INTRODUCTION

Metformin is used widely as an antidiabetic drug, but also is indicated in insulin-resistant conditions, such as polycystic ovary syndrome (PCOS), acanthosis nigricans, in the prevention of DM2 and in patients with MS. Inflammation and endothelial dysfunction are characteristic features of MS that contribute to its pathogenic cardiovascular profile. Until recently, most studies into the actions of metformin on oxidative stress, inflammation or endothelial dysfunction have been conducted in animal experiments, in patients with DM2 or in women with PCOS. There are limited and conflicting reports about the effect of metformin on several indices of inflammatory and endothelial dysfunction in MS (with or without DM2). Some studies have reported an improvement in several markers of endothelial function and/or inflammation, whereas others report amelioration of some markers of endothelial activation and coagulation without any effect on inflammatory markers.

The aim of the present study was to investigate the effect of a moderate dose of metformin in adults with MS who do not meet the criteria for diabetes mellitus, focusing on several standard clinical and metabolic variables, as well as carotid intima–media thickness and nitroxidation in metabolic syndrome: the Mefisto Study.

Eduardo Meaney,* Agustín Vela,* Virginia Samaniego,* Alejandra Meaney,* Juan Asbún,† Juan-Carlos Zempoalteca,‡ Zárate N Elisa,† Mendoza N Emma,* Martin Guzman,‡ Juan Hicks‡ and Guillermo Ceballos†

*Cardiovascular Unit, Regional Hospital ‘October 1st, ISSSTE’, †Postgraduate Studies and Research Section, School of Medicine National Polytechnique Institute, and ‡National Institute of Respiratory Diseases, Mexico City, Mexico

SUMMARY

1. Metabolic syndrome (MS) is one of the greatest public health problems in Mexico, where more than 75% of adults in urban populations are overweight or obese. Metabolic syndrome has several comorbidities, which result in a high cardiometabolic risk.

2. Some of the vasopathogenic phenomena in MS are caused by nitroxidant stress, secondary to cardiometabolic dysfunction.

3. The action of metformin to diminish or control MS remains a matter of debate.

4. In the present study, 60 patients with at least three diagnostic criteria for MS were divided into two groups. Both groups received similar dietary counselling, but one group was given 850 mg metformin daily.

5. The variables assessed were body mass index, waist circumference, systolic and diastolic blood pressures (SBP and DBP, respectively), total cholesterol (TC), high- and low-density lipoprotein–cholesterol, triglycerides (TG), fasting glucose, nitroxidant metabolites (free carbonyls, malondialdehyde, dityrosines and advanced oxidative protein products (AOPP)), nitric oxide (NO), carotid vascular stiffness, carotid intima–media thickness (IMT) and C-reactive protein (CRP).

6. After 1 year follow up, both groups reported weight loss, as well as decreases in waist circumference, SBP and DBP.

7. Patients on metformin exhibited reductions in TC and IMT and there were marked changes in nitroxidation: levels of carbonyls, dityrosines and AOPP were reduced, whereas those of NO were increased, indicating better endothelial function. In addition, in patients given metformin, CRP levels decreased.

8. In conclusion, metformin has a considerable beneficial effect on nitroxidation, endothelial function and IMT in patients with MS.

Key words: arterial stiffness, intima–media thickness, metabolic syndrome, metformin, nitroxidation.
thickness (IMT), arterial stiffness and some markers of oxidative stress and inflammation.

**METHODS**

All procedures were approved by institutional ethics and research committees and all patients provided written informed consent prior to their participation in the study.

Male and female patients, aged 35–60 years, were selected from the Risk Factors Outpatient Clinic of the Cardiovascular Unit, Regional Hospital ‘October 1st, ISSSTE’, if they fulfilled at least three of the five diagnostic criteria for MS. Sample size was calculated according to the reported changes in serum malondialdehyde (MDA), an oxidative molecule that is used as an index of liperoxidation, with the following formula:

\[ n = \frac{(Za + Zb)^2 \cdot \epsilon^2 \cdot d^2}{\epsilon^2 \cdot d^2} = 12.7 = 13 \text{ patients} \]

where \( Za \) is 1.96, \( Zb \) is 0.842, \( \epsilon \) is 0.18 mmol MDA/100 mg protein and \( d = 2.0 \text{ mmol MDA/100 mg protein} \). This estimate allowed for an alpha of 0.05 and a potency of 80%.

Patients were randomly assigned to one of two groups of 30 subjects each. The study was conducted following the procedures of good clinical practice and in accordance with international and Mexican official regulations for human clinical investigation.

Patients with overt diabetes, atherosclerotic coronary, cerebral or peripheral diseases, other forms of structural cardiac diseases, severe systemic diseases, malignancies, present or past episodes of heart failure, drug or alcohol abuse, acute or chronic inflammatory diseases or any other condition likely to interfere with the conduct of the trial were excluded. Elimination criteria included withdrawal of consent, lack of compliance with trial requirements, severe complications and insurmountable intolerance to metformin.

Weight, height and abdominal waist circumference were measured and body mass index (BMI) calculated. Systolic, diastolic and pulse blood pressures (SBP, DBP and PP, respectively) were determined using a mercury sphygmomanometer. During the fasting period, a venous blood sample was obtained to measure fasting glucose and creatinine, to enable liver function tests, and to determine total cholesterol (TC), high-density lipoprotein–cholesterol (HDL-C) and triglycerides (TG). Low-density lipoprotein–cholesterol (LDL-C) was estimated by the Friedwald formula.

The serum concentration of nitric oxide (NO) was quantified indirectly by measuring nitrites using the Griess reaction. To convert nitrites to nitrates, the 1775 Escherichia coli strain was used as a source of nitrate reductase. A 250 \( \mu \)L aliquot of plasma was incubated with 40 \( \mu \)L E. coli culture for 1 h at 37°C with constant agitation. The sample was centrifuged at 1000 \( \times \) g for 5 min and then 250 \( \mu \)L supernatant was mixed with 250 \( \mu \)L N-(1-naftil) ethylenediamine hydrochloride (0.1 g in 100 mL distilled water) and 250 \( \mu \)L sulfuramid (1 g in 100 mL orthophosphoric acid 5%) for 1 min. Optical density was determined at 540 nm.

**Advanced oxidative protein products**

Advanced oxidative protein products (AOPP) were determined using the method of Capellière-Blandin et al. A 200 \( \mu \)L aliquot of plasma (1 : 5 dilution in polybenzamine solution) was mixed with 20 \( \mu \)L glacial acetic acid. The mixture was stirred for 2 min and optical density was determined at 340 nm.

**C-Reactive protein**

High-sensitivity C-reactive protein (hsCRP) was measured in plasma in duplicate using an ELISA kit (n-hscrp; Dade Boering, Newark, NJ, USA).

**Intima–media thickness**

Intima–media thickness of the carotid wall was measured by high-resolution carotid artery B-mode echographic scans (Phillips Sonos 500 ultrasonograph; Phillips, Bothell, WA, USA) using a vascular transducer (7.5–10 MHz), which provided an axial resolution of 0.1 mm. Using a bidimensional approach, a segment 1 cm below the carotid bifurcation was selected and the image was frozen in a place free of gross atheromatosis. Two measurements were performed manually using electronic calipers from the intima–lumen to the intima–media interfaces in both near (GIM A) and far (GIM B) walls. In addition, 1 cm below the bifurcation, the internal systolic and diastolic diameters (CSD and CDD, respectively) of the main right carotid artery were measured with the electronic caliper using a reference electrocardiographic signal to define both systole and diastole. Carotid stiffness was calculated as \( \Delta P/\Delta \text{carotid diameter} \) using a modification of a previously validated formula, where \( \Delta P \) is differential pressure. Pulse pressure (PP) or \( \Delta P \) were determined by subtracting CDD from CSD (\( \Delta \text{carotid diameter} \)).
Table 1 Basal data for the two groups

<table>
<thead>
<tr>
<th>Variable</th>
<th>Metformin-treatment</th>
<th>Control group</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. men/women</td>
<td>10/20</td>
<td>7/21</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>49 ± 10</td>
<td>49 ± 8</td>
<td>NS</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>86 ± 17</td>
<td>83 ± 13</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>33.6 ± 4.6</td>
<td>33.7 ± 4.4</td>
<td>NS</td>
</tr>
<tr>
<td>Waist (cm)</td>
<td>105 ± 12</td>
<td>104 ± 12</td>
<td>NS</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>128 ± 13</td>
<td>128 ± 13</td>
<td>NS</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>81 ± 11</td>
<td>84 ± 10</td>
<td>NS</td>
</tr>
<tr>
<td>PP (mmHg)</td>
<td>47 ± 8</td>
<td>44 ± 9</td>
<td>NS</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>94 ± 16</td>
<td>98 ± 11</td>
<td>NS</td>
</tr>
<tr>
<td>TC (mg/dL)</td>
<td>214 ± 44</td>
<td>213 ± 36</td>
<td>NS</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>40 ± 11</td>
<td>43 ± 8</td>
<td>NS</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>242 ± 108</td>
<td>248 ± 160</td>
<td>NS</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>128 ± 44</td>
<td>125 ± 28</td>
<td>NS</td>
</tr>
<tr>
<td>Non-HDL-C (mg/dL)</td>
<td>173 ± 43</td>
<td>170 ± 35</td>
<td>NS</td>
</tr>
<tr>
<td>CRP (mg/mL)</td>
<td>0.58 ± 0.41</td>
<td>0.53 ± 0.54</td>
<td>NS</td>
</tr>
<tr>
<td>IMT 2 (mm)</td>
<td>1.02 ± 0.32</td>
<td>1.0 ± 0.4</td>
<td>NS</td>
</tr>
<tr>
<td>ΔP/ΔD (mmHg/mm)</td>
<td>138 ± 18</td>
<td>144 ± 16</td>
<td>NS</td>
</tr>
</tbody>
</table>

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides; LDL-C, low-density lipoprotein cholesterol; CRP, C-reactive protein; IMT 2, mean intima–media thickness; ΔP/ΔD, carotid stiffness.

Patient groups

Patients were randomly assigned to one of two groups. One group served as the control group; and the other group served as the experimental group and was given metformin. All patients received similar nutritional advice. Dietary treatment was supervised by a registered nutritionist. A Mediterranean-style diet was prescribed with a caloric content of 20 kcal/‘ideal weight’ (where ‘ideal weight’ (kg) = height (cm) – 100). According to the comorbidities identified in each patient, patients were treated with the usual antihypertensive and lipid-lowering therapies, using calcium antagonists, Angiotensin-converting enzyme inhibitors or Angiotensin II antagonists and statins to attain the standard goals for blood pressure and lipid profile in accordance with international recommendations.22,36 In addition, patients in the metformin group received 850 mg metformin once daily. All patients were followed up every 3 months. During each visit, blood pressure measurements, anthropometric variables and secondary unwanted effects were registered. Laboratory tests and ultrasonographic studies were repeated at the beginning and end of the study.

Statistical analysis

Data were analysed using SPSS 13 for Windows (SPSS, Chicago, IL, USA). For all numerical variables, the mean±SEM were calculated. One-way ANOVA was used to compare mean values and standard deviations. Statistical significance was set at P < 0.05.

RESULTS

Thirty-nine patients finished the study (29 women and 10 men): 17 patients in the control group and 22 in the metformin-treated group. Mean patient age was 49 ± 8 years in the control group and 49 ± 10 years in the metformin-treated group. Table 1 gives basal data for both groups. There were no significant differences in anthropometric, blood pressure or laboratory variables between the two groups, indicating homogeneity.
Carotid structural and functional changes

In both groups, combined IMT values were greater than reported normal values (0.6 mm; Table 5). With respect to the A measurement, a significant reduction of 0.099 mm (9%) was observed in the metformin-treated group compared with only a slight non-significant reduction of 0.021 mm in the control group. At point B, a similar trend for IMT was found: IMT was decreased slightly but significantly by 0.05 mm (4.8%) in the metformin-treated group, but remained unchanged in the control group. Carotid stiffness increased in the metformin-treated group (35 mmHg/mm, +24.5%) and diminished the control group (45.3, –26.7%); however, neither of these changes was significant following inter- or intragroup comparisons.

Nitrooxidative stress

Figures 1–6 show results for markers of nitrooxidative stress, inflammation and oxide nitrite availability, with the latter serving as an indirect sign of endothelial function.
Free carbonyls (Fig. 1) decreased significantly in the metformin-treated group (−51%, \( P < 0.01 \)), whereas the reduction in the control group was not significant (−3.76%). Malondialdehyde (Fig. 2) levels increased in both groups, but the increment was less marked in the metformin-treated group (+35%) compared with the control group (+59%). None of the differences, either between or within groups, was significant. Figure 3 shows levels of dityrosine, a marker of intermediate nitroxidation. Although levels of the marker decreased in both groups, the difference was significant only in the metformin-treated group. Figure 4 shows AOPP levels, a marker of advanced nitroxidation. Patients treated with metformin showed a significant reduction in AOPP concentrations (−49%), whereas no changes were seen in AOPP levels in control patients. Figure 5 shows changes in nitrate concentrations in control and metformin-treated patients.

Fig. 3  Intermediate nitroxidation. Although dityrosine levels decreased in both groups, the decrease was greater in the metformin-treated (■) compared with the control (□) group (34 vs 14%, respectively; \( P < 0.05 \)). Data are the mean±SEM.

Fig. 4  Advanced nitroxidation. After 1 year, levels of advanced oxidative protein products (AOPP) decreased by 49% in the metformin-treated group \( (P < 0.001; \square) \) but only by 11% in the control group \( (\text{NS}; \blacksquare) \). Data are the mean±SEM.

Fig. 5  Nitric oxide availability. Nitrite concentrations, as a marker of nitric oxide production, did not change in the control group (□) but increased significantly in the metformin-treated group (by 65%; \( P < 0.05; \square) \). Data are the mean±SEM.

Fig. 6  Inflammation. Mean values of C-reactive protein (CRP), a non-specific maker of inflammation, did not differ from normal at baseline. However, after 1 year, metformin treatment \( (\square) \) significantly \( (P < 0.02) \) reduced the CRP concentrations. \( (\blacksquare) \), control group. Data are the mean±SEM.
Although nitrate concentrations did not change in the control group, a significant increase was seen in the metformin-treated group. Finally, Fig. 6 shows the concentration of hsCRP, a non-specific marker of inflammation. Basal values of hsCRP were less than 0.6 mg/dL, the lower limit of normal. At the end of the study period (1 year), the mean CRP value had decreased significantly in the metformin-treated group, but not in the control group.

**DISCUSSION**

Abdominal obesity is often linked to a cluster of comorbidities that comprise the so-called MS. Metabolic syndrome is associated with a very high cardiometabolic risk profile, leading to direct vascular damage secondary to nitrooxidative stress, inflammation and reparative tissue healing. These, in turn, may lead to endothelial dysfunction, the common vasopathogenic route of vascular lesions.42,43

Endothelial dysfunction is characterized by a reduced capacity to produce NO and other molecules necessary for the correct functioning and structure of the endothelium.44 Dysfunctional endothelium produces pro-inflammatory and pro-apoptotic cytokines, adhesion proteins, monocytes chemotactic molecules and several pro-thrombotic and proliferative factors. The accumulation of asymmetric dimethylarginine (ADMA), which competitively inhibits endothelial NO synthase (eNOS), thus interfering with normal NO generation, is another indicator of endothelial dysfunction.45 The enzyme dimethylarginine dimethylamino-hydrolase (DDAH) metabolises ADMA, preserving endothelial function. Significant nitrooxidation occurs in both MS and DM2 and oxidation inhibits DDAH by modifying the sulphydryl portion of its chemical structure,46 contributing to the development of vascular lesions.41 In conditions such as dyslipidaemia, subarachnoid haemorrhage, hyperinsulinaemia, advanced age and renal failure, DDAH activity decreases and the concentration of ADMA increases, resulting in diminished endothelial function.42,43

The present study has identified several important issues regarding the clinical and therapeutic management of MS. Our data confirm the relative failure of dietary treatment, even if it is strictly supervised, as in the present study. As reported previously, dietary counselling produces disappointing results.44 Treatment of obesity places too much emphasis on the psychological and behavioural aspects, instead of paying attention to the essential biological aspects of the disease, which are the biochemistry and molecular determinants of appetite control.46 Despite the fact that metformin causes weight loss, in the present study there was no marked effect on weight and BMI in the metformin-treated group, even though waist circumference in both groups was reduced at the end of the study. In addition, our data indicate that this slight decrease in weight was associated with a modest reduction in blood pressure, which was significant in both groups, as expected. Thus, the purported antihypertensive effects47 of metformin were not evident in the treated group. There is conflicting evidence regarding the antihypertensive effect of metformin and some of the mechanisms suggested to account for it include less Ca2+ entering vascular myocytes, release of endothelium-derived hyperpolarizing factor, antagonism of insulin pressor activity, less noradrenergic flow, improvements in endothelium function and greater availability NO.47–49 However, it has proven difficult to establish the real effect of metformin on human blood pressure.

No effect on fasting glycaemia was noted in either group in the present study. Because less than 50% of patients had fasting glucose levels above 100 mg/dL, the hypoglycaemic effect of metformin was less obvious than that typically observed in subjects without overt dysglycaemia.40

There was a slight modification of blood lipids in both groups. Total cholesterol was decreased to a greater extent in the metformin-treated group compared with control, whereas HDL-C levels increased in both groups, but more so in the control group. There was a marginal increase in TG in the metformin-treated group, whereas TG levels tended to decrease (not significantly) in the control group. There were no changes in LDL-C in either group, but non-HDL-C levels were reduced significantly and to the same extent in both groups. These subtle changes seem to be secondary to the weight reduction rather that the result of the pharmacological action of metformin. The negligible effects of metformin on lipids in the present study are in general agreement with previous reports.51

In the metformin-treated group, a significant reduction was observed in carotid IMT at the two points selected below the bifurcation. Previous reports have described an anti-atherosclerotic effect of glitazones secondary to enhanced insulin tissue action, a reduction of hyperinsulinaemia, interruption of the transcription of pro-inflammatory signals and an improvement in endothelial function.53 Manifestation of the effects of metformin effects on IMT has been reported in patients with MS,54 but the effect of metformin on IMT has been described only in patients with MS associated with POCS.55 Carotid IMT is a sensitive marker of subclinical atherosclerosis, with considerable power to predict the development of atherosclerotic plaques, myocardial infarction and stroke.56 Stopping increases in or even reducing carotid IMT could be the consequence of improvements in blood pressure, lipids, dysglycaemia and nitrooxidative stress. In our patients, a reduction in nitrooxidative stress seems to be the main explanation for the anti-atherogenic effect of metformin.

Carotid stiffness was reduced in the control group, but increased (albeit not significantly) in the metformin-treated group. It has been reported that metformin increases vascular stiffness.59 Vascular stiffness is regulated by a complex interaction of structural and dynamic factors, including hemodynamic forces, anatomical composition of the middle arterial tunica, sodium and calcium content in both the vascular wall and interstitium, adrenergic tone, angiotensin II, hormones and oxidative stress, among others. In any case, the action of metformin on arterial stiffness remains highly speculative.

The most striking difference between the two groups was nitrooxidative stress. Compared with controls, patients treated with metformin showed a significant improvement in oxidation, nitrooxidation, inflammation and NO availability.

Under physiological conditions, reactive oxygen species (ROS) are produced in all tissues, where they play a critical role as molecular signals with the capacity to activate diverse local defence systems. The main ROS is superoxide anion (O2·−), produced mainly by NADPH.60 In the normal endothelium, the production of NO exceeds the production of O2·− and the latter is rapidly neutralized by anti-oxidant enzymes. Oxidative stress develops after the crucial and early phenomena of endothelial dysfunction take place, as described before. In the case of endothelial dysfunction, the production of O2·− is greater than the production of NO, superoxide oxidizes the NO, forming peroxinitrite (ONOO−), and starts a cascade of ROS production capable of oxidizing biomolecules such as carbohydrates, lipids, proteins and DNA. The end-products of the oxidation (peroxinitroso acid, OH radicals and nitrogen dioxide) are extremely
Nitroxidation in metabolic syndrome

It has also been shown that part of the anti-oxidant effect of metformin resides in its scavenging properties, eliminating some free radicals. Metformin cannot be oxidized by superoxide or hydrogen peroxide, but it is the target of oxidation attack by ·OH radicals.71,72 Some clinical studies have shown that metformin improves several indices of inflammation, such as the amount of vascular cells adhesion molecules (vascular cell adhesion molecule-1, intercellular adhesion molecule-1), macrophage migration inhibition factor and CRP. Activation of NF-κB increases the expression of several pro-inflammatory molecules, such as IL-6, which, in turn, stimulates the hepatic production of CRP and IL-8, which stimulate monocyte recruitment and adhesion to the vascular wall and, at the same time, stimulate neutrophil chemotaxis. Metformin inhibits the expression of NF-κB by blocking phosphatidylinositol 3-kinase, thus decreasing the production of pro-inflammatory cytokines.69 However, some studies have reported no significant effect of metformin on CRP.22,76 One of the consequences of carbonyl stress is the formation of AGE in the plasma, cells and interstitium, where they stimulate specific AGE receptors (RAGE), leading to increased production of oxidant molecules, toxic cytokines and greater collagen deposition in the extracellular matrix.77 The action of metformin on the concentration of free carbonyl highlights the drug's effect on methylglyoxal and glyoxal, with which metformin reacts, forming guanidine-carbonyl molecules and thereby decreasing the deleterious consequences of carbonyl stress.78 The action of metformin on lipoxygenation is less robust. Malondialdehyde levels increased in both the metformin-treated and control groups, even though the increase was less obvious in the metformin-treated group. The relative failure of metformin to affect lipid oxidation, compared with tiazolidinediones, has been reported previously.79 In the present study, metformin showed a marked capacity to reduce intermediate and advanced nitroxidation, abating both dityrosine and AOPP. We believe that this is the first report of the action of metformin on advanced nitroxidation.

Conclusions

Metformin is an anti-diabetic drug with multiple pleiotropic effects that is particularly suitable for correcting the cardiometabolic abnormalities of the insulin resistance syndrome. However, the effects of the drug on weight, fasting glucose levels, blood pressure and lipids are rather modest or even absent. Conversely, in the present study we observed a profound effect of metformin on several nitroxidation molecules and a modest anti-atherogenic effect (IMT).

Our therapeutic strategy should be based on the fact that the oxidation of biomolecules is part of a sequential cascade, better reflecting vascular functional state than the classical markers of inflammation and endothelial dysfunction. The concentration of several metabolites derived from nitroxidative stress demonstrates the consequences of oxidative attack on different biological substrates: carbohydrates, lipids and proteins. Our data provide evidence that metformin considerably reduces several indices of nitroxidation in MS, improving endothelial function and decreasing IMT in the carotid arteries. These findings support the use of metformin as a powerful therapeutic tool in MS, that not only prevents the development of DM2, but also counteracts nitroxidation, inflammation and atherogenesis, which are promoters of the vascular catastrophes associated with the syndrome.

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