Chapter 6

Dimethylformamide Reduces Cerebral Ischaemia in Diabetic Rats Hours after Its Occurrence; A New Horizon

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Abstract

The antioxidant properties of dimethylformamide (DMF) depend on its interference with the hydroxyl-radical-transduction pathway. Diabetes is a risk factor of cerebral ischaemia (CI), and both entities are associated with oxidative stress (OS). We evaluated DMF's effects on CI in non-diabetic rats (NDRs) and in diabetic rats (DRs). One hour after CI, the animals were divided into two treatment groups (300 μl subcutaneous): either DMF or isotonic saline solution. Treatment effects were analysed in NDRs or DRs without CI. Eight hours after CI, a neurophysiologic score (NS) was determined; CI and OS biomarkers were measured in the ischaemic cerebral hemisphere. Infarct/oedema volumes were measured on dyed brain slices. DMF reduced infarct volume in NDRs and DRs but only improved the NS in DRs. Basal concentrations of all the biomarkers were similar in the NDRs and DRs. Metalloproteinase 9 (MMP9) did not change with DMF. Malondialdehyde (MDA) increased with CI, and DMF only reduced it in DRs. RAGE, nitrite/nitrate and nitrotyrosine increased with CI only in DRs (all prevented by DMF). We conclude that DMF's benefits on CI were greater in the DRs due to a higher susceptibility of diabetic animals to the OS produced by CI. The results open a new horizon in CI treatment since DMF has not been investigated before.

Keywords: cerebral ischaemia, diabetes, oxidative stress, dimethylformamide, antioxidant

1. Introduction

Stroke is one of the leading causes of death and disability worldwide [1]. To date, thrombolysis is the only approved therapy for acute stroke, but the window of time for treatment is short (3–4 h after a stroke). There is an associated risk of haemorrhagic transformation with thrombolysis, which increases the morbidity and mortality of ischaemic stroke [2], and the efficacy of this treatment is small. There is an obvious need for pursuing new treatment strategies to address these deficiencies. Being diabetic is an important risk of suffering a stroke [3]. Oxidative stress (OS) has been associated with both diabetes [4] and stroke [5, 6]. Some antioxidants have shown beneficial effects in animal models of diabetes [7]. However, exogenous antioxidant effects on humans are difficult to evaluate. Indeed, the long-term ingestion of antioxidants included in one's diet seems to provide beneficial health effects [8, 9], whereas there is still some controversy with respect to the usefulness of long-term antioxidant supplements [10].

Most of the positive effects of antioxidants on stroke models are the result of prophylactic strategies (treatment administered before stroke) [11]. The applicability of such approaches in humans yields dubious results because, although there are risk factors that could identify stroke-susceptible patients, it is virtually impossible to predict who would actually suffer a stroke.

N-N-dimethylformamide (DMF) is an amphipathic solvent significantly miscible with water and most organic solvents. It has been shown that DMF significantly protected against neuronal damage produced by dopamine or hydrogen peroxide. Even though the results were attributed to an increase in antioxidant enzymes [12], it seems that DMF interferes with the signal transduction pathway originated by the hydroxyl radical, which includes changes in permeability and ion exchange in the different compartments of mitochondria [13]. Moreover, DMF seems to have anti-ageing effects associated with protein homeostasis, which are independent of its antioxidant properties [14].

Signal transduction mediated by free radicals (including the hydroxyl radical) acts as a double-edge sword. It could be harmful (*i.e.* oxidative damage to biomolecules—proteins, lipids, RNA/DNA-) or beneficial (leading to the synthesis of antioxidant enzymes through nuclear factor kappa B activation) [10]. Free radicals generated during cerebral ischaemia (a first wave within minutes followed by a second wave shortly thereafter) participate in the cascade of events generating neuronal damage, which includes changes in mitochondria function [6]. We postulate that signal transduction mediated by the hydroxyl radical (one of those free radicals liberated during cerebral ischaemia) is one of the harmful events in cerebral ischaemia, and DMF would reduce such ischaemic damage. Therefore, the goal of this study was to precisely investigate the effects of a single dose of DMF, administered 1 h after induced cerebral ischaemia in rats.

2. Material and methods

The Institutional Ethical Committee approved the present work.

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2.1. Diabetes model

Type 1 diabetes mellitus was induced in 4-week old male Wistar rats (79.7 ± 1.8 g), through the intraperitoneal (ip) administration of streptozotocin (STZ, 75 mg/kg, Sigma St. Louis, USA). Diabetes was confirmed 3 days later by the onset of hyperglycaemia (>200 mg/dL). A control group (same characteristics, non-diabetic) was also maintained during the experiment. All the animals were fed Purina chow and water *ad libitum* and kept in light-darkness cycles of 12×12 h. The evolution of diabetes was evaluated by: (1) weighing the rats weekly and comparing the weights of the diabetic and the control animals and (2) measuring glycaemia and glucosylated haemoglobin at the end of the experiment.

2.2. Cerebral ischaemia model

Six weeks after diabetes induction, cerebral ischaemia was produced in diabetic as well as the control animals by slightly modifying the model reported by De Cristóbal et al. [15]. In short, the animals were anaesthetised through intramuscular administration of an anaesthesia cocktail containing acepromazine (5 mg/kg), ketamine (5 mg/kg) and butorphanol (13 mg/kg). The right carotid and the middle cerebral arteries were permanently ligated. One hour later the animals were randomly divided into two treatment groups (eight control and eight diabetic animals per group).

2.3. Experimental groups

1. Isotonic saline solution (ISS, 300 μl) administered subcutaneously (sc)
2. DMF (300 μl, equivalent to 0.9 g/kg, Sigma, St. Louis, USA) also administered sc

2.4. Neurophysiological test

Eight hours after cerebral ischaemia was induced, the neurological signs were scored as follows (sum of all): consciousness (0–3, where 3 was the response only after stimulation), decreased grip of the left forelimb when the tail was pulled (0–3, 3 was complete gripping), failure to grab a bar with the left forelimb (0–3, 0 was strongly grabbing with both forelimbs, 3 was no grabbing at all with the left forelimb), and walking in a circle to the left side (0–3, 3 was walking spontaneously in circles). If the animal did not respond to any stimulus, the score was 12 points.

2.5. Infarct and oedema evaluation

The animals were anaesthetised (pentobarbital, 75 mg/kg, ip). A blood sample (at least 5 ml) was taken from the heart and anticoagulated with ethylenediaminetetraacetic acid (EDTA, Sigma, St. Louis, USA). The blood glucose and glucosylated haemoglobin were measured. The animals were decapitated after taking the blood samples, and each brain was immediately extracted and sliced (2 mm slices). The slices were incubated in a 1% triphenyltetrazolium chloride (Sigma, St. Louis, USA)/ISS solution, at 37°C for 5 min). Both sides of each slice were photographed against

a millimetre paper background and measured using the ImageJ software (National Institutes of Health, USA). Areas were integrated as volume in mm^3 and infarct volume quantified as a percentage of the right hemisphere volume. Oedema was calculated (in percentage of the left brain hemisphere) by subtracting the left from the right hemisphere volumes.

2.6. Stroke and oxidative stress biomarkers' evaluation

Two groups of animals (diabetic or non-diabetic, n = 12 per group) submitted to cerebral ischaemia (treatments—6 per group—and sacrifice as mentioned earlier) were used to measure biomarkers. Three hundred milligrams of the right hemispheres were homogenised in 1.5 ml of phosphate saline buffer (Sigma, St. Louis, USA; 0.1 M, pH 7.4). The homogenised tissue was centrifuged (13,000 ×*g*, 4°C, 10 min). The supernatant was isolated and kept at −80°C until measurement of the biomarkers was taken (within a month of taking the samples). Ten milligrams of the right hemisphere was homogenised in 0.5 ml of malondialdehyde (MDA) lysis buffer (Abcam, MA, USA) for the measurement of MDA.

Biomarkers evaluated were:

1. Metalloproteinase 9 (MMP 9): It was evaluated through ELISA using a commercial kit (R&D Systems, MN, USA). The concentrations were expressed in ng/ml per mg of protein (proteins were measured using a commercial kit from Cayman Chemical Co, MI, USA).
2. Advanced glycation end-product receptor (RAGE) was measured through ELISA using a commercial kit (Abcam MA, USA). The concentrations were expressed in ng/ml per mg of protein.
3. Nitrite/nitrate (NO_2/NO_3 ratios, an indirect measurement of nitric oxide) were measured by the modified Griess method, using a commercial kit (Cayman Chemical Co, MI, USA). Results were expressed as μM per mg of protein.
4. Nitrotyrosine (NT) was measured through ELISA using a commercial kit (Abcam MA, USA). The concentrations were expressed in ng/ml per mg of protein.
5. MDA was measured using a commercial kit (Abcam MA, USA). The results were expressed in nmol per mg of protein.

To establish reference values, and evaluate the effects of DMF, the biomarkers were measured in the brains of four non-diabetic and four diabetic rats that were not submitted to cerebral ischaemia.

2.7. Statistical analysis

The Kolmogorov-Smirnov test was used to analyse the normality of distribution. The unpaired "t" test was used to compare glycaemia and glucosylated haemoglobin between the non-diabetic and diabetic animals. The two-way ANOVA test (Bonferroni post-hoc) was used to analyse the time-course of weights (6 weeks, diabetic and non-diabetic animals). The one-way

ANOVA test (Tukey post-hoc) was used to compare biomarkers (diabetic, non-diabetic, with or without ischaemia). The neurophysiological score was analysed using the Kruskal-Wallis test (Dunn's multiple comparison post-hoc). Data are shown as the mean ± standard error of the mean (SEM). The $p < 0.05$ was considered significant.

3. Results

3.1. Metabolic evolution

Non-diabetic and diabetic animals had the same weights at the beginning of the experiment (81.6 ± 0.8 g *vs.* 78.8 ± 2.7 g, respectively, $p > 0.05$). The time-course of their weights is shown in **Figure 1**. There was a significant difference beginning the third week after diabetes induction. Glycaemia at the beginning of the experiment (after diabetes induction) was higher in the diabetic animals compared with the non-diabetic animals (456.1 ± 2.3 mg/dL *vs.* 91.1 ± 2.3 mg/dL respectively, $p < 0.0001$). At the end of the experiment both glycaemia and glucosylated haemoglobin were also different: glycaemia 88.7 ± 3.0 mg/dL versus 467.5 ± 12.6 mg/dL (non-diabetic *vs.* diabetic, $p < 0.0001$); glucosylated haemoglobin 4.3 ± 0.1% versus 9.3 ± 0.1% (non-diabetic *vs.* diabetic, $p < 0.0001$).

3.2. Cerebral ischaemia evaluation

3.2.1. Neurophysiological score

The neurophysiological score is shown in **Figure 2**. DMF significantly ($p < 0.05$) improves the neurophysiological scores only in diabetic rats.

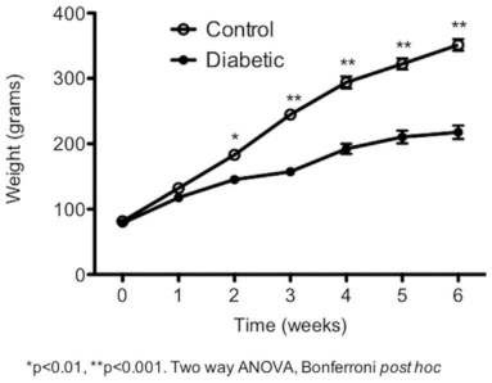

Figure 1. Time-course of the weight. The time-course of the weight was significantly different. Control (non-diabetic rats) gained more weight than diabetic animals during the experiment. The media + SEM are shown. Zero was at the beginning of the experiment, when the rats were 4 weeks old.

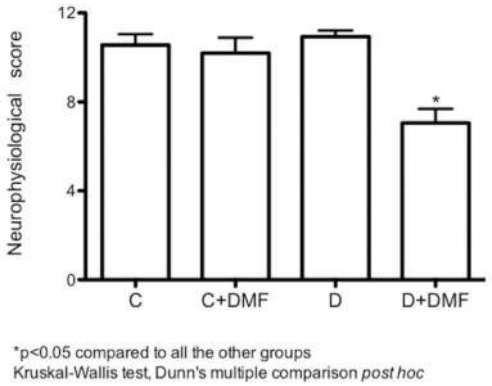

Figure 2. Neurophysiological scores: the neurophysiological score was similar in the control (non-diabetic) rats without (C) or with dimethylformamide treatment (C + DMF), as well as in the diabetic animals without DMF treatment (D). DMF significantly decreased the score in diabetic rats treated with DMF (D + DMF). The media + SEM are shown.

3.2.2. Infarct and oedema volume

DMF significantly decreased the infarct volume in both non-diabetic ($p = 0.02$, **Figure 3**) and diabetic ($p = 0.007$) rats. However, oedema was the same in non-diabetic rats compared to diabetic rats and was not changed by DMF (not shown). **Figure 4** shows an example of the slices, dyed with triphenyltetrazolium.

3.2.3. Cerebral ischaemia and stroke biomarkers

Results are shown in **Figures 5–9**. The results in non-diabetic (Control, C) rats are shown in the left panels whereas results in the diabetic (D) animals are in the right panels. The figures

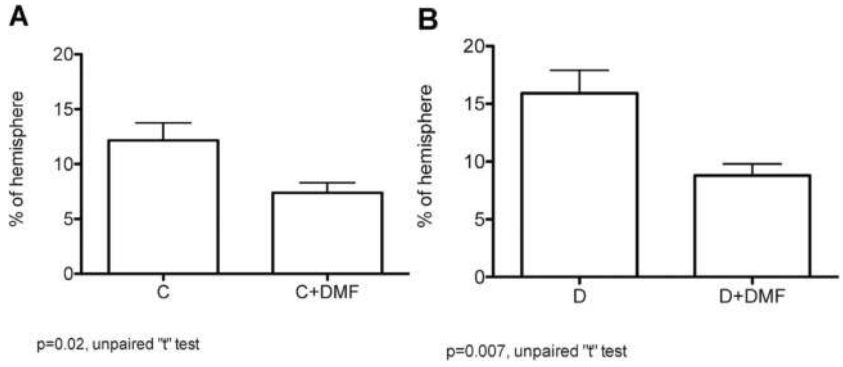

Figure 3. Infarct volume: this figure shows the infarct volume (expressed as percentage of the right hemisphere) in non-diabetic (C, panel A) and diabetic (D, panel B) animals. Dimethylformamide (DMF) significantly reduced infarct volume in non-diabetic as well as in diabetic rats.

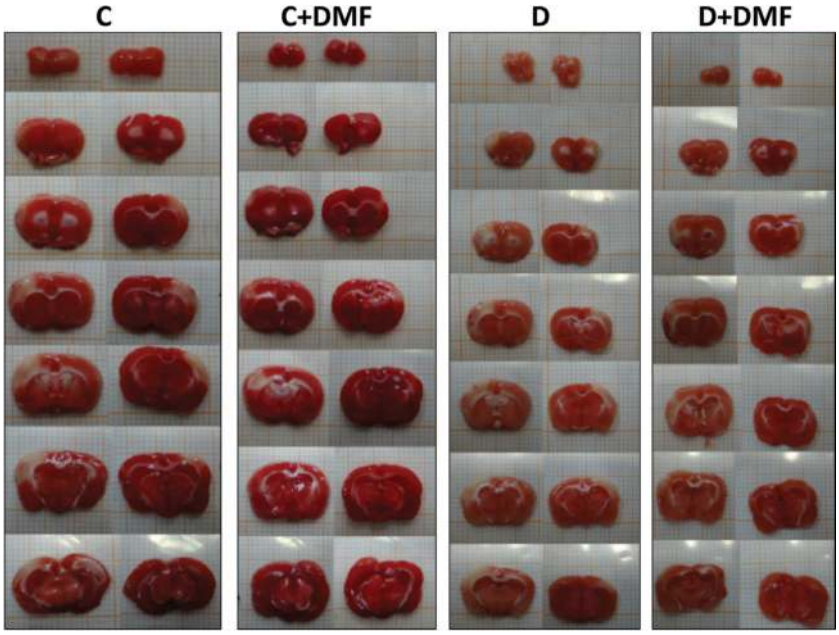

Figure 4. Both sides of the slices of a representative animal per group are shown: C (non-diabetic without treatment), C + DMF (non-diabetic treated with DMF), D (diabetic without treatment), D + DMF (diabetic treated with DMF). The infarct shows no colour (in white), whereas the tissue that is not affected has a red colour. It is noticeable that animals treated with DMF (control and diabetic) have a smaller infarct.

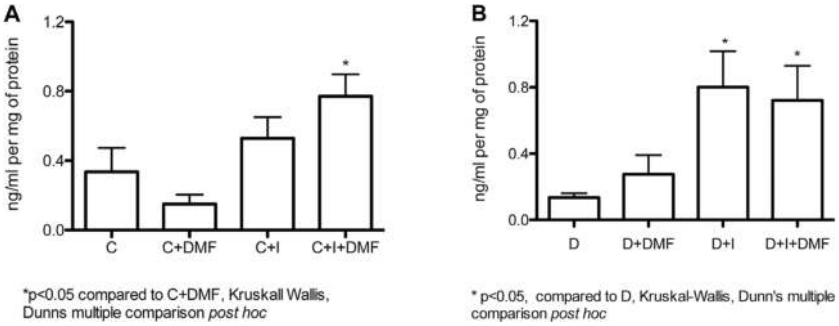

Figure 5. Concentration of metalloproteinase 9 (MMP9) in the brain: The concentration of MMP9 showed significant incremental increases in the brains of diabetic (D, panel B) animals with ischaemia with or without DMF treatment (D + I and D + I + DMF, respectively). DMF did not change MMP9 either in non-diabetic (C + DMF, panel A) or in diabetic (D + DMF, panel B) rats. Non-diabetic (C, panel A) animals with ischaemia and treated with DMF (C + I + DMF) had significantly higher concentrations of MMP9 than non-diabetic animals treated with DMF (C + DMF).

show the basal data (without ischaemia) of animals without (C, D) or with treatment (C + DMF, D + DMF). Animals with ischaemia and without treatment are represented as C + I or D + I. The animals with ischaemia and treatment are shown as C + I + DMF or D + I + DM.

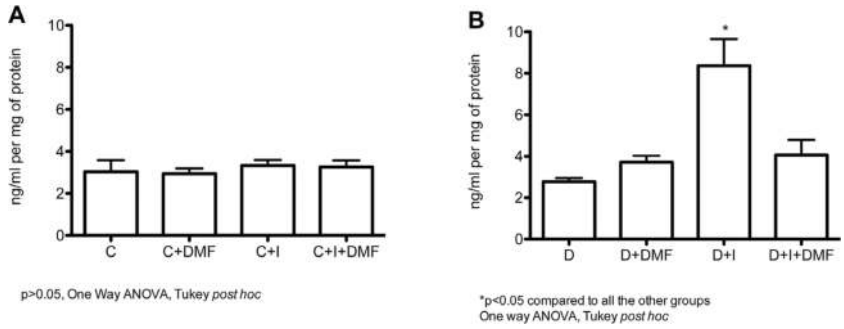

Figure 6. Concentration of the receptor for advanced glycation end products (RAGE) in the brain: RAGE did not change in the brains of non-diabetic (control, C) animals with or without ischaemia (I), either with or without dimethylformamide (C + DMF) treatment (panel A), whereas this biomarker significantly increased in the brains of diabetic animals submitted to ischaemia (D + I, panel B). DMF prevented the effect of ischaemia in diabetic animals (D + I + DMF). The mean + SEM are shown.

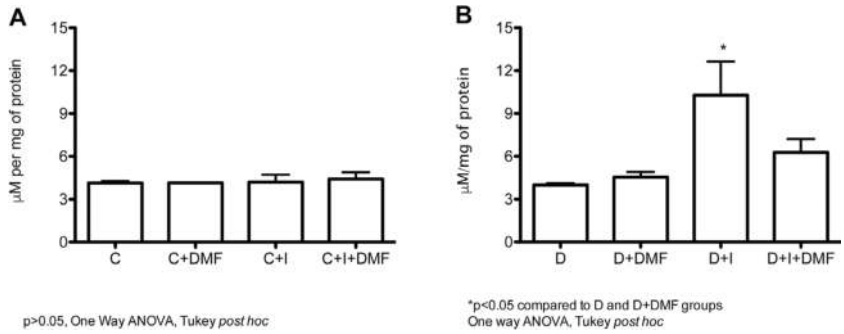

Figure 7. Nitrite and nitrate (NO_2/NO_3) in the brain: NO_2/NO_3 did not change in the brains of non-diabetic (control, C) animals with or without ischaemia (I), neither with nor without dimethylformamide (DMF) treatment (panel A) whereas this biomarker increased significantly in the brains of diabetic (D) rats submitted to ischaemia (panel B). DMF completely prevented the effect of ischaemia in diabetic animals. The mean + SEM are shown.

3.2.3.1. MMP9

Figure 5 shows that MMP9 increased significantly ($p < 0.05$) in the right hemisphere of diabetic rats, and DMF did not prevent such increase. There was a slight (non-significant) increase in the right hemisphere of the non-diabetic rats with ischaemia. Likewise, DMF did not reduce MMP9 in non-diabetic animals with ischaemia. Interestingly, DMF slightly reduced MMP9 in non-diabetic animals without ischaemia; because of that change, non-diabetic animals treated with DMF had different concentrations (significantly higher with than without ischaemia).

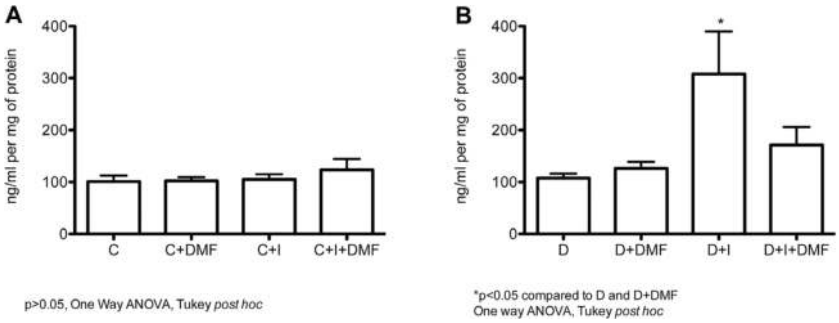

Figure 8. Nitrotyrosine (NT) in the brain: NT did not change in the brains of non-diabetic rats (control, C, panel A), with or without ischaemia (I), neither with nor without dimethylformamide (DMF) treatment, whereas this biomarker increased significantly in the brains of diabetic (D, panel B) rats submitted to ischaemia. DMF prevented the effect of ischaemia in diabetic animals. The mean + SEM are shown.

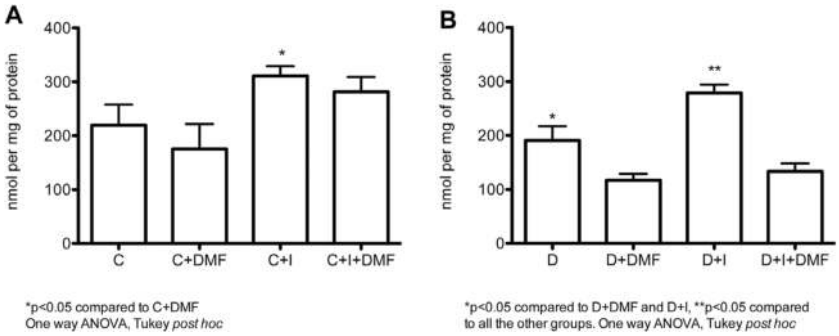

Figure 9. Malondialdehyde (MDA) in the brain: dimethylformamide (DMF) non-significantly reduced MDA in the brains of non-diabetic (control, C, panel A) rats. Ischaemia (I) did not significantly increase MDA in control animals resulting in a significant difference between control rats without ischaemia with DMF treatment (C + DMF) and control rats with ischaemia (C + I). DMF significantly reduced DMF in diabetic (D, panel B) rats without ischaemia (D + DMF). Ischaemia significantly increased MDA in diabetic rats and the change was prevented by DMF. The mean + SEM are shown.

3.2.3.2. RAGE

Figure 6 shows that RAGE did not change in non-diabetic rats, with or without treatment or ischaemia, whereas it significantly increased in diabetic rats with ischaemia ($p < 0.05$), and the effect was prevented by DMF.

3.2.3.3. NO_2/NO_3 and NT

Nitrite/nitrate ratios and nitrotyrosine results are shown in **Figures 7** and **8**. Results were similar to those of RAGE (**Figure 6**). Neither ischaemia nor DMF changes the basal concentrations

of NO_2/NO_3 or NT in the brains of non-diabetic rats, whereas in the diabetic animals nitrates/nitrates as well as NT significantly increased with ischaemia, and the effect was prevented by DMF.

3.2.3.4. MDA

Figure 9 shows the concentrations of MDA in the brains. Basal (no ischaemia, no treatment) concentrations were similar in diabetic and non-diabetic animals. DMF slightly (non-significantly) reduced MDA in non-diabetic rats, whereas it significantly reduced MDA in diabetic animals ($p < 0.05$). Cerebral ischaemia increased MDA in both diabetic and non-diabetic animals but the change was significant only in diabetic rats ($p < 0.05$). DMF suppressed the effect of ischaemia in diabetic rats ($p < 0.05$). Due to the non-significant changes produced by DMF in non-diabetic animals without ischaemia, there was a significant difference ($p < 0.05$) between this group and non-diabetic animals with ischaemia.

4. Discussion

This is the first time that DMF has been used and shown to be beneficial in treating cerebral ischaemia. In the present study DMF significantly reduced infarct volume in both diabetic and non-diabetic animals (similar reductions). It is important to note that infarct volume was evaluated just 8 h after cerebral ischaemia induction; thus, it could be interesting to observe if the similar effect occurs after a longer time (24 or 48 h). It is also important to note that treatment was administered 1 h after the onset of ischaemia, which is different from the experimental strategies that have been used for antioxidants (they usually are administered as a pre-treatment). One hour is a long period and results of the present study open a new horizon in the evaluation of therapeutic strategies that eventually could be used after the therapeutic window of thrombolysis, which is the current treatment (and not very efficient) in cerebral ischaemia.

Even though the reduction of infarct volume was similar in non-diabetic as in diabetic animals, DMF seems to have a better effect in diabetic rats because it significantly improved the neurophysiological score. One of the concerns of using DMF is the possibility of toxic effects. However, the toxic effect noted in workers in the synthetic leather industry is the result of chronic long-term exposure [16, 17]. Moreover, the single dose that we used in the present study (0.9 g/kg) is far from the reported LD_{50} (7.2 g/kg) [18]. Since no apparent toxic effect was noted, it seems that DMF was safe and beneficial. Moreover, the treatment was administered 1 h after ischaemia in non-diabetic as well as diabetic animals, which places the study closer to clinical setting conditions where diabetes is a risk factor for stroke, and it is virtually impossible to predict who would suffer cerebral ischaemia.

The difference of DMF effects between groups seems to be related to special conditions of diabetes pathology. It is known that long-term inflammation and oxidative stress play an important role in the cardiovascular complications of diabetes [8, 19]. Indeed, it was reported that cardiovascular, renal, and neurological complications become apparent at least 2 months after diabetes induction in the rat streptozotocin model and that those complications were attenu-

ated by inhibitors of the renin-angiotensin system, whose effects include the reduction in oxidative stress and probably inflammation [20–22]. In the present study, the diabetic animals had low weights, hyperglycaemia, and high glucosylated haemoglobin. Cerebral ischaemia was produced 5 weeks after the induction of diabetes. This means that diabetic animals were not supposed to have apparent cardiovascular complications of diabetes, although the pathophysiological events were certainly already triggered. Indeed, this could explain why even in basal conditions (brains of animals without ischaemia and without treatment) all the biomarkers had the same concentrations, and notably cerebral ischaemia significantly increased all the biomarkers only in diabetic animals, whereas MDA (biomarkers or OS effects on lipids) was the only biomarker increased in non-diabetic rats with ischaemia.

The expression of MMP9 has been related to various steps of the pathophysiology of cerebral ischaemia, such as inflammation, excitotoxicity, neuronal damage, and blood brain barrier (BBB) disturbance [6, 23]. MMP9 increased clearly in diabetic but not in non-diabetic animals with ischaemia. DMF did not prevent MMP9 incremental increases. The lack of an increment in non-diabetic animals could be due to the short time that passed after ischaemia was induced and the sacrifice (8 h).

Nitrotyrosine (NT) is the fingerprint of peroxynitrite, a nitrogen active species produced by the interaction of superoxide and nitric oxide (NO) [24]. NT is a biomarker of inflammation and OS [24]. NT increases in the brain mitochondria of diabetic animals 21 days after STZ administration [25]. NT also increases in the brains 24 h after ischaemia induction [26]. In the present study, NT was evaluated in homogenised tissue, which could explain why the increment of this biomarker was not observed in the brains of diabetic animals without ischaemia. No increment of NT was observed in the brains of non-diabetic animals, whereas it significantly increased after cerebral ischaemia in diabetic animals. Nitrotyrosine changes in diabetic animals were completely prevented by DMF. Interestingly, nitrite/nitrate (indirect measurement of NO production) and RAGE (a biomarker of neuroinflammation in cerebral ischaemia [27]) changed similarly to NT; they increased after ischaemia only in diabetic animals and such increment was prevented by DMF.

The evaluation of cerebral ischaemia was performed early after arterial occlusion (8 h). It could be the reason for not observing changes in non-diabetic animals (only MDA increment). Diabetic animals were probably more susceptible to inflammation and OS after ischaemia due to the basal conditions produced by hyperglycaemia. Such basal conditions could explain the greater effects of DMF in diabetic animals. It is necessary to study the effects of DMF for longer periods after ischaemia and on other biomarkers. The results of this study open a new horizon in the design of therapeutic strategies for the treatment of stroke.

5. Conclusion

We conclude that DMF has beneficial effects on cerebral ischaemia produced in rats. The protective effects of DMF in diabetic rats could be the result of interference with oxidative and inflammation-triggered pathways, which are exacerbated by diabetes. That could explain the

reason for the better results in diabetic rather than in non-diabetic animals. The mechanisms of the protective effects in non-diabetic rats were not clear. It is necessary to explore other biomarkers and longer periods to elucidate those effects.

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