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EXPERIMENTAL STUDY

Protective effect of ischemic preconditioning on testis injury following transient focal cerebral ischemia in diabetic rats

Kumas M[1], Altintas O[2], Esrefoglu M[3]

BezmiAlem Vakif University, Vocational School of Health Services, Medical Laboratory Techniques, Istanbul, Turkey. **kumasmeltem@gmail.com**

ABSTRACT

OBJECTIVES: We aim to evaluate the effect of ischemic preconditioning (IPreC) on testicular tissue after transient middle cerebral artery occlusion (MCAo) in Streptozotocin-induced diabetic (STZ) and non-diabetic rats.

METHODS: Testis injury and alterations of testosterone levels were evaluated histologically.. Testicular damage was detected by using hematoxylin- eosin and periodic acid- schiff staining and apoptosis was identified by terminal-deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL).

RESULTS: Total mean serum testosterone levels decreased in all diabetic groups ($p < 0.05$) although remote ischemia and IPreC did not have any effect. Serious testicular tubular damage was observed in both, diabetic and ischemic groups ($p < 0.0001$). STZ-induced diabetes and MCAo decreased mean number of apoptotic cells (MNAC) ($p > 0.05$). Otherwise, remote IPreC induced MNAC instead of any changes in histopathological architecture. Moreover, remote IPreC reduced the tubular degeneration against synergistic damage of both ischemia and diabetes ($p < 0.05$).

CONCLUSIONS: It can be suggested that remote ischemic preconditioning prevents testicular damage by improving histopathological alterations and inducing apoptosis, probably temporarily, after focal transient middle cerebral artery occlusion in both, diabetic and non-diabetic rats. This is the first report demonstrating protective effects of ischemic preconditioning on remote testis injury after transient middle cerebral artery occlusion in diabetic rats *(Fig. 6, Ref. 23)*. Text in PDF *www.elis.sk.*

KEY WORDS: diabetes mellitus, experimental, cerebral infarction, experimental, ischemic preconditioning, testes, remote organ.

Introduction

Remote ischemic preconditioning (rIPreC) is the appearance of anearlier stress response that occurs during repeated episodes of brief ischemia and reperfusion of the target organ, it is an adaptational response that can restore various target organs, including the brain, kidneys, myocardioum and intestine, to tolerate a potential lethal ischemic injury (1, 2, 3). Recent studies showed that brief ischemia-reperfusion induced in non-target tissue could provide tissue-protective effect at a remote site by anti-inflammatory, neuronal, and humoral signaling pathways (4, 5). The systemic inflammatory response determines the effect on remote organs (liver, lung, kidney, myocardium and testis) structure and function (6). The development of remote organ dysfunction was observed only following reperfusion, which implies that humoral and/or cellular mediators produced locally in the target ischemic tissue were responsible for mediating remote organ injury (3, 4, 5).

Diabetes contributes to various microvascular complications related to endothelial dysfunction in the blood-barriers (7). Hyperglycemia can induce apoptosis by increasing reactive oxygen species and contributes to diabetes related target organ damage (8). Clinical studies showed that hyperglycemia increased the size of ischemic infarct and worsened the clinical outcome following a stroke (9). Moreover, diabetes can impair male reproductive functions in both humans and animals (10). Diabetes also impairs spermatogenesis and reduces sperm count, sperm motility, seminal fluid volume, and testosterone levels (11, 12).

Based on these observations, the aim of the present study was to evaluate the remote organ injury in testis tissue against IPreC following focal cerebral ischemia induced by transient middle cerebral artery occlusion in streptozotocin (STZ)-induced diabetic rat model and to explore the possible associations between the level of testosterone, testicular damage and apoptosis in diabetic and non-diabetic rats. To our knowledge, this is the first report demonstrating protective effects of ischemic preconditioning on remote testis injury after transient middle cerebral artery occlusion in STZ-induced diabetic rats.

[1]BezmiAlem Vakif University, Vocational School of Health Services, Medical Laboratory Techniques, Istanbul, Turkey, [2]Kirklareli State Hospital, Neurology Clinic, Kirklareli, Turkey and [3]Bezmialem Vakif University, Faculty of Medicine, Department of Histology and Embryology, Istanbul, Turkey

Address for correspondence: M. Kumas, PhD, BezmiAlem Vakif University, Vocational School of Health Services, Medical Laboratory Techniques, Adnan Menderes Bulvari, 34093 Fatih, Istanbul, Turkey.
Phone: + 90.555.5962824

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Methods

Animals

All data about the study design including the induction of experimental diabetes mellitus, experimental ischemic preconditioning (IPreC) and Middle Cerebral Artery Occlusion (MCAo) methods, and assessment of infarct volume was given in our previous study (13).

Determination of total serum testosterone level

Rat Testosterone ELISA kit (CK-E90243, Eastbiopharm, Hangzou) was used for the quantitative measurement of testosterone in blood samples. The samples and standards were added to appropriate wells, which was pre-coated with Anti-Human monoclonal antibody before incubation; then, biotin was added to all wells, and combined with Streptavidin-HRP to form an immune complex and then the samples were incubated and washed to remove the uncombined enzyme. Chromogen Solution A and B were added so the color of the liquid would change to blue. With the effect of acid, the color finally became yellow. Optical density was read on a standard automated plate reader at 450 nm (Perkin Elmer, 1420 Victor3). The detection range of kit was between 0.5 – 100 nmol/L.

Histological analysis and evaluation of apoptotic cells

Testicular damage was detected by using hematoxylin and eosin staining and periodic acid- Schiff staining and apoptosis identified by terminal-deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL). To our knowledge, estimation of the average radius or length of seminiferous tubules on the cross-sections for the morphometric analysis might not be a useful parameter due to the change during dehydration and embedding in tissue blocks and specific stages of seminiferous cycle for each rat. Then, we decided to estimate the histopathological scores by calculating the number of the degenerative tubules and total seminiferous tubules in twenty different areas under light microscope at 20X magnifications. The percentage of the number of degenerative seminiferous tubules was calculated as [100x (number of dejenerative tubule/ number of total tubule)].

TUNEL fluorescence (Roche® - 11 684 795 910-kit) detection kit was used for determination of apoptotic cells. Twenty successive areas in each section were examined for the presence of TUNEL positive apoptotic cells under fluorescence microscope at 20X magnification. All of the analysis and imaging procedures were also performed by using Nikon Eclipse i5 microscope and Nikon NIS Elements version 4.0 imaging and analysis systems (Nikon® Instruments Inc., Tokyo, Japan).

Statistical analysis

Continuous measurements were expressed as mean and standard deviation for each group. ANOVA and Kruskal–Wallis tests were used to assess the effectiveness of intervention and group differences in infarct size and weights of rats, along with levels of testesteron and glucose. Dunn's test (non-parametric) and Tukey (parametric) test was used for multiple comparisons after Kruskal–Wallis and ANOVA, respectively. p values < 0.05 were considered statistically significant.

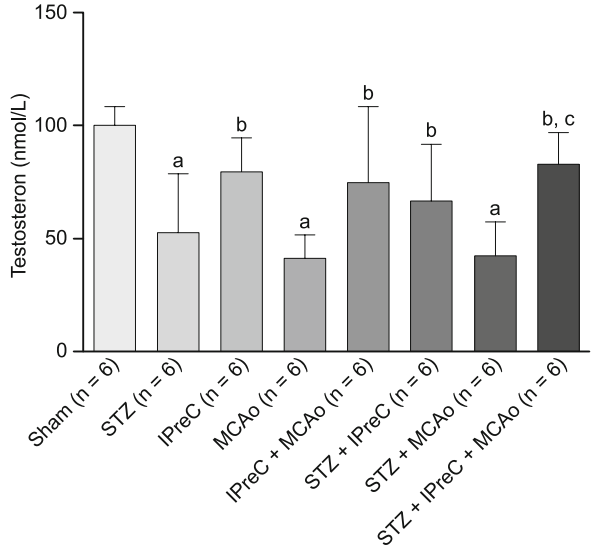

Fig. 1. Total serum testosterone levels, and unilateral variance analysis in groups. (One way ANOVA, Post-hoc: Tukey F(7,40) = 6.434 p < 0.0001. A: vs sham group, B: vs MCAo group, C: vs STZ + MCAo group, p≤0.05). (STZ, Streptozocin-induced diabetes; IPreC, ischemic preconditioning; MCAo, middle cerebral artery occlusion).

Results

General characteristics of the study groups

None of the animals died during the study period. After surgical procedures, carotid ligation did not cause any ptosis in the animals, presumably as aresult of damage to the sympathetic nerve trunk during isolation of the carotid artery.

The general characteristics of STZ-treated rats included reduced body weight and elevated blood glucose levels compared to non-diabetic ones. We observed that ischemic preconditioning had no effect on blood glucose levels (STZ + IPreC + MCAo versus (vs) STZ + MCAo; p > 0.05).

The body weight loss of STZ-induced diabetic rats and ischemia/reperfusion injury induced rats was significantly higher compared with the sham group (p < 0.0001). Moreover, it was shown that ischemic preconditioning did not have any effect on body weight (p = 0.154, IPreC vs Sham).

Total serum testosterone level

The mean serum testosterone level was 100.04 ± 8.35 nmol/L in sham group. Therefore, the testosterone levels in IPreC (79.61 ± 15.44 nmol/L) and MCAo (82.73 ± 13.85 nmol/L) groups were similar with sham group (p > 0.05). The mean testosterone levels were considerably decreased in diabetic related groups including STZ (52.52 ± 26.40 nmol/L), STZ + IPreC (66.36 ± 25.15 nmol/L), STZ + MCAo (48.78 ± 14.88 nmol/L) and STZ + IPreC + MCAo (52.01 ± 18.34 nmol/L) groups when compared to sham group (p

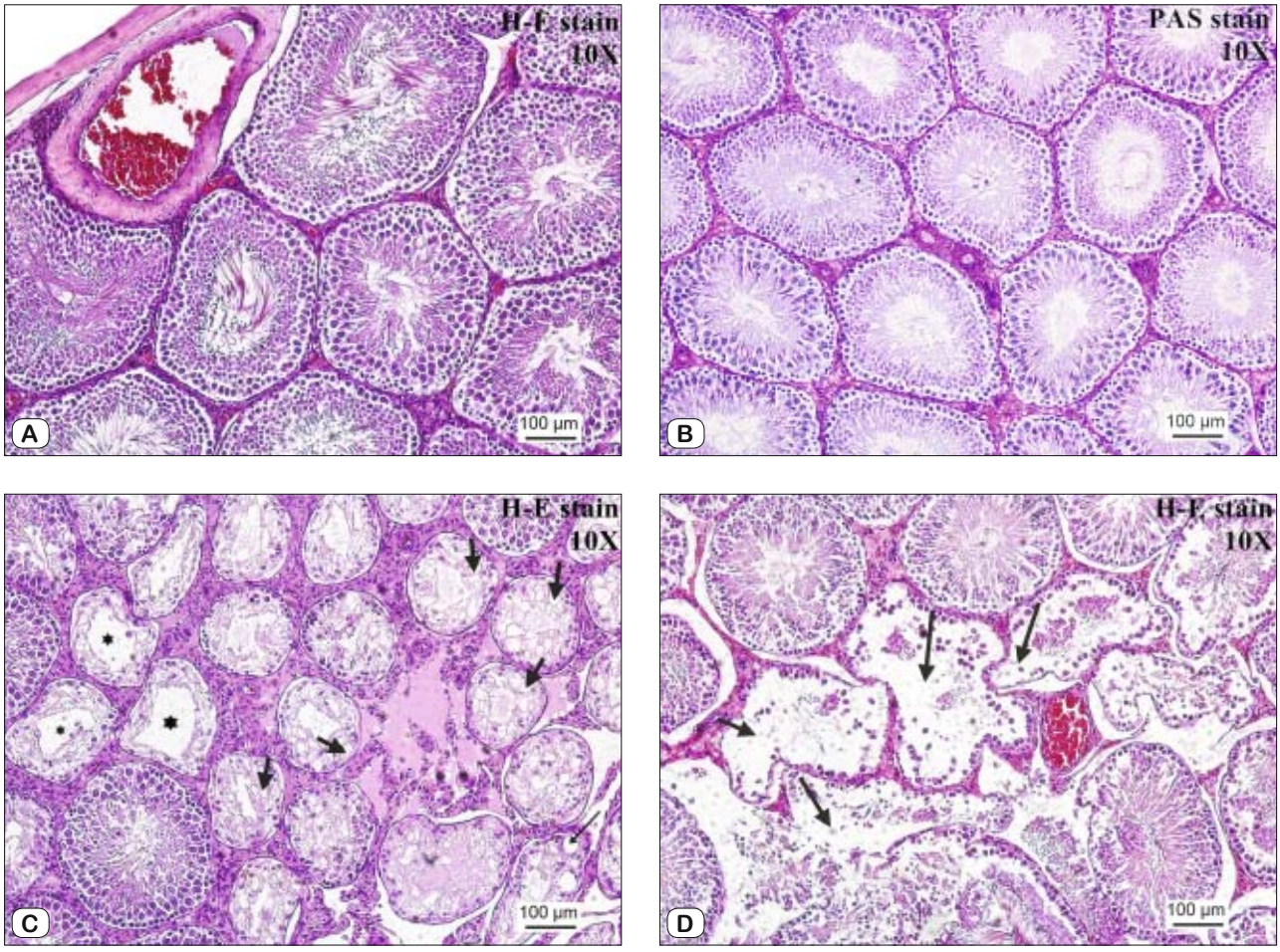

Fig. 2. Testicular histological results. (A, C, D: Hematoxylin–eosin x10 and B: Periodic acid- Schiff x10). A–B) Normal testicular histology was observed in sham-operated and IPreC groups, respectively. C) Vacuolization (arrows) and devoid of spermatids in the tubular lumen (stars) were obtained in STZ group were. D) Disruption of seminiferous tubular structure was labelled by arrows. In MCAo group, desquamation of the germ cells and devoid of spermatids in the tubular lumen were observed. (STZ, Streptozocin-induced diabetic; IPreC, ischemic preconditioning; MCAo, middle cerebral artery occlusion)

< 0.0001, for all comparisons). Moreover, we showed that remote ischemic preconditioning had protective effect on remote testicular tissue injury due to maintaining the mean testosterone level in STZ + IPreC + MCAo group higher than in the STZ + MCAo group ($p = 0.024$). The mean testosterone levels in all groups are shown in Figure 1.

Histopathological evaluation

Testis tissues showed normal histological appearance in sham and ischemic preconditioning groups (Figs 2A–2B). Serious histopathological alterations, including tubular vacuolization and devoid of spermatids in the tubular lumen were observed in diabetic rats (STZ) group (Fig. 2C), and tubular atrophy, degeneration of spermatogenetic cells were observed in ischemic rat (MCAo) groups (Fig. 2D) when compared to sham group ($p <$ 0.0001, for both).

In STZ + MCAo group, severe testicular damage including degeneration in tubular cells, atrophy of seminiferous tubules, and expansion of the interstitial spaces were observed. Thus, devoid of spermatids in the tubular lumen at some sections was evaluated in the group (Fig. 3A–3B). Remote ischemic preconditioning decreased the degeneration of tubular cells in IPreC + MCAo group when compared to MCAo groups. Also, histological appearance of tubular cells seemed to be generally normal in IPreC + MCAo and STZ + IPreC + MCAo groups (Figs 3C–3D). The highest mean of degenerative tubules (MRDT) was observed in STZ (11.31 ± 2.27 %), MCAo (9.75 ± 2.63 %), and STZ + MCAo (9.18 ± 1.56 %) groups and the lowest MRDT was observed in IPreC group among the study groups. Remote ischemic preconditioning was significantly decreased MRDT in IPreC + MCAo (4.80 ± 0.96 %) and STZ + IPreC + MCAo (6.01 ± 1.61 %) groups compared to MCAo and STZ + MCAo groups ($p < 0.0001$, $p = 0.002$; respectively). The percentage of the degenerative tubules to the total tubules in the study groups is given in Figure 4.

Assessment of apoptotic cell death

The mean number of apoptotic cells (MNAC) was not significantly increased in STZ-induced (36.6 ± 19.98) diabetic groups

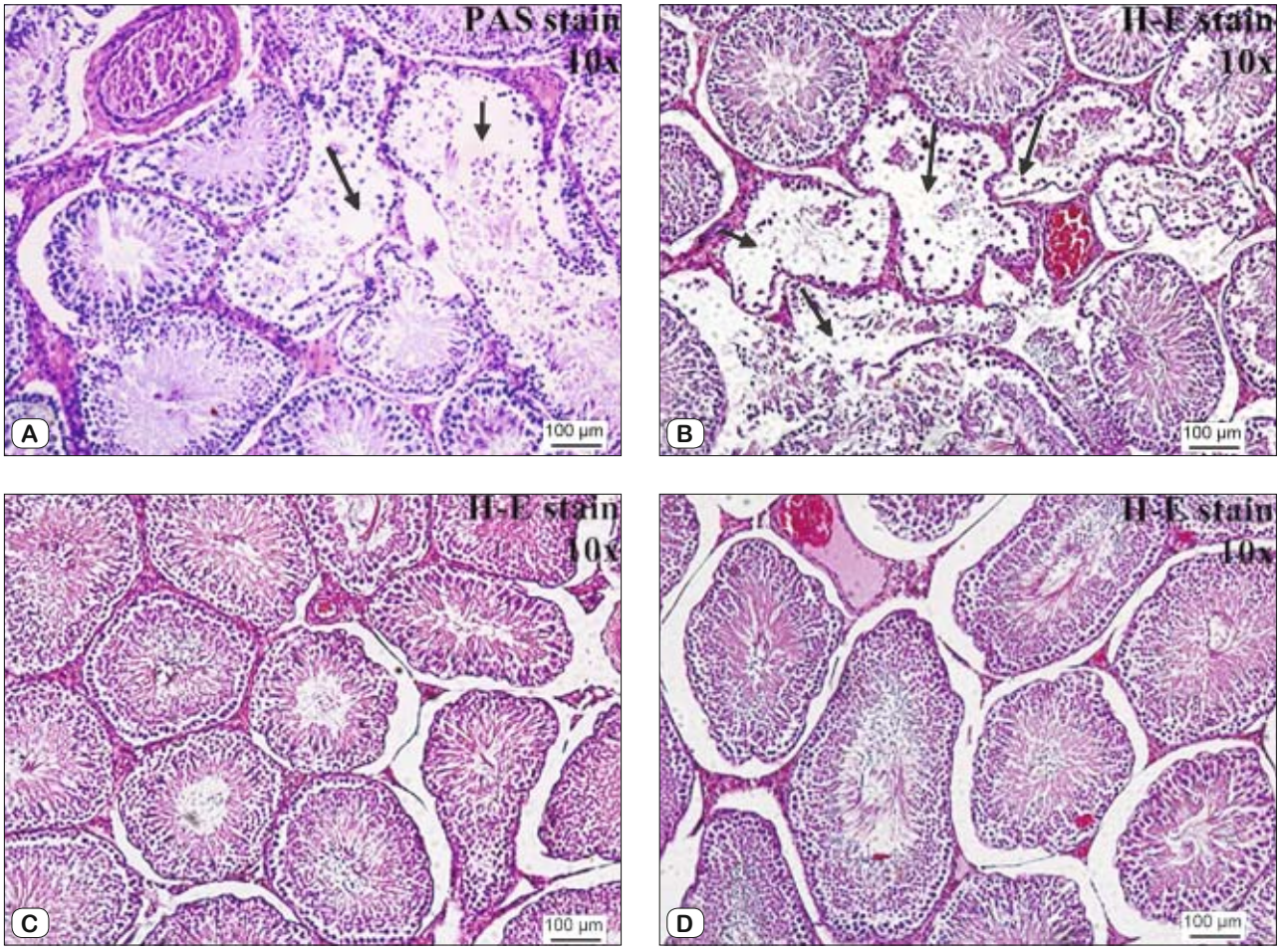

Fig. 3. Testicular histological results. (A: Periodic acid- Schiff x10 and B, C, D: Hematoxylin–eosin x10). A–B) The seminiferous tubules labelled by arrows that were destroyed and seemed to lack most of germ cells in STZ + MCAo group. (C–D) Testis had normal tubular histology, although some of interstitial area extented between the seminiferous tubules in IPreC + MCAo and STZ + IPreC + MCAo groups, respectively. (STZ, Streptozocin-induced diabetes; IPreC, ischemic preconditioning; MCAo, middle cerebral artery occlusion).

when compared to sham group (41.00 ± 28.54), ($p > 0.05$). Remote ischemic preconditioning significantly increased MNAC in IPreC + MCAo (123.6 ± 33.00) and STZ + IPreC + MCAo (119.4 ± 15.08) groups rather than MCAo (45.60 ± 33.89) and STZ + MCAo (57.60 ± 30.12) groups, respectively ($p = 0.002$, for both). Moreover, the highest MNAC was 163.4 ± 31.75 in STZ + IPreC group. TUNEL positive cells observed in the study groups are shown in Figures 5 and 6.

Ischemic preconditioning reduces the total infarct volume

We did not observe any infarct area on the TTC-stained brain sections of STZ, Sham, IPreC and STZ + IPreC groups. Ischemic preconditioning before cerebral ischemia significantly reduced infarction size compared with the other groups [IPreC + MCAo (27.26 ± 10.04 mm^3) vs MCAo (109.07 ± 15.28 mm^3) $p < 0.001$; STZ + IPreC + MCAo (38.70 ± 9.59 mm^3) vs STZ + MCAo (165.87 ± 41 mm^3) $p < 0.001$, respectively]. Also, we detected that ischemic preconditioning could improve the ischemic injury in diabetes [STZ + IPreC + MCAo vs MCAo $p < 0.001$].

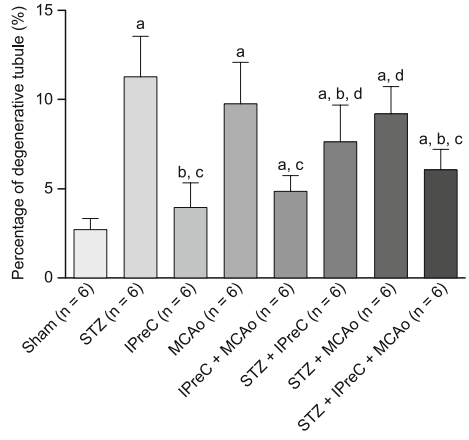

Fig. 4. The percentage of degenerative seminiferous tubules to total seminiferous tubules was shown in each group. (One way ANOVA, Post-hoc: Tukey; $F = (7, 40) = 20.314$, $p < 0.0001$. A: vs sham group $p \leq 0.05$, B: vs STZ group, C: vs MCAo group, D: vs IPreC group, e: vs STZ + MCAo group, $p \leq 0.05$) (STZ, Streptozocin-induced diabetic; IPreC, ischemic preconditioning; MCAo, middle cerebral artery occlusion).

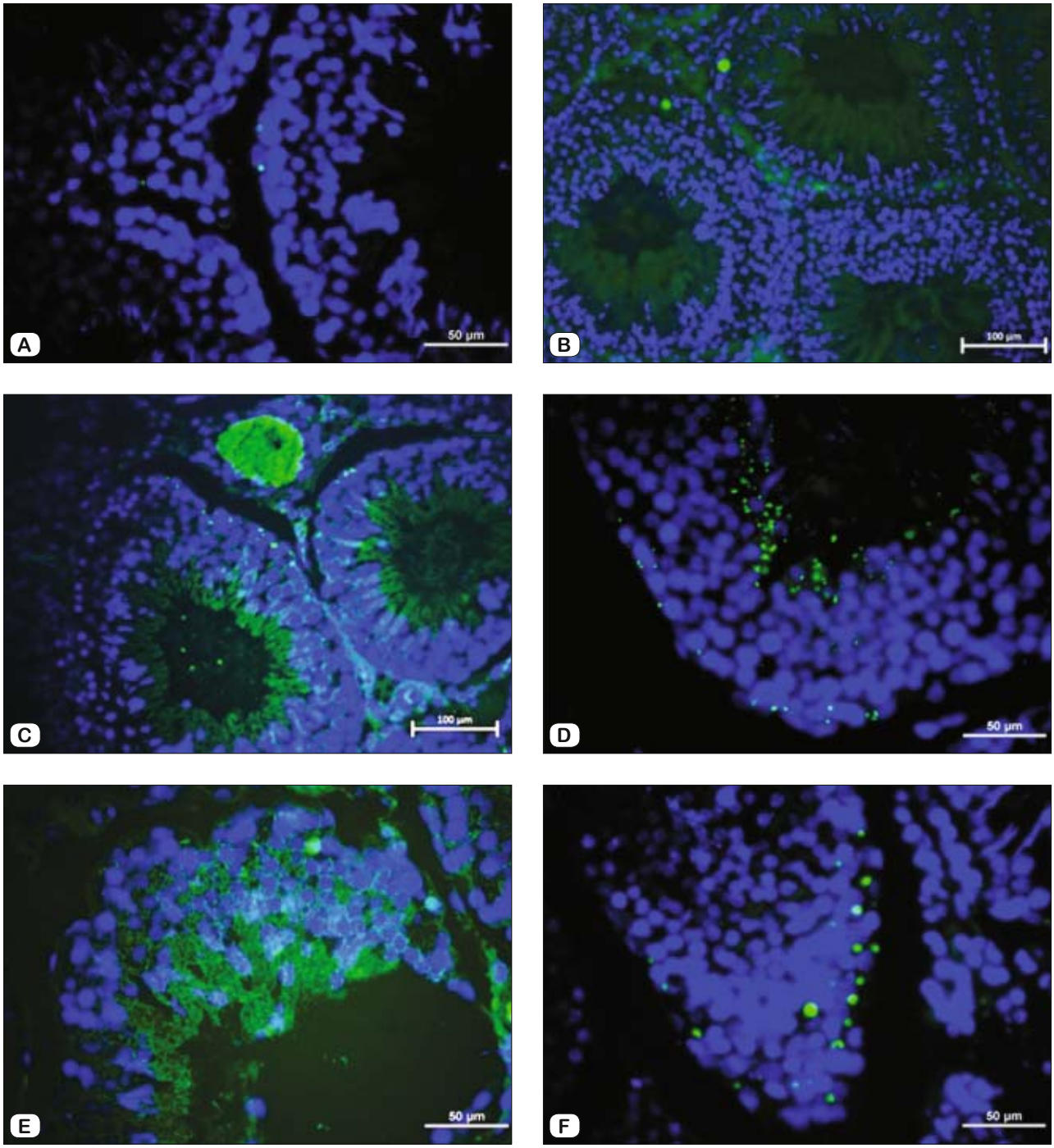

Fig. 5. TUNEL (terminal-deoxynucleotidyl-transferase-mediated dUTP nick end labeling) positive cells were shown in the groups, at 20x (A: STZ group B: MCAo group C: IPreC + MCAo group D: STZ + IPreC group E: STZ + MCAo group F: STZ + IPreC + MCAo group). Apoptotic nuclei were stained in green, while normal nuclei were stained in blue by DAPI (4',6-diamidino-2-phenylindole). (STZ, Streptozocin-induced diabetic; IPreC, ischemic preconditioning; MCAo, middle cerebral artery occlusion)

Discussion

Ischemia reperfusion injury not only affects local tissue but can also result in life-threatening damage to remote organs such as lungs, heart, liver, kidneys and testis (2, 6). Previous studies suggested that myocardial ischemia, skeletal muscle ischemia and unilateral hind limb ischemia caused non-target severe testicular injury along with sloughing germinal cells within the seminiferous tubules, reduced sperm motility, intracellular vacuolization, interstitial edema, neutrophil infiltration and coagulative necrosis

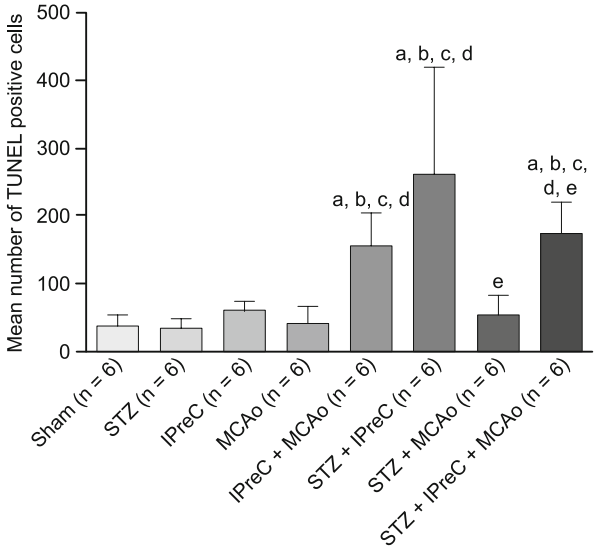

Fig. 6. Mean number of TUNEL positive cells in the study groups [One way ANOVA, Post-hoc: Tukey F (7, 40) = 11.297 p < 0.0001. A: vs sham group, B: vs STZ group, C: vs MCAo group, D: vs IPreC group, E: vs STZ + IPreC group, p ≤ 0.05]. (STZ, Streptozocin-induced diabetes; IPreC, ischemic preconditioning; MCAo, middle cerebral artery occlusion).

(14, 15). In the present study, remote ischemia-reperfusion injury increased severely tubular damage when compared to other study groups ($p < 0.05$).

Diabetes induces structural and functional alterations in the micro- and macrovascular compartments (7). The recent experimental study indicated that diabetes could reduce the diameters of seminiferous tubules and spermatogenic cells and damage to the epithelium in diabetic rats (16). We observed significantly serious tubular damage in diabetes related rat groups when compared to sham group ($p < 0.0001$, for each). In rats, it has been reported that diabetes impairs sperm motility and spermatogenesis, also decreases sperm number as well as the level of testosterone (11). Similarly to our study, several case-control studies have shown decreased levels of endogenous testosterone in male survivors of myocardial infarction, whereas testosterone levels in men who subsequently died from cardiovascular disease were normal (17,18). Another experimental study found that transient cerebral ischemia in the brain results in lower expression levels of steroidogenesis-related genes and thus lower serum testosterone level (19). Also after acute stroke, the researchers found that when the level of total testosterone was decreased, the initial loss of neural function and size of the cerebral infarction were increased (20). The possible reason to explain the decreased level of testosterone could be an acute stress reaction which is known to occur in several forms of stress, including myocardial infarction, surgery, and head trauma (20). On the other hand the reaction could be a protective mechanism against stroke progression due to lowering fibrinolytic activity, which would delay the lysis of a preformed thrombus. The present study has shown that total serum testosterone levels in STZ-induced diabetic groups were significantly lower than in non-diabetic groups ($p < 0.05$). Therefore, further experimental studies are needed to investigate alternations of hormone levels by labeling specific cells in testis tissue against the effect of diabetes or IPreC.

The induction of apoptosis following brief ischemia is largely a reperfusion-associated event (5). A recent study suggested that the appearance of apoptotic cells increased up to 72 hours of reperfusion, which resulted in providing energy supply to allow apoptotic pathway (21). At our study, we observed increased apoptotic cells in IPreC related groups due to state of the time interval from IPreC up 72 hours to MCAo. On the other hand, observations from experimental studies indicate that ischemic preconditioning is a protective mechanism due to reduction of both, necrosis and apoptosis (22). Thus, it has been shown previously that a chemical can induce apoptosis or necrosis depending on the applied dose because of bioaccumulation, genomic effect or oxidative stress (8). Indeed, we observed that apoptosis was induced in ischemic preconditioning group without histologicall changes on the testis tissue. Although, tubular degeneration was lower in IPreC + MCAo and STZ + IPreC + MCAo groups than MCAo and STZ + MCAo groups, apoptotic cell numbers were higher than that of groups ($p < 0.05$). In the present studz remote ischemic preconditioning dramatically improved histopathological findings caused by diabetes and cerebral ischemia injury ($p<0.05$). Our findings might be related to the time interval between onset of ischemic preconditioning and transient ischemia effects on remote testis tissue damage. Therefore, the evaluation of the necrotic and apoptotic cells simultaneously might be good criteria to observe the apoptotic/ necrotic index value in further experimental studies. The type of cell death induced in seminiferous tubules could depend on duration of ischemia reperfusion injury or hyperglycemia. In the recent study, application of a single dose of cadmium chloride for 4 weeks resulted in necrosis and degeneration of seminiferous tubules, although acute administration of cadmium caused the apoptotic cell death (23). Another possibility to explain the results is that remote ischemic preconditioning may have caused a temporary increase in tubular cell apoptosis, and also it could be too early to make any statement about the long-term effect. Thus, further studies are necessary to determine the critical time beyond which cell death pathways occur after induction of ischemia or diabetes and clarify the controversies in the pattern of cell death through necrosis or apoptosis. As the period of induction of ischemia (3 hours) –reperfusion (3 hours) injury in the present study was limited, we could hypothesize that remote ischemic preconditioning protects seminiferous tubules morphology while inducing apoptosis.

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