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Does Metformin Protect Against Brain Injury After Subarachnoid Hemorrhage? An Experimental Study

Subaraknoid Kanama Sonrası Metformin Erken Beyin Hasarını Önleyebilir mi? Deneysel Çalışma

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ABSTRACT Objective: Hyperglycemia is one of the main risk factors for early brain injury and an outcome in patients with subarachnoid hemorrhage (SAH). Agents that regulate blood glucose levels can prevent the development of early brain injury. The present experimental study aimed to investigate the histological and anti-apoptotic effects of metformin on SAH in a rat model. **Material and Methods:** An SAH model was applied in 28 male Wistar rats who were randomly grouped as follows: control (Group 1), SAH alone (Group 2), SAH with hyperglycemia (Group 3), and metformin-treated SAH with hyperglycemia (Group 4). Intraperitoneal streptozotocin (50 mg/kg) was administered for 7 d to induce hyperglycemia before conducting the experiment. All rats were sacrified 72 hours after SAH. Histopathological and immunohistochemical evaluations were also conducted. **Results:** The thickness of the basilar artery wall was significantly different among the four groups (p=0.003). The thickness of the basilar artery wall was higher in Group 3 than in Groups 1 and 2. Blood glucose values were significantly different among the four groups (p<0.001). The number of neurons (glial cells) stained with terminal deoxynucleotidyl transferase dUTP nick end labeling was lower in Group 4 than in Group 3 (p=0.025). **Conclusion:** The main findings of our study indicate that metformin regulates blood glucose levels in hyperglycemic rats with SAH. Our results suggest that metformin may protect neurons against apoptosis.

Keywords: Early brain injury; hyperglycemia; metformin; subarachnoid hemorrhage

ÖZET Amaç: Subaraknoid kanama (SAK) sonrası gelişen hiperglisemi, erken beyin hasarı ve sağkalım için ana risk faktörlerinden biridir. Kan glukoz seviyesini düzenleyen ajanlar erken beyin hasarını önleyebilirler. Bu deneysel çalışma ratlarda SAK modelinde metforminin histolojik ve anti-apoptotik etkilerini etkilerini ortaya koymayı amaçlamıştır. **Gereç ve Yöntemler:** Toplam 28 adet Wistar albino cinsi rata deneysel SAK modeli uygulanarak rastgele 4 gruba ayrıldı: kontrol (Grup1), sadece SAK uygulananlar (Grup 2), SAK ve hiperglisemi geliştirilenler (Grup 3), SAK ve hiperglisemi uygulanıp metformin verilenler (Grup 4). Deney öncesi ratlarda hiperglisemi intraperitoneal streptozosin (50 mg/kg) ile sağlandı. SAK sonrası 72. saatte ratlar sakrifiye edildi. Histopatolojik ve immünohistokimyasal incelemeler yapıldı. **Bulgular:** Dört grupta baziller arter kalınlıklarının anlamlı farklı olarak saptandı (p=0,003). Grup 3'te baziller arter duvar kalınlıkları grup 1 ve 2'den daha fazla bulundu. Kan glukoz seviye değerleri dört grup arasında farklı olarak saptandı (p<0,001). TUNEL terminal deoksinükleotidil transferaz-aracılı dUTP işaretleme testinde grup 4'te grup 3'e göre daha düşük sonuçlar bulundu (p=0,025). **Sonuç:** Çalışmanın ana bulgusu hiperglisemik ratlarda metformin kullanımının kan glukoz düzeylerini düzenlediği yönünde. Sonuçlarımız metforminin nöronları apoptoza karşı koruyabileceğini göstermektedir.

Anahtar Kelimeler: Erken beyin hasarı; hiperglisemi; metformin; subaraknoid kanama

Aneurysimal subarachnoidal hemorrhage is a cerebrovascular disease which is known with serious permanent sequelae and mortality.[1,2] Annual incidence of subarachnoid hemorrhage (SAH) is reported to be 10/100000 individuals worldwide.[3] Vasoconstriction of the vasculature develops due to arterial

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narrowing in the main perforating arteries in the subarachnoid space, and this "vasospasm" occurs within 3–14 d after SAH.[4] Early surgical treatment helps to remove blood from the cisterns to reduce the risk of vasospasm.

Vasospasms leading to cerebral ischemia and early brain injury continue to be the most important factors affecting morbidity and mortality;[5-9] therefore studies on SAH have focused on this factor. However, these studies have not identified sufficient agents for the treatment of vasospasms. Not all morbidity is caused by vasospasms; therefore, several studies have shown the relationship between early brain injury and patient mortality and morbidity.[10,11] Vasospasms and early brain injury are interrelated, and ischemia, inflammation, dysfunction of blood–brain barrier , cerebral edema, and oxidative stress cascades constitute the main steps in the mechanisms leading to apoptosis. Apoptosis is known to be induced by various pathways following SAH; [12] however, the relationship between SAH and apoptosis is poorly understood.

Blood glucose monitoring is an important parameter for patients diagnosed with SAH. Serum glucose levels are known to increase after SAH. Several studies have highlighted the relationship between hyperglycemia and poor neurological outcomes in patients with SAH, and the effects of hyperglycemia-associated vasospasms and brain injury occured in early stages of SAH are believed to be one of the main responsibles of this neurological decline.[13-17]

Metformin is a biguanide group drug used to decrease serum glucose levels in the treatment of diabetes mellitus. It is an antihyperglycemic drug that suppresses hepatic glucose output and increases insulin sensitivity and peripheral glucose uptake. Metformin adequately reduces blood glucose levels by creating a consistent mean glucose level throughout the day.[18] It also decreases the inflammatory response against insulin resistance and endothelial activation.[18]

Our study eveluated the histological changes and effects on apoptotic pathways of metformin on SAH in a rat model. We hypothesized that metformin reduces hyperglycemia-induced apoptosis and brain injury after SAH.

MATERIAL AND METHODS

Local Animal Research Ethics Committee approved the study (approval number/date 2017-094/ 04.10.2017), and the experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* and *Helsinki Declaration principles* in the university's Experimental Animal Laboratory using a hyperglycemia and SAH model similar to that used by Huang et al. [19]

STUDY ANIMALS

Twenty-eight Wistar albino male rats weighing 300-350 g (Kobay D.H.L.A.S. Research Laboratory Animal Center, Turkey) were used in the study. The rats were treated with 50 mg/kg intraperitoneal (IP) streptozotocin (STZ) for 7 d to induce hyperglycemia, and daily blood glucose controls were monitored using a calibrated glucometer (Roche Accu Chek Performa Nano, Roche Diagnostics Ltd, Basel, Switzerland). Level of >300 mg/dL blood glucose after 7 d of STZ treatment was the hyperglycemia limit of this study.[20] The one-shot SAH model was applied on the hyperglycemic rats at the end of the 7-d STZ treatment. The rats were anesthetized with 5-10 mg/kg IP ketamine hydrochloride-xylazine combination, and subcutaneous prophylactic15 g/kg cefazolin sodium was administered just before the surgery. After fixation to the operating table, surgical procedures were performed following sterility conditions with a loupe microscope. A previously used method was modified and used for injecting the cerebellomedullary cistern.[20] A linear midline incision (approximately 1 cm) was made into the rats' suboccipital area. The cervical muscles were bluntly dissected laterally to expose the craniocervical junction. A 27-gage dental needle was advanced into the cleft through the occiput, and the atlas vertebra in the midline. About 0.3 mL cerebrospinal fluid was withdrawn from the cerebellomdullary cistern and replaced with fresh arterial blood which was collected from the central tail artery.

Four random groups each containing 7 rats were created randomly: control, (Group 1); only SAH performed, (Group 2); SAH with hyperglycemia, (Group

3); SAH with hyperglycemia treated with a 200 mg/kg/day peroral metformin, which was dissolved in serum (Group 4). Rats were treated with dissolved metformine with the help of a gastric lavage. The metformin treatment was initiated 30 minutes after SAH till all were sacrificed 72 hours after SAH. The dose of metformin was based on experimental studies for the protective effects of metformin on cerebral ischemia.[21,22]

HISTOPATHOLOGICAL ASSESSMENT

Brain and cerebellar tissues were extracted from the sacrificed rats and immediately suspended in 10% formaldehyde solution. After overnight fixation, the tissues were removed from the formalin solution, dehydrated using a graded ethanol, immersed in xylene. The next step was that the tissues were embedded in paraffin. And then, these tissues were cut approximately 3-4 μm thick, thereby it could be spotted with hematoxylin and eosin. Histological preparations were examined by a central nervous system experienced pathologist using the Olympus BX51 light microscope, and photographed with an Olympus DP72 camera. Changes in vascular structures, neuronal and glial cells, necrosis, and lymphocytic infiltration of the interstitium as well as other changes in the tissues were investigated and rated using scores ranging from 1 to 3. One meant there are no changes, 2 meant there are minor changes, 3 meant there are medium changes, 3 meant there are major changes. Ten high-power fields (HPFs; magnification, 400×) for each section were preferred to analyze samples. We investigated the basilar arter diameter and wall thickness, and also other differences about the basilar artery.

IMMUNOHISTOCHEMICAL ANALYSIS

We wanted to detect the DNA fragments during apoptosis in situ. For this reason, we preferred terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method and the ApopTag In Situ Apoptosis Detection Kit (Millipore, USA). Deparaffinized sections with xylene were rehydrated using ethanol following incubation with proteinase K (20 μg/mL) for 30 minutes at 20˚C and rinsed in dH_2O. Sections were incubated with 3% H_2O_2 in phosphate-buffered saline (PBS) at 20˚C throughout 15 min to inhibate the endogenous peroxidase activity. Equilibration buffer was used to incubate the sections for 3-5 minutes and with TdT-enzyme at 37˚C for 120 min. Thereafter, we put the sections in a heated wash bumper at 20˚C throughout 10 min following incubation with antidigoxigenin conjugate for 60 min. Washes with PBS was performed between the steps. A peroxidase substrate was used to reveal labeling. We stained the section's floor using hematoxylin, afterward dehydrated, cleared, and mounted. The ratio of TUNEL-positive cells to total HPF cells was determined as the index of apoptosis. At least ten microscopic fields were evaluated for the each section to determine the apoptotic cell numbers.

STATISTICAL ANALYSIS

Descriptive statistics are reported as the mean ± standard deviation for parametric variables and the median and interquartile range (IQR) for nonparametric variables. The data were analyzed using the Shapiro–Wilk test for normal distribution. One-way analysis of variance was used to compare the groups for parametric variables and Kruskal-Wallis tests for nonparametric variables. Post hoc test was conducted to put forth the difference of pairwise-groups. SPSS v. 21.0 (I.B.M. Corporation, Armonk, New York, U.S.A.) was used to perform statistical analyses. A p value of <0.05 was considered statistically significant.

RESULTS

The thickness of the basilar artery wall was significantly different among the four groups (p=0.003); it was greater in Group 3 than in Groups 1 and 2 (Table 1, Figure 1).

Blood glucose values were significantly different among the four groups (p<0.001); they were higher in Group 3 than in the other groups (p<0.001), and those in Group 2 were higher than those in Group 1 (p<0.001; Figure 2).

Histological sections of the TUNEL staining in the experimental groups are shown in Figure 3. The median (IQR) number of neurons and/or glial cells stained with TUNEL was 0 (0) in Group 1, 1 (0) in Group 2, 1 (3) in Group 3, and 0 (0) in Group 4. The number of neurons stained with TUNEL was signif-

TABLE 1: Results of TUNEL staining reported as median (interquartile range [IQR]) and wall thickness.

						Post hoc p					
	Group 1	Group 2	Group 3	Group 4	P	Group 1-2	Group 1-3	Group 1-4	Group 2-3	Group 2-4	Group 3-4
Neurons stained with TUNEL*	0 (0)	1 (0)	1 (3)	0 (0)	0.001	0.027	0.003	1.000	1.000	0.170	0.025
Wall thickness**	20.9±2.4	15.9±2.5	15.3±1.7	17.8±3.6	0.003	0.983	0.005	0.369	0.009	0.546	0.152

*After Kruskal Wallis test was performed, post hoc p value was calculated for pairwise comparisons. Data was shown as median and interquartile range [IQR].

**After one-way analysis of variance was performed, p value using Tukey's post hoc test was calculated for pairwise comparisons.

Data was shown as mean and standard deviation [SD].

p<0.05 was considered statistically significant and marked as bold.

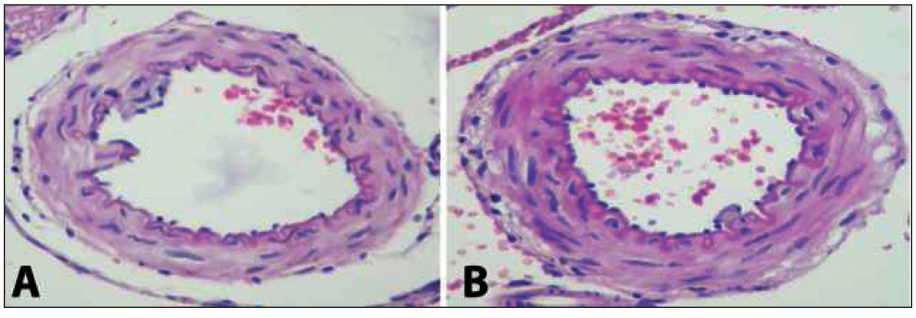

FIGURE 1: Representative histologic sections of the basilar artery after hematoxylin and eosin (HE) staining in the 2 experimental groups: (A) Group 4; and (B) Group 2. Compared with control samples, basilar artery specimens in Group 2 showed swelled endothelial and smooth muscle cells, decreased luminal cross-sectional area, and increased the thickness of the basilar artery wall. These morphologic changes occurred to a much lesser extent in Group 4 (HE 100×).

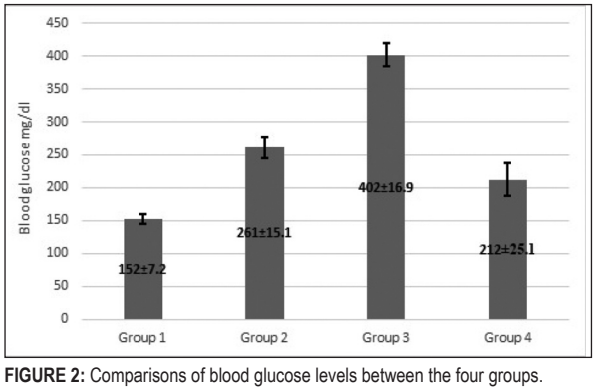

FIGURE 2: Comparisons of blood glucose levels between the four groups.

icantly different among the four groups ($p < 0.001$); it was lower in Group 1 than in Groups 2 and 3 and lower in Group 4 than in Group 3 (Table 1).

DISCUSSION

The treatment strategies available currently for ischemic brain damage and vasospasm after SAH are insufficient. Although several medications have been explored, only a few can be used in the routine treatment of these conditions. Several studies have used experimental SAH models in rats wherein

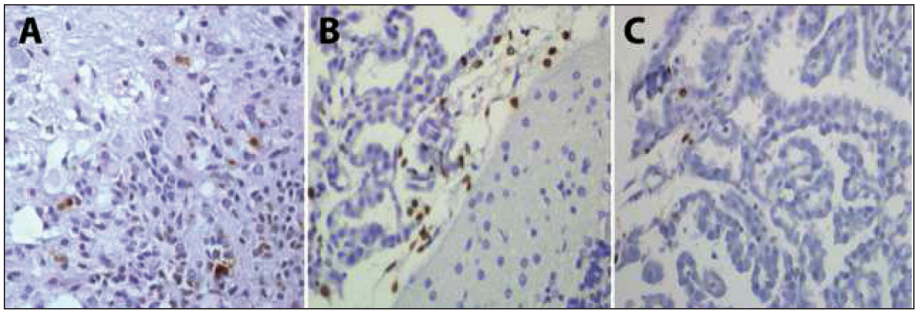

FIGURE 3: Histological sections of TUNEL staining in the three experimental groups: (A) Group 3; (B) Group 2; and (C) Group 4. Compared with Group 1 and 4 samples, apoptosis levels were markedly greater in Group 2 and 3 samples (DABX 100×).

blood obtained from rat tails was injected into the cisterna magna as the preferred method. In this context, for the first time, we studied the effects of metformin, which is a medication used for controlling blood glucose levels in type 2 diabetes. Metformin acts by inhibiting gluconeogenesis and glycogenolysis, thereby reducing liver glucose production, peripheral glucose intake, and increasing insulin sensitivity in the muscles. Therefore, we hypothesized that this medication is useful for treating hyperglycemia caused by stress hormones during SAH or other conditions.

Hyperglycemia after SAH is known to be triggered via several pathways; it is a well-known detrimental factor for the prognosis of patients with SAH, leading to secondary brain injury and increased mortality and morbidity rates.[19,23,24] Blood glucose control is often provided with insulin to such patients in intensive care units (ICUs); however, this protocol might cause hypoglycemia even with good control of blood glucose levels.[25] In particular, hypoglycemia can be overlooked in unconscious patients, thereby resulting in neurologic deterioration. It is believed that after bleeding, the sympathetic autonomic nervous system is activated and increases in stress-related hormones (e.g., cortisol and catecholamine) are responsible for the onset of hyperglycemia, especially in SAH, through their catabolic effects.[16,23] Insulin resistance and hyperinsulinemia are also important metabolic conditions that develop after SAH. Some studies have suggested that acute reversible pancreatic β-cell dysfunction is responsible for this condition, which leads to insulin resistance during the first week SAH.[26] The use of insulin to regulate blood glucose might also be dangerous for patients with neurological diseases and those treated in ICUs, with hypoglycemia being the most serious risk. It is known that neurons are insensitive to insulin, and systemically administered insulin might immediatedly decrease glucose levels in the extracellular space of the brain.[24] Considering these findings, we suggested that metformin has positive effects on SAH by reducing plasma insulin levels and the amount of insulin necessary for metabolic control as well as by preserving β-cell function.[27]

Metformin is a well known and frequently used drug that is known to bypass easily through the barrier of blood brain and reach the central nervous system. Gui et al. have also highlighted the benefits of metformin in their systematic review, and other studies have demonstrated its apoptosis-reducing effects.[25,28,29] Elmadhun et al. have reported that metformin supress the apoptosis in ischemic and nonischemic myocardium and promotes the survival of protein expression.[29] Similarly, Chen et al. have also demonstrated the benefits of metformin for apoptosis in a dose- and time-dependent manner in the nucleus pulposus by stimulating autophagy.[28]

In the present study, our results show that metformin helps in regulating blood glucose levels in rats with SAH and conducted TUNEL staining to determine whether metformin affects programmed cell death. We found that apoptosis was significantly lower in the metformin group than in the other groups. In their experimental study, Ge et al. also demonstrated that metformin protects the brain against ischemia via the PI3K/Akt1/JNK3 signaling pathways.[22]

To the best our knowledge, our experimental study is the first to investigate the neuroprotective effects of metformin on SAH. Undoubtedly, it is difficult to determine whether these protective effects are caused directly by metformin, by decreased blood glucose levels, or by the dual action of these two factors. Controlled studies with other antihyperglycemic agents will shed light to elucidate this issue.

CONCLUSION

The main finding of our study is that metformin regulates blood glucose levels, thereby protecting neurons against apoptosis in hyperglycemic rats with SAH.

Source of Finance

During this study, no financial or spiritual support was received neither from any pharmaceutical company that has a direct connection with the research subject, nor from a company that provides or produces medical instruments and materials which may negatively affect the evaluation process of this study.

Conflict of Interest

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

Authorship Contributions

Idea/Concept: *Mahmut Çamlar;* ***Design:*** *Çağlar Türk, Mahmut Çağlar, Emrah Akçay;* ***Control/Supervision:*** *Füsun Özer, Gülden Diniz;* ***Data Collection and/or Processing:*** *Çağlar Türk, Meryem Merve Ören;* ***Analysis and/or Interpretation:*** *Gülden Diniz, Meryem Merve Ören, Fatma Demet Arslan;* ***Literature Review:*** *Emrah Akçay;* ***Writing the Article:*** *Mahmut Çamlar, Emrah Akçay, Gülden Diniz, Füsun Özer;* ***Critical Review:*** *Füsun Özer, Fatma Demet Arslan;* ***References and Fundings:*** *Mahmut Çamlar, Füsun Özer.*

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