EVALUATION OF THE ANTI-ISCHEMIC EFFECT OF THE PPARγ AGONIST, (ROSIGLITAZONE) IN PERMANENT FOCAL CEREBRAL ISCHEMIA IN DIABETIC RATS.

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ABSTRACT
Diabetes mellitus (DM) is a clear risk factor for stroke. Furthermore, diabetes has been shown to be a strong determinant for the presence of multiple lacunar infarcts in stroke patients. There has been a recent appreciation that peroxisome proliferator-activated receptors (PPARs) and their ligands may play an important role in the brain. An increasing number of studies have reported on the effects of PPAR agonists in animal models of neurological damage and disease, including the excitatory damage that occurs in stroke. The PPARγ agonist, rosiglitazone, a synthetic ligand of the thiazolidinedione class was currently used as an antidiabetic agent because of its insulin-sensitizing effect. So, the purpose of this study was to determine whether rosiglitazone may be neuroprotective in a model of permanent focal cerebral ischemia in diabetic rats. Methods: This study was carried on 96 albino rats. Rats were divided into 8 equal groups: Group (1): Control group, Group (2): Diabetic control group, Group (3): Non-treated ischemic group, Group (4): Diabetic and non-treated sham operated group, Group (5): Diabetic and non-treated permanent left middle cerebral artery occlusion group, Group (6): Treated ischemic group, Group (7): Diabetic and treated sham operated group, Group (8): Diabetic and treated permanent middle cerebral artery occlusion group. Treated groups

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ry responses in the brain [6] and to reduce infarction size against transient focal ischemia [5,7]. Cerebral ischemia is frequently accompanied by inflammation, which can worsen neuronal injury. Activation of PPAR reduces inflammation and the expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [8]. In addition, PPAR activators increase levels of Cu Zn-superoxide dismutase (SOD) in cultured endothelium, suggesting an additional mechanism by which it may exert protective effects within the brain [9].

Diabetes is a clear risk factor for stroke [10]. Diabetes mellitus (DM) was significant independent predictors of recurrent stroke in different studies of stroke [11, 12]. In the evaluation of 2-year stroke recurrence in the Stroke Data Bank, patients at the lowest risk had no history of diabetes [13]. Furthermore, diabetes has been shown to be a strong determinant for the presence of multiple lacunar infarcts in 2 different stroke cohorts [14, 15]. Glycemic control had shown to reduce the occurrence of microvascular complications (nephropathy, retinopathy, and peripheral neuropathy) in several clinical trials [16]. Data on the efficacy of glycemic control on macrovascular complications, including stroke, are more limited.

The purpose of this study was to determine whether a PPAR agonist may be neuroprotective in a model of permanent focal cerebral ischemia in diabetic rats. So that, we used the PPAR agonist rosiglitazone which is a synthetic ligand of the thiazolidinedione class and currently used as an antidiabetic agent because of its insulin-sensitizing effect. Rosiglitazone is soluble in water and can cross the blood brain barrier [17].

MATERIAL AND METHODS

Drug used:
Selective PPAR agonist: rosiglitazone, (Avanda 4 mg- GlaxoSmith Kline)

Animals used:
This study was carried on 96 male albino rats weighting 250 grams/rat. Animals were having free access to food and water. They were exposed to the same housing conditions of heat and humidity.

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mia groups but without diathermic occlusion of the middle cerebral artery.

The animals received saline or medications within 5 minutes after surgery and then once per day for successive seven days intraperitoneally. The doses of drugs were dissolved in saline in order to obtain a dose required for each animal in 1c.c saline. The dose of rosiglitazone was 3mg /kg /day IP for successive 7 days [21].

Consequent steps after left MCA occlusion:

1- Neurobehavioral evaluation:

Neurobehavioral evaluation was started on the next day after surgery and carried out for successive 6 days. It consisted of the following six tests [23].

1- spontaneous activity: The animal was observed for 5 minutes in its normal cage. The rat’s activity was assessed by its ability to approach all four walls of the cage. Scores indicate the following: Score 3: Rat moved around, explored environment, and approached at least three walls of the cage. Score 2: Slightly affected rat moved about the cage but did not approach all sides and hesitated to move, although it reached at least one upper rim of the cage. Score 1: Severely affected rat did not rise up at all and barely moved in the cage. Score 0: Rat did not move at all.

2- Symmetry in the movement of four limbs: The rat was held in the air by the tail to observe symmetry in the movement of the four limbs. Scores indicate the following: Score 3: All four limbs extended symmetrically. Score 2: Limbs on right side extended more or less slowly than those on left. Score 1: Limbs on right side showed minimal movement. Score 0: Limbs on right side did not move at all.

3- Forepaw out stretching: The rat was brought up to the edge of the table and to walk on forelimbs while being held by the tail. Symmetry in the outstretching of both forelimbs was observed while the rat reached the table and the hindlimbs were kept in the air. Scores indicate the following: Score 3: Both forelimbs were outstretched, and the rat walked symmetrically on forepaws. Score 2: Right side
each group were quickly removed and placed in ice-cold saline for 5 minutes. Both hemispheres were cut into 2-mm. coronal slices. Sections were incubated in TTC-containing saline solution (Sigma chemical Co.) for 20 minutes. Then, the slices were refrigerated in 10% formalin overnight. The infarcted areas were outlined in white [25]. The longitudinal and transverse axes were measured in mm. between the farthest two points. The percentage of infarcted size was measured by adobe photoshop-5 program.

IV- Assay of oxidative stress markers in the forebrain tissue:

Parasagittal brain slices of both hemispheres of each forebrain of the other six rats were weighed and equally divided for the assay of nitric oxide (NO) and malondialdehyde (MDA).

A. Assay of nitric oxide metabolites (nitrate and nitrite) level in the forebrain tissue:

The parasagittal slices were placed in a 5c.c plastic tube containing 1 ml Krebs-Ringer's solution and preincubated in a water bath at 37° for 1 hour with continuous carbogen (5% O₂ / 95% CO₂) aeration. Krebs-Ringer's solution had the following composition (mM): NaCl 129, MgSO₄ 1.3, NaHCO₃ 22.4, KH₂PO₃ 1.2, KCl 4.2, glucose 10.0 and CaCl₂ 1.5. Following the completion of incubation, the slices were transferred into another 5c.c plastic tube containing 0.5 ml of ice-cold tris buffer (50mM, pH 7.4). The slices were homogenized, sonified and the homogenates were centrifuged at 2500 xg (Heraeus Pepsatech Biofuge 17RS) for 30 min at 4°C. Resultant supernatants were kept at -40°C until assay within 6 weeks [26]. NO was quantified in the brain tissue via the nitrite method based on the Griess reaction [27]. The nitrate present in the sample is reduced to nitrite by reduced nicotinamide adenine dinucleotide phosphate in the presence of the nitrate reductase. The nitrite formed reacts with sulfanilamide and N-(1-naphthyl)-ethylenediamine dihydrochloride to yield a red violet diazo dye, which is measured on the basis of its absorbance in the range of 546 nm using UV. visible spectrophotometer. Known concentrations of sodium nitrite (1μmol/μL) were included as standards.
of follow up (7 days). The mean neurological scores were significantly decreased in the focal cerebral ischaemic group as compared to sham groups starting from the second postoperative day until time of sacrifice. There was a significant increase in the mean neurological scores starting on the 3rd day of occlusion and manifest until time of sacrifice in comparison to the neurological score on the 2nd postoperative day in all focal cerebral ischaemic groups (either treated or non-treated). Treatment with rosiglitazone caused significant increase in the mean neurological score starting on the 3rd postoperative day and till the time of sacrifice in the ischaemic groups (diabetic or non diabetic; gps 6,8) as compared to the non-treated ischaemic groups (gps 3,5).

II- Effect of rosiglitazone on the mean levels of fasting venous blood glucose in permanent focal cerebral ischemia (MCA occlusion): [table 2]

There was significant increase in the mean level of fasting venous blood glucose in the non-treated diabetic groups (gps 2,4,5) in comparison to that of control group (gp 1). There were non significant differences in the mean levels of fasting blood glucose between the non-treated control, sham or ischaemic diabetic groups.

There were significant decrease in the mean levels of fasting blood glucose in the rosiglitazone treated diabetic sham or ischemic groups (gps 7,8) in comparison to that of the non-treated diabetic groups (gps 4,5). This decreased level of fasting blood glucose was not significantly differed than that of the control group.

III- Effect of rosiglitazone on the infarction size [stained by 2,3,5 triphenyl tetrazolium chloride (TTC)] of brain sections in permanent focal cerebral ischemia (MCA occlusion) [Table 3, fig.1]

The infarcted size was significantly decreased in the diabetic or non diabetic MCA occluded groups treated with rosiglitazone (gps 6,8) versus that of non-treated MCA occluded groups (gps 3,5).

IV- Effect of rosiglitazone on the mean levels of oxidative stress markers (nitric oxide or malondialdehyde)
Table (1): Effect of rosiglitazone (3mg/Kg IP for successive 7 days) on neurological score after 7 days of permanent focal cerebral ischemia (MCA occlusion) in diabetic rats (Mean±SEM, n=12)

<table>
<thead>
<tr>
<th></th>
<th>Non-ischemic groups (gps 1,2,4,7)</th>
<th>Non-treated ischemic group (gp3)</th>
<th>Non-treated diabetic ischemic group (gp 5)</th>
<th>Rosiglitazone treated ischemic group (gp6)</th>
<th>Rosiglitazone treated diabetic ischemic group (gp8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2nd day</td>
<td>18±0</td>
<td>4.0±0.2</td>
<td>3.9±0.3</td>
<td>4.3±0.1</td>
<td>4.5±0.3</td>
</tr>
<tr>
<td>3rd day</td>
<td>18±0</td>
<td>5.6±0.3</td>
<td>5.5±0.2</td>
<td>8.6±0.7</td>
<td>8.8±0.6</td>
</tr>
<tr>
<td>4th day</td>
<td>18±0</td>
<td>5.6±0.3</td>
<td>5.5±0.2</td>
<td>11.9±0.8</td>
<td>12.1±0.4</td>
</tr>
<tr>
<td>5th - 7th day</td>
<td>18±0</td>
<td>5.6±0.3</td>
<td>5.5±0.2</td>
<td>12.8±0.9</td>
<td>13.2±0.3</td>
</tr>
</tbody>
</table>

P<0.05

a versus non-ischemic group
b versus non-treated ischemic group
c versus non-treated diabetic ischemic group
* versus the 2nd day # versus the 3rd day
Table (3): Effect of rosiglitazone (3 mg/Kg IP for successive 7 days) on the infarcted size of brain sections stained by 2,3,5 triphenyl tetrazolium chloride (TTC) stain after 7 days of permanent focal cerebral ischemia (MCA occlusion) in diabetic rats (Mean±SEM, n=6)

<table>
<thead>
<tr>
<th></th>
<th>Percentage of total infarcted area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-treated ischemic group (gp 3)</td>
<td>17.2 ± 0.8</td>
</tr>
<tr>
<td>Non-treated diabetic ischemic group (gp 5)</td>
<td>16.7 ± 0.9</td>
</tr>
<tr>
<td>Rosiglitazone-treated ischemic group (gp 6)</td>
<td>6.7 ± 0.5 *+</td>
</tr>
<tr>
<td>Rosiglitazone-treated diabetic ischemic group (gp 8)</td>
<td>6.3 ± 0.2*</td>
</tr>
</tbody>
</table>

P<0.05  * versus non-treated ischemic group  
+ versus non-treated diabetic ischemic group

Fig [1]: TTC stain of non-treated(A), rosiglitazone treated (B) ischemic forebrain section of rats.

A  B
can be beneficial in the neurological conditions e.g stroke [3] where glucose availability is reduced through modification of astrocyte metabolism and mitochondrial function. Also, Sundararajan et al., [8] reported that administration of troglitazone or pioglitazone 24 h before and at the time of cerebral infarction dramatically reduced infarction volume and improved neurological function following middle cerebral artery occlusion in rats. They argued that the beneficial effects of these drugs were likely due to reduced expression of the inflammatory mediators such as interleukin-1beta, cyclooxygenase-2 and inducible nitric oxide synthase which were known to exacerbate ischemic injury following stroke.

Treatment with rosiglitazone also caused significant decrease in the infarcted size in the diabetic or non-diabetic ischemic groups versus the non-treated ischemic groups. This result was in accordance to Sundararajan et al.,[38] who administered troglitazone, a PPAR- agonist, to non diabetic rats twenty-four hours before and again at the time of MCA occlusion in doses of 35, 70 or 100 mg/kg. They found that there was significant reduction in infarct volume in rats treated with 35 mg/kg and 70 mg/kg troglitazone. Troglitazone, reduces infarct size following cerebral ischemia was likely due to the drug anti-inflammatory properties. As the immunoreactivity against the proinflammatory cytokines II-1β and TNF α was reduced in the peri-infarct region of troglitazone treated rats. Furthermore, immunoreactivity against other markers of inflammation, intracellular adhesion molecule, major histocompatibility complex antigen I and cyclooxygenase-2 was also reduced in troglitazone treated animals compared with vehicle treated animals. Also, Shimazu et al., [7] noted that treatment with pioglitazone reduced the total infarct volume after transient MCAO compared with vehicle-treated rats. They hypothesized that the PPAR- agonist pioglitazone induces CuZn-superoxide dismutase, and this increase in the antioxidant CuZn-SOD is responsible for the decrease in infarct size in cerebral ischemia. Moreover, Pereira et al., [39] tested the effect of TZD-unrelated PPAR gamma agonist L-796,449 after permanent middle cerebral artery occlu-
on oxidative stress and inflammatory response induced by ischemia/reperfusion in the rat hippocampus after occlusion of common carotid artery for 30 min followed by 1 h reperfusion. The ischemia resulted in a significant increase in the generation of reactive oxygen species, nitric oxide and the end products of lipid peroxidation as well as markedly reduced endogenous antioxidant glutathione levels and up-regulated superoxide dismutase activity. Pre-treatment with either rosiglitazone or pioglitazone significantly reduced oxidative stress and nitric oxide. Also, Heneka et al., [44] demonstrated that 7 day oral treatment of PPAR agonist pioglitazone resulted in a reduction in the number of activated microglia and reactive astrocytes in the hippocampus and cortex of mice. Drug treatment reduced the expression of the proinflammatory enzyme inducible nitric oxide synthase (iNOS) in parallel to the suppression of inflammatory markers of neurodegenerative diseases. It is becoming increasingly clear that oxidative stress and excessive inflammatory response are implicated in the pathogenesis of ischemia and reperfusion injury to many organs, including the brain [45]. The brain is very susceptible to the damage caused by oxidative stress, due to the high rate of oxidative metabolic activity, high polyunsaturated fatty acid contents, relatively low antioxidant capacity and inadequate neuronal cell repair activity [46]. Overproduction of reactive oxygen species results in oxidative damage, including lipid peroxidation, protein oxidation and DNA damage, which can lead to cell death [47]. Furthermore, reactive oxygen species can activate diverse downstream signalling pathways, such as mitogen-activated protein kinases (MAPKs) or the transcription factor nuclear factor-κB (NF-κB), thus regulating expression of genes encoding a variety of proinflammatory proteins. Overexpression of cyclooxygenase-2 (COX-2) and of inducible nitric oxide synthase (iNOS) have recently emerged as important determinants of post-ischemic inflammation, which contributes to the progression of brain damage [48].

Finally, we noted that the improvement of the neurological score was the same in both non-diabetic and diabetic ischemic groups (gps 6&8 re-
ed to inhibition of ischemia-induced inflammatory markers (interleukin-1β, COX-2 and inducible nitric oxide synthase) [43]. There is a link between PPAR-induced modulation of oxidative stress and inflammation, since prevention of COX-2 induction results from oxidative stress inhibition [40]. The cellular target of these anti-inflammatory effects is probably microglial cells, since PPARγ agonists, such pioglitazone, are able to decrease microglial activation when administered intracerebrally [51]. The key target of this anti-inflammatory effect is NF-kB, which plays a crucial role in neuronal death [52]. PPARγ activation is responsible for inhibition of the NF-kB p65 monomer as well as induction of IkBa (inhibitory kB) [39]. The role of suppression of activation of p38 mitogen-activated protein kinase has also been demonstrated recently [43].

Beyond this direct effect on ischemia-induced deleterious pathways explaining neuroprotection, the challenge will be to demonstrate that a part of the neurological improvement induced by PPAR activators could be the result of neurorepair, since PPARγs are also involved in the regulation of neural stem cell proliferation and differentiation [53].

In conclusion, we showed that the PPAR-γ agonists rosiglitazone exert neuroprotective effects against cerebral ischemic injury by reducing oxidative stress and inflammatory response. This anti-ischemic effect does not depend on its anti-diabetic effect. So, this protective effects of the PPAR-γ agonists rosiglitazone might suggest a potential role of PPAR-γ agonists in modulating the events occurring late after permanent ischemic attacks that occur as a vascular complication of diabetes mellitus.

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1- مجموعة مصابة بقصور بؤرى دائم للدورة الدموية ومعالجة بدواء روزيجليتازون
2- مجموعة قياسية مصابة بمرض البوس السكري و غير مصابة بقصور بؤرى دائم للدورة الدموية المخية ومعالجة بدواء روزيجليتازون
3- مجموعة مصابة بقصور بؤرى دائم للدورة الدموية المخية في الفئران المصابين بمرض البوس السكري معالجة بدواء روزيجليتازون

وقد تم تقييم تأثير الدواء المستخدم على الخلل الناتج في الوظائف نتيجة لقصور الدورة الدموية للمخ في هذا النموذج التجيري في الفئران بدراسة:

1- السلوك الحركي للفئران من اليوم التالي لربط الشريان المخ الأيمن في VII أيام متتالية وذلك لتقييم القدرة القيادية للمخ على الجهاز الحركي.

2- في اليوم السابع، قياس نسبة السكر الصائم في الدم ثم اخذ المخ من كل مجموعة وتقسيمهم إلى مجموعتين تحتوي كل منها على 6 عينات: أحدثهما لتحديد حجم التكرر باستخدام صبغة 0.32% تراي فينيل تترازوليم كلورايد، والأخرى لقياس كل من الأكسيد النتريكي والمالونديالدهيد كدلائل الجهد الأكسيدى الناتج في أنسيمة المخ.

وأن كانت النتائج هي نجاح الروزيجليتازون في خفض ذو دلالة إحصائية في تقليل الإصابة سواء من ناحية السلوك الحركي أو حجم التكرر أو دلالات الجهد الأكسيدى هذا إلى جانب تقليل نسبة السكر في الدم. لذا كنا هذا التأثير الفعال مدعمة لأهمية دواء الروزيجليتازون في تقليل نسبة الإصابة بمضاعفات مرض البوس السكري مثل السكتة الدماغية.