calcium Binding Protein, also known as S-100β, is a member of the S-100 protein family and especially found in the cytoplasm and nucleus of astrocytes and neurons [16]. While S-100β acts as a neurotrophic factor in the developing CNS, it plays an important role in the glial and neuronal survival of the CNS in adult cells [17]. It has been reported that the mechanisms responsible for neuronal survival include activating the PI3K/Akt/p21 pathway, and reducing oxidative stress-related apoptosis by inhibiting GSK-3β. [18, 19]. The relationship between the roles of S-100β and GSK-3β on neuronal survival are simply summarized in Fig. 1.

In this study, it was investigated whether the effects of metformin on neuroprotection and neuronal survival were mediated by zinc-dependent inhibition of GSK-3β in rats with glutamate neurotoxicity. Neuronal survival was evaluated via intracellular S-100β levels which is related to GSK-3β activation.

2. Materials And Methods

2.1 Study Design

40 male, healthy, non-genetically modified, non-immunocomprimised, 6–8 weeks old rats of *Sprague Dawley* breed (mean weight 300 ± 50 g) obtained from Istanbul University Aziz Sancar Experimental Medicine Research Institute were included in the study. The use of female rats was avoided in our study to rule out hormonal fluactations due to menstrual cycles. During the experiment, each animal was housed in seperate cages with labeled stating the experimental unit. Feeding and living conditions were provided at a room temperature of 20-22^{o}C, in an environment containing 55–60% humidity, and with standard and low-zinc pellet feed in a 12-hour daylight/12-hour dark cycle. Zinc content of standard pellet is 85mg/kg, nutritional content is 20% protein, 6% cellulose, 2% fat, metabolizable energy value is 2600 kcal/kg. Low-zinc feed is specially produced (MBD Yem Ticareti, Gebze, Turkey) adhering to the "Altromin C 1040 Zinc deficiency diet" (Altromin International, Lage, Germany) formula. Its zinc content is 4mg/kg

and other contents are the same as a standard pellet. The experiment groups were created by block randomization of rats and summarized as follows:

I. Control (C) (n = 8)

Rats were fed with standard pellets ad-lib and given a single daily dose of 3 ml saline by gavage for 35 days. On the 35th day, 2.5 ml saline was injected intraperitoneally.

II. Glutamate group (G) (n = 8)

Rats were fed with standard pellets ad-lib and given a single daily dose of 3 ml saline by gavage for 35 days. On the 35th day, 4mg/kg D-glutamic acid was dissolved in 2.5 ml saline and injected intraperitoneally.

III. Metformin + Glutamate group (MG) (n = 8)

Rats were fed with standard pellets ad-lib and given a single dose of 100 mg/kg metformin 3 ml daily by gavage for 35 days. On the 35th day, 4 mg/kg D-glutamic acid was dissolved in 2.5 ml saline and injected intraperitoneally.

IV. Zinc deficiency + Glutamate group (ZG) (n = 8)

To induce zinc deficiency, rats were fed with a zinc-poor pellet (containing 5mg/kg of zinc) ad-lib and given a single daily dose of 3 ml saline by gavage for 35 days. On the 35th day, 4mg/kg D-glutamic acid was dissolved in 2.5 ml saline and injected intraperitoneally.

V. Zinc deficiency + Metformin + Glutamate group (ZMG) (n = 8)

To induce zinc deficiency, rats were fed with a zinc-poor pellet (containing 5mg/kg of zinc) ad-lib and given a single daily dose of 100 mg/kg metformin dissolved in 3 ml saline by gavage for 35 days. On the 35th day, 4 mg/kg D-glutamic acid was dissolved in 2.5 ml saline and injected intraperitoneally.

2.2 Gavage

The rats were gavaged with an 18-gauge metal feeding probe between 9:00 am and 10:00 am every day for 35 days. Metformin at a dose of 100 mg/kg was given to the MG and ZMG groups by gavage in 3 ml volume. The dose selection of administered metformin was made considering the pharmacokinetics and oral bioavailability of metformin in rats. In studies, after calculating the intestinal and hepatic first-pass effects of metformin in rats, its oral bioavailability is stated to be 29.9% [20]. Considering this situation, the 100 mg/kg dose we planned to give in our study was close to 2550 mg/day, which is considered the upper limit of the maximum daily dose specified by the Food and Drug Administration (FDA) in humans. When determining the 35-day application period, studies showing that the neuroprotective effect of metformin occurs between 21–35 days for the shortest duration were taken into consideration [21, 22].

To rule out the effects of stress caused by gavage, rats in other groups (C, G, ZG) were given 3 ml saline by gavage for 35 days.

2.3 Intraperitoneal Injection

The rats were injected intraperitoneally in the lower abdominal outer quadrant on the 35th day. Rats in G, MG, ZG, and ZMG groups were injected intraperitoneally after 4 mg/kg D-glutamic acid (powder) was dissolved in 2.5 ml isotonic saline. The reason for using D-glutamic acid to create glutamate-induced neurotoxicity was that D-glutamic acid could not deaminate in rats and could pass through the blood-brain barrier [23]. While determining the intraperitoneal administration dose of D-glutamic acid, it was taken into consideration that the dose in question in the literature is the dose that causes convulsions but is considered to have a minimum mortality risk [23, 24].

In order that the stress caused by intraperitoneal injection on rats did not affect the results of our study, 2.5 ml saline was given to the control group by intraperitoneal injection.

2.4 Collection of Blood and Tissue Samples

On the 38th day, 2 days after D-glutamic acid administration [25] an anesthetic combination of ketamine/xylazine (50mg/kg / 10mg/kg) was administered to the rats intraperitoneally and sacrification was performed due to hypovolemia caused by intracardiac blood collection. Blood samples were centrifuged at + 4 ºC for 10 minutes and serum was obtained. The frontal cortices and hippocampi, where the glutamate neurotoxicity is seen intensely can be seen [26], were removed bilaterally from post-mortem rats by paying attention to the cold chain, weighed, taken into tubes and stored at -80 ^{o}C. Right frontal cortex and hippocampus samples were marked numerically for blinding to reduce the observer bias and transferred for immunohystochemical and hystopathological examinations. Left frontal cortex and hippocampus samples were turned into 20% tissue homogenates with the help of a glass homogenizer in ice molds using phosphate buffer (pH 7.4). Tissue homogenates were taken into previously labeled tubes and centrifuged at 5000 g at + 40 ^{o}C for 5 minutes. The resulting supernatants were divided into tubes, marked numerically for blinding to reduce the observer bias and stored in a deep freezer at -80 ^{o}C until the study.

2.5 Measurement of Non-phosphorylated (ser9) GSK-3β Levels by ELISA Method

The non-phosphorylated (ser9) GSK-3β ELISA kit was obtained from Aviva Systems Biology (MA, USA). The non-phosphorylated (ser9) GSK-3β ELISA kit works on the basis of the enzyme immunoassay method using biotinylated non-phosphorylated (ser9) GSK-3β antibody and Avidin-HRP conjugate. The analyzed supernatant was added to the anti- non-phosphorylated-GSK-3β coated plates and incubated for 2 hours at 37^{o}C. After the incubation period, the wells were emptied and a biotinylated non-phosphorylated (ser9) GSK3-Beta detector antibody was added. It was washed 3 times after incubation at 37^{o}C for 1 hour. Then the wells were emptied and incubated with Avidin-HRP conjugate for 1 hour at

37^{O}C. After incubation, the wells were washed 5 times. Afterward, 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was added to the wells and incubated for another 15 minutes. The enzyme-substrate reaction produced a blue-colored end-product. Finally, the blue-colored product turned yellow with the stop solution added to stop the reaction. The optical density of the colored product was measured spectrophotometrically at 540 nm in an optical reader. The non-phosphorylated (ser9) GSK-3β concentration of each sample was calculated from the standard curve.

2.6 Measurement of Zinc Levels

Zinc levels in acid-dissolved frontal cortex and hippocampus tissues and serum were measured in Thermo iCAP 600 series Inductively Coupled Plasma-Optical Emission Spectrophotometer (ICP-OES) (Thermo Fisher, MA, USA) at 206-nanometer wavelength.

2.7 Pathological Examinations

2.7.1 Histopathology

After the brain tissues were fixed in 20 ml of 10% formalin for 24 hours, macroscopic sections were taken to include the cortical and subcortical tissues of the frontal and temporal lobes. Paraffin blocking was then performed. Nissl staining was applied to each block by taking 4 μm sections. The covering solution was dripped onto the stained slides and covered with a coverslip. In the evaluation of Nissl staining, the rate of degenerated cells was determined in three fields in cortical and subcortical areas using x40 and x100 objectives using Image Pro Plus 7 software (Media Cybernetics, MD, USA).

2.7.2 Immunohistochemistry

Sections of 4 μm obtained from paraffin blocks were taken on slides previously coated with poly-L-Lysin. The sections were kept in an oven at 56°C overnight. The EP32 clone of the S-100β antibody to be used for immunohistochemical staining was obtained from Cell Marque (CA, USA) and the secondary Bond Polymer Refine 3,3'-Diaminobenzidine (DAB) kit was obtained from Leica (Newcastle, United Kingdom). Staining was completed by incubating S-100β immunohistochemical stain at 1:400 dilution in Leica Bond Max IHC Stainer (Leica, Newcastle, United Kingdom) for 32 minutes.

An Olympus BX51 light microscope (Tokyo, Japan) was used for the examination.

Nuclear staining at any level was considered immunoreactive in the immunohistochemical evaluation of S-100β. Cells with immunoreactivity in three areas in the cortical and subcortical areas were determined using x40 and x100 objectives. Then, using the x400 objective, cells with positive nuclear reaction in a total of 100 cells were detected and the number of positively stained cells in 100 cells was determined.

2.8 Biochemical Colorimetric Analytical Methods

2.8.1 Advanced oxidation protein products

Analysis of advanced oxidation protein products (AOPP) levels was performed by making modifications to the spectrophotometric method of Hanasand et al. [27].

2.8.2 Malondialdehyde

Malondialdehyde (MDA) reacts with thiobarbituric acid (TBA) to form a colored product with maximum absorbance at 535 nm. The efficiency of lipid peroxidation was studied with the colorimetric method of Buege and Aust [28].

2.8.3 Lipid hydroperoxides

The Lipid hydroperoxide levels was studied with the colorimetric method of Jiang et al. [29]. In the presence of lipid hydroperoxides (L-OOH), ferrous (Fe^{+2}) ions in an acidic medium are oxidized to ferric (Fe^{+3}) ions. The ferric ions formed form a one-to-one, colored, soluble complex (blue-violet) with ferric xylenol orange 2 (FOX2) The absorbance of the supernatant fraction was read at 560 nm against a reagent blank. $\varepsilon = 4.46 \pm 0.23 \times 104\ M^{-1}\ cm^{-1}$ value was used as the molar extinction coefficient in the calculations.

2.8.4 Analysis of protein-bound advanced glycation end-products

Analysis of protein-bound advanced glycation end-products (AGEs) was performed according to the spectrofluorimetric method. To prevent fluorescence interference, the samples were pretreated according to the method of Yanar et al. [30]. In the next step, protein-containing pellets were dissolved in 6 M guanidine-HCl and fluorescence values were read [30]. The fluorescence intensities of the samples at 350 nm excitation and maximum 440 nm emission were recorded. Fluorescence intensity was expressed as FU/mg protein.

2.8.5 Cu-Zn superoxide dismutase activity

Cu-Zn superoxide dismutase (SOD) (EC 1.15.1.1) activity in the samples was determined by the method of Sun and Oberley [31]. The absorbance value was read at 560 nm against a reagent blank. One unit of Cu-Zn SOD is defined as the enzyme activity that dismutes the superoxide radical anion by 50%.

2.8.6 Catalase activity

Catalase (CAT) activity (E.C.1.11.1.6) was determined according to the Aebi method [32]. The degradation rate of H_2O_2 was observed kinetically at 240 nm. 750 µL of PBS containing 30 mM H_2O_2 was added to 10 µL of the sample, and the change in absorbance was recorded for 1 minute. The molar absorptivity value of 43.6 $M^{-1}cm^{-1}$ was used to calculate the activity. One unit of catalase is defined as 1 µmol H_2O_2 broken down per minute, CAT activity expressed as U/mg protein.

2.9 Evaluation of total protein amount

Measurement of protein levels in tissue samples was performed fluorometrically with a Qubit analyzer (Invitrogen, Carlsbad, CA). The absorbance of the purple color formed in the samples was read at 550 nm. The protein concentration of the samples was calculated through the standard curve formed with albumin solutions at different concentrations (2.5–80 mg/ml) prepared by serial dilution.

2.10 Statistical Analysis

Statistical analysis was performed using the Statistical Package of Social Sciences (SPSS) program (SPSS Inc.: IL, USA, version 12.0). According to the results of the power analysis (mean 10 units difference and 5 units standard deviation at 80% power and 95% confidence level), it was determined that a minimum of 8 rats were required for each group. Kolmogrov Smirnov test was used for the normality distribution of the data. Parametric or non-parametric tests were selected according to the number of groups, normality distribution and homogeneity (M:50, SD:10 at CI:%95). Non-parametric tests were used because our data did not fit the normality distribution. Pairwise comparisons between groups were made using the Mann Whitney-U test. The analysis of numerical data for which standard deviation could not be calculated was performed using a One-Sample t-test. The p 0.05 value was considered significant for all statistical analyses. The data of our study were expressed as mean ± standard deviation.

3. Results

3.1 Zinc Levels

Serum zinc levels were found to be significantly lower in the ZM and ZMG groups compared to the other groups ($p < 0.05$). Similarly, tissue zinc levels were found to be significantly lower in the ZM and ZMG groups compared to the other groups ($p < 0.05$) (Table 1*).*

Table 1
Serum and tissue Zinc levels

	C *(n = 8)*	G *(n = 8)*	MG *(n = 8)*	ZG *(n = 8)*	ZMG *(n = 8)*
Serum Zinc (ppm)	0,999 ± 0,078	1,01 ± 0,089	1,005 ± 0,083	0,827 ± 0,076^{a,b,c}	0,821 ± 0,085^{a,b,c}
Tissue Zinc (ppm)	15,74 ± 0,52	15,332 ± 1,02	15,633 ± 0,72	12,52 ± 0,96 ^{a,b,c}	12,289 ± 0,58^{a,b,c}
Values are expressed as means ± SD. The letters indicate significant difference ($p < 0.05$): ^{a}C; ^{b}G; ^{c}MG. (%95 CI)					

Abbreveiations: C, Control; G, Glutamate, MG, Metformin + Glutamate; ZG, Zinc deficiency + Glutamate; ZMG, Zinc deficiency + Metformin + Glutamate, ppm; parts per million.

3.2 Active GSK-3β Levels

Non-phosphorylated (Ser9) GSK-3β (the so-called “active” form) levels in serum were found to be significantly higher in group G than in group C ($p < 0.01$). It was found to be significantly lower in the MG group compared to the G group ($p < 0.01$). It was found to be significantly higher in the ZG group compared to the G group ($p < 0.01$). It was found to be significantly lower in the ZMG group compared to the ZG group ($p < 0.01$). It was found to be significantly lower in the MG group than in the ZMG group ($p < 0.05$) (Table 2).

Active GSK-3β levels in tissue homogenates were found to be significantly higher in group G than in group C ($p < 0.01$). It was found to be significantly lower in the MG group compared to the G group ($p < 0.01$). It was found to be significantly higher in the ZG group compared to the G group ($p < 0.01$). It was found to be significantly lower in the ZMG group compared to the ZG group ($p < 0.01$). It was found to be significantly lower in the MG group than in the ZMG group ($p < 0.05$) (Table 2).

Table 2
Serum and tissue active GSK-3-Beta levels

	C (n = 8)	G (n = 8)	MG (n = 8)	ZG (n = 8)	ZMG (n = 8)
Serum active GSK 3-β (pg/mL)	2060,916 ± 0,11	7049,855 ± 0,14[a]	3233,578 ± 0,34[b]	10749,355 ± 0,15[b]	4249,855 ± 0,032[c,d]
Tissue active GSK 3-β (pg/mL)	3556,916 ± 0,06	8329,855 ± 0,06[a]	4253,578 ± 0,21[b]	12827,355 ± 0,01[b]	5368,425 ± 0,23[c,d]

Values are expressed as means ± SD. The letters indicate significant difference ($p < 0.01$): [a]C; [b]G; [c]MG, ($p < 0.05$): [d]ZMG. (%95 CI)

Abbreveiations: C, Control; G, Glutamate, MG, Metformin + Glutamate; ZG, Zinc deficiency + Glutamate; ZMG, Zinc deficiency + Metformin + Glutamate; GSK 3-β, Glukogen synthase kinase 3-β

3.3. S-100β Staining Rates

Cortical intracellular S-100β staining rates were significantly lower in the C group than in the G group ($p < 0.05$). It was found to be significantly lower in the G group than in the MG group ($p < 0.01$). It was found to be significantly lower in the ZG group than in the G group ($p < 0.05$). It was found to be significantly lower in the ZG group than in the ZMG group ($p < 0.05$). In the ZMG group, it was significantly lower than the MG group ($p < 0.001$) (Table 3).

Hippocampal intracellular S-100β staining rates were significantly lower in the G group than in the MG group ($p < 0.01$). It was found to be significantly lower in the ZG group than in the G group ($p < 0.05$). It was found to be significantly lower in the ZG group than in the ZMG group ($p < 0.05$). In the ZMG group, it was found to be significantly lower than the MG group ($p < 0.01$). (Fig. 2, Table 3)

Table 3
Cortical and hippocampal intracellular S-100 Beta staining ratios (%)

	C (n = 8)	G (n = 8)	MG (n = 8)	ZG (n = 8)	ZMG (n = 8)
Cortical	16,45	23,06[a]	33,02[d]	17,01[b]	20,01[f]
Hippocampal	15,03	26,11[a]	39[d]	17,02[b]	26,01[e]

Values are expressed as means. The letters indicate significant difference ($p < 0.05$): [a]C; [b]G; [c]ZG, ($p < 0.01$): [d]G; [e]MG, ($p < 0.001$): [f]MG. (%95 CI)

Abbreveiations: C, Control; G, Glutamate, MG, Metformin + Glutamate; ZG, Zinc deficiency + Glutamate; ZMG, Zinc deficiency + Metformin + Glutamate

3.4. Neurodegeneration rates

Cortical degenerated neuron rates seen in Nissl staining were found to be significantly higher in the G group than in the C and MG group ($p < 0.01$). No statistically significant difference was found between C and MG group ($p > 0.05$). It was found to be significantly higher in the ZG group than in the G group ($p < 0.05$). It was found to be significantly higher in the ZG group than in the ZMG group ($p < 0.05$). It was found to be significantly higher in the ZMG group than in the MG group ($p < 0.01$) (Table 4).

Hippocampal degenerated neuron rates seen in Nissl staining were found to be significantly higher in the G group than in the C and MG group ($p < 0.01$). No statistically significant difference was found between C and MG group ($p > 0.05$). It was found to be significantly higher in the ZG group than in the G group ($p < 0.05$). It was found to be significantly higher in the ZG group than in the ZMG group ($p < 0.05$). It was found to be significantly higher in the ZMG group than in the MG group ($p < 0.01$) (Table 4).

Table 4
Cortical and hippocampal neurodegeneration ratios (%)

	C (n = 8)	G (n = 8)	MG (n = 8)	ZG (n = 8)	ZMG (n = 8)
Cortical	7,04	24,1[f]	8,07[g]	32,01[a]	22,03[b,e]
Hippocampal	6,03	13,2[c]	6,5[d]	21,75[a]	16,01[b,e]

Values are expressed as means. The letters indicate significant difference ($p < 0.05$): [a]G; [b]ZG; [c]C, ($p < 0.01$): [d]G; [e]MG, ($p < 0.001$): [f]C; [g]G. (%95 CI)

Abbreveiations: C, Control; G, Glutamate, MG, Metformin + Glutamate; ZG, Zinc deficiency + Glutamate; ZMG, Zinc deficiency + Metformin + Glutamate

3.5 Biochemical Colorimetric Analytical Methods

3.5.1 Advanced oxidation protein products

AOPP levels measured in serum were found to be significantly higher in group G than in group C ($p < 0.05$). It was found to be significantly lower in the MG group compared to the G group ($p < 0.05$). It was found to be significantly lower in the ZMG group than in the ZG group ($p < 0.05$). It was found to be significantly higher in the ZG group than in the G group ($p < 0.05$). It was found to be significantly higher in the ZMG group compared to the MG group ($p < 0.01$) (Table 5).

The AOPP levels measured in the tissue were found to be significantly higher in the G group than in the C group ($p < 0.01$). It was found to be significantly lower in the MG group compared to the G group ($p < 0.01$). It was found to be significantly lower in the ZMG group compared to the ZG group ($p < 0.01$). It was found to be significantly higher in the ZG group compared to the G group ($p < 0.01$). It was found to be significantly higher in the ZMG group compared to the MG group ($p < 0.01$) (Table 5).

Table 5
Biochemical marker levels

	C *(n = 8)*	G *(n = 8)*	MG *(n = 8)*	ZG *(n = 8)*	ZMG *(n = 8)*
Advanced oxidation protein products (µmol/mg protein)					
Serum AOPP	43,1425 ± 0,23	64,92625 ± 0,37[a]	48,09625 ± 0,53[b]	61,09125 ± 0,789[b]	42,846 ± 1,82[c,d]
Tissue AOPP	23,35 ± 1,28	36,74 ± 1,36[e]	30,428 ± 1,19[f]	37,669 ± 0,96[f]	31,164 ± 1,13[g,d]
Lipid peroxidation markers (µmol/mg protein)					
Malondialdehyde					
Serum MDA	4,31125 ± 0,56	6,34625 ± 0,57[a]	5,02375 ± 0,93[f]	6,66375 ± 0,82	5,2929 ± 0,76[c,d]
Tissue MDA	15,3725 ± 2,36	18,62875 ± 1,63[a]	14,495 ± 1,34[f]	18,1 ± 0,98	16,037143 ± 0,99[d]
Lipid Hydroperoxides					
Serum L-OOH	0,97625 ± 0,08	1,135 ± 0,06[a]	1,03 ± 0,18[f]	1,21875 ± 0,007	1,1243 ± 0,06[c,d]
Tissue L-OOH	1,7225 ± 0,53	2,395 ± 0,58[e]	1,67 ± 0,98[f]	2,53375 ± 0,32	1,9643 ± 0,24[d]
Protein bound advanced glycation end products (µmol/mg protein)					
Serum AGE	163,25 ± 0,35	247,125 ± 0,38[e]	165,625 ± 1,02[b]	235,5 ± 0,77	147 ± 1,34[g,d]
Tissue AGE	77,25 ± 0,68	112,25 ± 0,87	81,5 ± 0,59	109,25 ± 0,72	94 ± 0,73
Enzymatic redox homeostasis biomarkers (µmol/min/mg protein)					
Catalase					
Serum CAT (U/mg)	13,734 ± 1,02	9,02375 ± 0,98[a]	13,5425 ± 0,83[b]	9,64 ± 1,07	11,28286 ± 0,953[d]
Cu-Zn Superoxide Dismutase					

Values are expressed as means ± SD. The letters indicate significant difference ($p < 0.05$): [a]C; [b]G; [c]ZG, ($p < 0.01$): [d]MG; [e]C, [f]G. (%95 CI)

Abbreveiations: C, Control; G, Glutamate, MG, Metformin + Glutamate; ZG, Zinc deficiency + Glutamate; ZMG, Zinc deficiency + Metformin + Glutamate; GSK-3β, Glukogen synthase kinase-3β

	C *(n = 8)*	G *(n = 8)*	MG *(n = 8)*	ZG *(n = 8)*	ZMG *(n = 8)*
Advanced oxidation protein products (µmol/mg protein)					
Serum Cu-Zn SOD	11,56 ± 0,94	9,34125 ± 0,85[a]	12,8975 ± 0,83[f]	9,37 ± 0,91	11,2029 ± 1,03[d]
Tissue Cu-Zn SOD	10,735 ± 0,23	10,5613 ± 0,462	10,5275 ± 0,583	10,4525 ± 0,32	10,5228 ± 0,45
Values are expressed as means ± SD. The letters indicate significant difference ($p < 0.05$): [a]C; [b]G; [c]ZG, ($p < 0.01$): [d]MG; [e]C, [f]G. (%95 CI)					
Abbreveiations: C, Control; G, Glutamate, MG, Metformin + Glutamate; ZG, Zinc deficiency + Glutamate; ZMG, Zinc deficiency + Metformin + Glutamate; GSK-3β, Glukogen synthase kinase-3β					

3.5.2 Malondialdehyde

MDA levels measured in serum were found to be significantly higher in group G than in group C ($p < 0.05$). It was found to be significantly lower in the MG group compared to the G group ($p < 0.01$). It was found to be significantly lower in the ZMG group than in the ZG group ($p < 0.05$). There was no significant difference in the ZG group compared to the G group ($p > 0.05$). It was found to be significantly higher in the ZMG group compared to the MG group ($p < 0.01$) (Table 5).

MDA levels measured in tissue were found to be significantly higher in group G than in group C ($p < 0.05$). It was found to be significantly lower in the MG group compared to the G group ($p < 0.01$). No significant difference was found in the ZMG group compared to the ZG group ($p > 0.05$). There was no significant difference in the ZG group compared to the G group ($p > 0.05$). It was found to be significantly higher in the ZMG group compared to the MG group ($p < 0.01$) (Table 5)

3.5.3 Lipid hydroperoxide

L-OOH levels measured in serum were found to be significantly higher in group G than in group C ($p < 0.05$). It was found to be significantly lower in the MG group compared to the G group ($p < 0.01$). It was found to be significantly lower in the ZMG group than in the ZG group ($p < 0.05$). There was no significant difference in the ZG group compared to the G group ($p > 0.05$). It was found to be significantly higher in the ZMG group compared to the MG group ($p < 0.01$) (Table 5).

L-OOH levels measured in tissue were found to be significantly higher in group G than in group C ($p < 0.01$). It was found to be significantly lower in the MG group compared to the G group ($p < 0.01$). No significant difference was found in the ZMG group compared to the ZG group ($p > 0.05$). There was no significant difference in the ZG group compared to the G group ($p > 0.05$). It was found to be significantly higher in the ZMG group compared to the MG group ($p < 0.01$) (Table 5).

3.5.4 Protein-bound advanced glycation end products

AGE levels measured in serum were found to be significantly higher in group G than in group C ($p < 0.01$). It was found to be significantly lower in the MG group compared to the G group ($p < 0.05$). It was found to be significantly lower in the ZMG group compared to the ZG group ($p < 0.01$). There was no significant difference in the ZG group compared to the G group ($p > 0.05$). It was found to be significantly higher in the ZMG group compared to the MG group ($p < 0.01$) (Table 5).

There was no significant difference between the groups in the AGE levels measured in the tissue (Table 5).

3.5.5 Cu-Zn superoxide dismutase activity

Cu-Zn-SOD activity measured in serum was found to be significantly lower in group G than in group C ($p < 0.05$). It was found to be significantly higher in the MG group compared to the G group ($p < 0.01$). No significant difference was found in the ZMG group compared to the ZG group ($p > 0.05$). There was no significant difference in the ZG group compared to the G group ($p > 0.05$). It was found to be significantly higher in the ZMG group compared to the MG group ($p < 0.01$) (Table 5).

There was no significant difference between the groups in the levels of Cu-Zn-SOD activity measured in the tissue (Table 5).

3.5.6 Catalase activity

Catalase activity measured in serum was found to be significantly lower in group G than in group C ($p < 0.05$). It was found to be significantly higher in the MG group than in the G group ($p < 0.05$). There was no significant difference in the ZMG group compared to the ZG group ($p > 0.05$). There was no significant difference in the ZG group compared to the G group ($p > 0.05$). It was found to be significantly higher in the ZMG group than in the MG group ($p < 0.01$) (Table 5).

Measurement of catalase activity in tissue samples could not be performed due to technical problems.

4. Discussion

Our research shows that metformin has a protective role in neuronal damage caused by glutamate. It is suggested that this neuroprotective effect is mediated by a zinc-dependent inhibition of GSK-3β.

Our histopathological findings indicate that significant damage occurred in the cortical and hippocampal regions of rats administered D-glutamic acid. It was concluded that the neuronal degeneration caused by D-glutamic acid was even more prominent in rats with zinc deficiency (Table 4) and this is parallel with the studies [14, 33, 34]. Some studies have reported that zinc inhibits NMDA receptors [35, 36] and glutamate release [37, 38], by interacting with the zinc-binding site on the post-synaptic and pre-synaptic receptors respectively. Thus, the disappearance of inhibition on NMDA receptors and the increase in glutamate secretion from the presynaptic neuron in zinc deficiency may be responsible for the increased damage in the cortical and hippocampal regions of the rats fed with a zinc-deficient diet. Also, Ilouz *et al.*

have shown that the increase in zinc in the cell inhibits GSK-3β [15]. The occurrence of similar events in zinc deficiency and GSK-3β activation may be related to increased GSK-3β activity in zinc deficiency.

In our study, it was determined that there were significant increases in active GSK-3β levels in both blood and brain tissue of rats in groups with D-glutamic acid induced neurodegeneration (G and ZG). Similarly, Hanumanthappa *et al.* suggest that GSK-3β plays a role in brain damage with increased glutamate secretion as a result of ischemia [9].

Our research suggests that metformin has a neuroprotective effect by inhibiting GSK-3β activity in neurodegeneration caused by glutamate increase. Our histopathological findings reveal that glutamate-induced neurodegeneration (Table 4) and active GSK-3β levels were significantly reduced (Table 2) in metformin-treated groups compared to the non-treated groups. Similarly, Leng *et al.* showed that inhibition of GSK-3β is protective against cerebral ischemia and prevents glutamate-induced neurodegeneration [39, 40]. In another study, Cross *et al.* showed that inhibition of GSK-3β weakened apoptotic signals and prevented neuronal death [41].

In addition, intracellular S-100β staining rates, which is an indicator of neuronal survival, were found to be higher in metformin-treated groups (MG, ZMG) than in non-treated groups (G, ZG). (Table 3). Our findings also reveal that these protective and survival effects of metformin on neurons are less in the groups fed with a zinc-deficient diet than in the groups fed with a normal diet (Table 3). These findings suggest that metformin inhibits GSK-3β not only through AMPK-Akt pathways but also by using zinc-dependent pathways. Similarly, Miranda *et al.* suggested that IRS-1 phosphorylation, which causes Akt activation, may occur not only through AMPK but also directly through intracellular zinc [42]. Metformin may cause zinc to contribute additionally to IRS-1 phosphorylation at this stage. Therefore, the decrease in intracellular zinc levels in rats fed a deficient zinc diet may be responsible for the lower protective and survival effects of metformin. On the other hand, S6K, a zinc-dependent protein kinase, has been reported to inhibit GSK-3β in some studies, while to cause activation in some studies [43, 44]. It has been suggested that the effect of S6K on GSK-3β may differ between tissues [45]. We think that metformin can create GSK-3β inhibition by directly increasing S6K expression in the CNS. Further studies are needed to investigate the effect of metformin on the expression or activation of S6K and its relationship with GSK-3β.

In our study, the increase in blood AGE levels in D-glutamic acid administrated rats is remarkable. Huang *et al.* stated that increased glutamate may impair the function of β-cells and accelerates their apoptosis by over-activation of NMDA receptors [46]. Also, Shen *et al.* suggested that inhibition of GSK-3β induces human pancreatic β-cell proliferation [47]. In line with this information, it is suggested that over-stimulation of NMDA receptors in pancreatic beta cells may be responsible for increased blood AGE levels by increasing active GSK-3β levels in D-glutamic acid administrated rats.

AGEs activate reactive oxygen species (ROS) and advanced glycation end-products receptors (AGER) that produce an inflammatory response [48]. This interaction between the AGERs and AGEs causes an increase in the synthesis of the superoxide radical anion. This reduces the activity of catalase and

superoxide dismutase [48]. We think that the decrease in Cu-Zn SOD and catalase activities in the blood of D-glutamic acid administrated rats may have developed due to the increased use of these antioxidants in order to remove the increased ROS in peripheral tissues.

Our findings suggest that the increase in oxidative stress observed in glutamate administrated rats (G, ZG) may be related to the increased active GSK-3β levels (Tables 2 and 5). Similarly, Li *et al.* have reported that GSK-3β increases ROS formation by causing mitochondrial dysfunction in neurons [49]. On the other hand, oxidative stress parameters in blood and tissue were found to be significantly decreased in rats treated with metformin (GM, ZGM) compared to rats not treated (G, ZG). It was determined that there were also significant increases in Cu-Zn SOD and catalase activities in the groups treated with metformin. This suggests that the protective effects of metformin are due to decreased ROS production and the regulation of the balance between pro-oxidants and antioxidants. These findings are parallel to other studies suggesting that metformin has a similar effect in other tissues [50, 51]. Similarly, Zhao *et al*. suggested that metformin treatment provided cognitive protection in mice with pentylenetetrazole (PTZ)-induced epilepsy models and showed antioxidant properties by reducing oxidative stress [52].

Finally, it is remarkable in our findings that metformin administration to rats fed an insufficient zinc diet could not reduce lipid peroxidation caused by glutamate toxicity in brain tissue. This shows us that metformin needs zinc to prevent glutamate-induced lipid peroxidation, especially in brain tissue.

In conclusion, our study is the first to investigate the relationship of metformin with GSK-3β and zinc in the brain. We can say that the increase in glutamate secretion may cause an increase in oxidative stress via GSK-3β. Our findings confirm that metformin can inhibit glutamate-mediated neurodegeneration and increase neuronal survival by reducing the active GSK-3β levels with zinc-dependent pathways. Further studies are needed to investigate which advanced intracellular pathways metformin effects on GSK-3β in brain tissue.

Limitations

We could not perform measurement of catalase activity in tissue samples due to technical problems during storage but considering other results, we think that the results in the tissue samples would be parallel to results obtained from the serum samples.

Declarations

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COMPETING INTERESTS

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

All authors contributed significantly to this research.

Conceived and designed the experiments and wrote the manuscript: AO, GS. Performed the experiments and analyzed the data: AO, KYO, KY, MM, MA, AC, BOK, OFS, UC, HU, GS. The corresponding authors: AO. All authors have been involved in the drafting, critical revision and final approval of the manuscript for publication. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

DATA AVAILABILITY STATEMENT

The raw data supporting this study will be available by authors to any qualified researcher upon request.

ETHICS APPROVAL

Ethical approval was obtained for the project from Istanbul University Animal Experiments Local Ethics Committee with the number 35980450-050.99. Guide for the Care and Use of Laboratory Animals of National Academy of Sciences was followed for caring animals during the experiment.

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Figures

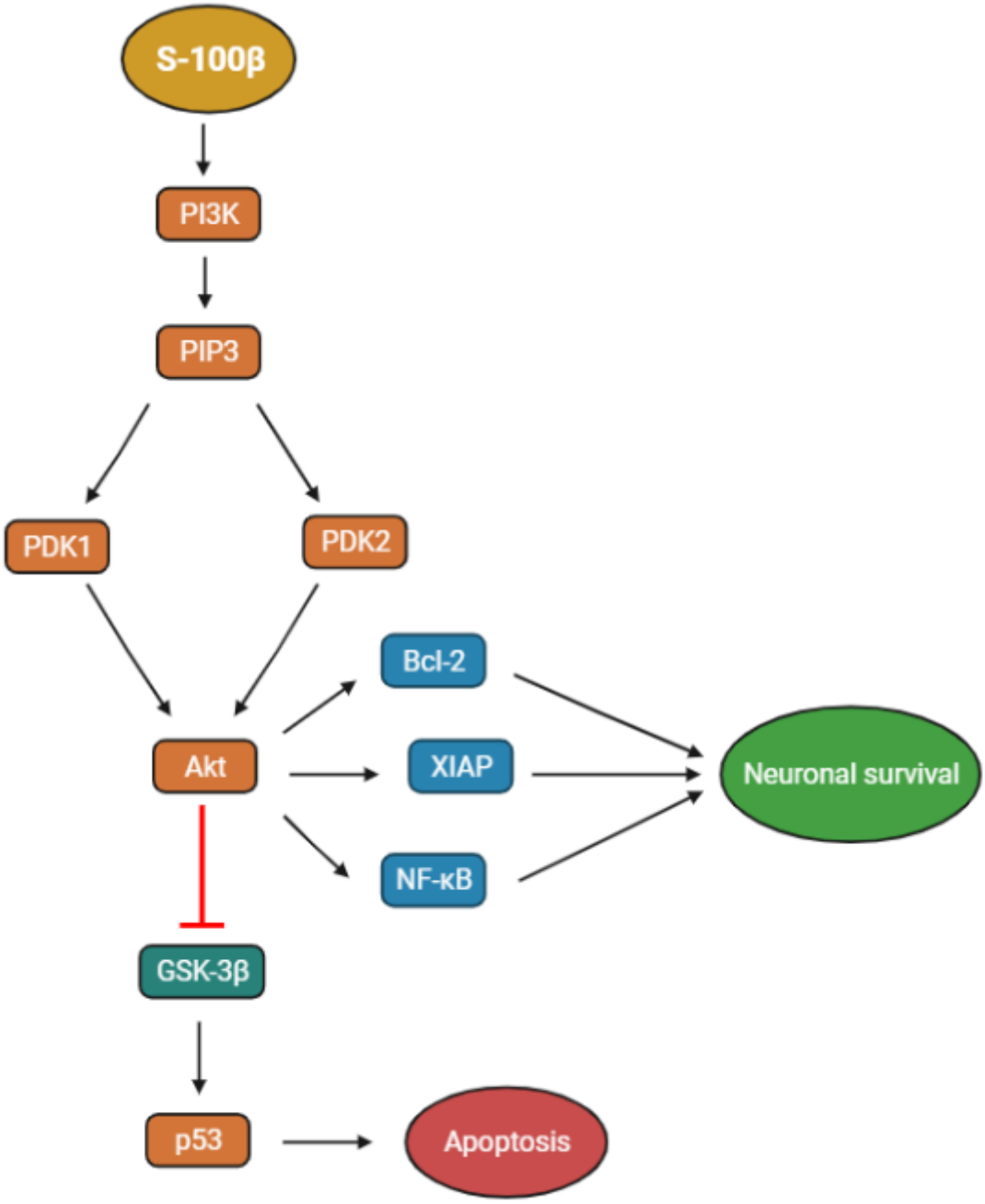

Figure 1

Mechanism of action of intracellular S-100β on neuronal survival (illustrated by Adobe Illustrator 27.2)

Intracellular S-100β activates PI3K/PIP3/Akt pathway and this inhibits stress-related apoptosis by phosphorylation and tetramerization of p53 via inhibiting GSK-3β. PI3K/PIP3/Akt pathwayalso increase neuronal survival by activation of Bcl-2, XIAP, and NF-κB during a cellular stress. Pointed arrows show stimulation, blunt arrow shows inhibition.

Abbreveiations: PI3K; phosphoinositide 3-kinase, PIP3; phosphoinositide 3-kinase, PDK; Phosphoinositide-dependent kinase, XIAP; X-linked inhibitor of apoptosis protein, NF-κB, Nuclear factor kappa B; GSK-3β,

Glycogen synthase kinase-3 beta.

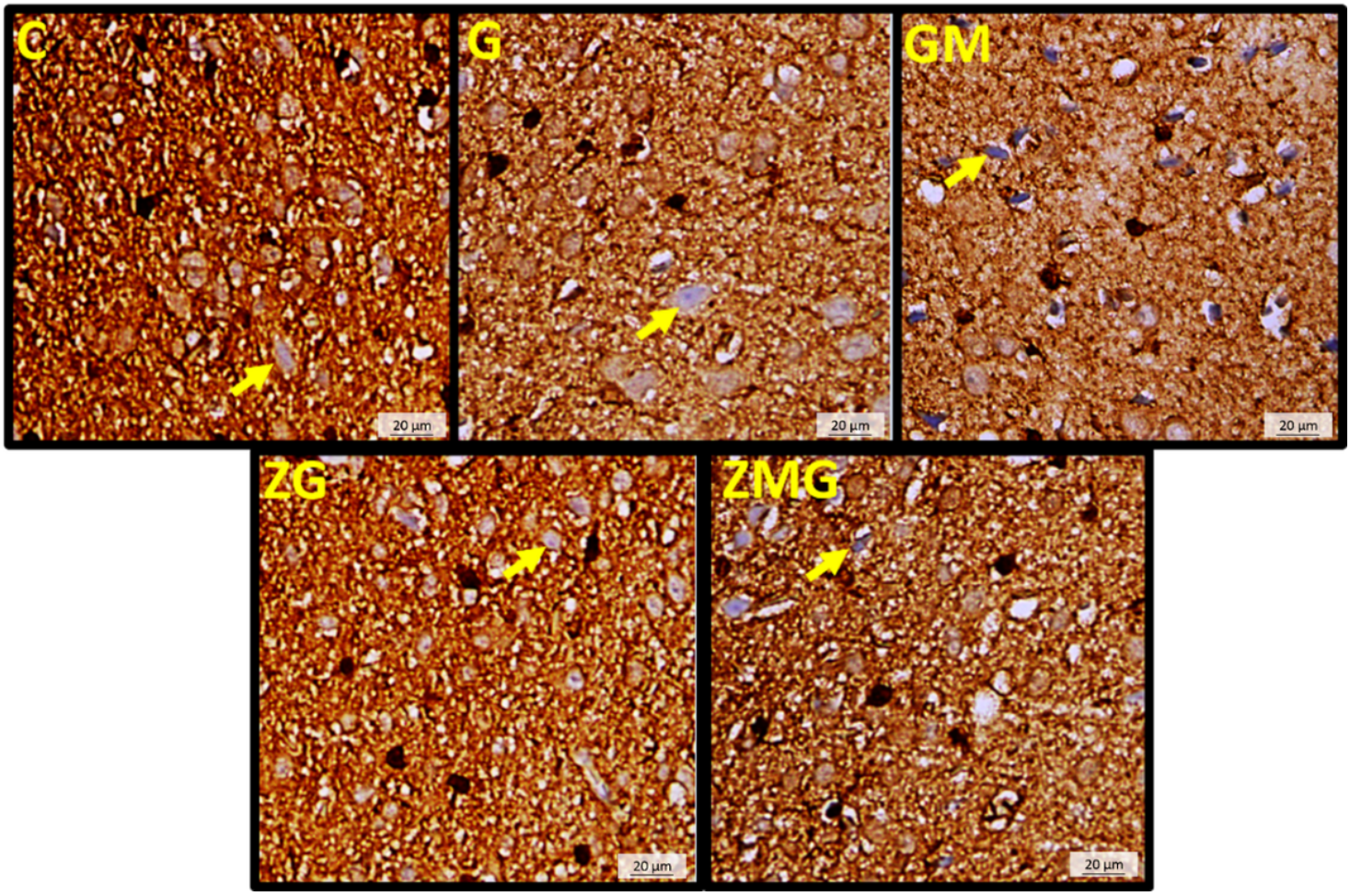

Figure 2

Hippocampal multipolar neuron samples stained immunohistochemically with S-100β

Images taken under 400x magnification and randomly selected from among the groups. Tips of the yellow arrow shows S-100β stained cells.

Abbreveiations: C, Control; G, Glutamate, MG, Metformin + Glutamate; ZG, Zinc deficiency + Glutamate; ZMG, Zinc deficiency + Metformin + Glutamate