Protective Effect of Metformin Against Walker 256 Tumor Growth is Not Dependent on Metabolism Improvement

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Key Words
Metformin • Walker 256 tumor • Apoptosis • Metabolism

Abstract
Background/Aims: The objective of the current work was to test the effect of metformin on the tumor growth in rats with metabolic syndrome. Methods: We obtained pre-diabetic hyperinsulinemic rats by neonatal treatment with monosodium L-glutamate (MSG), which were chronically treated every day, from weaning to 100 day old, with dose of metformin (250 mg/kg body weight). After the end of metformin treatment, the control and MSG rats, treated or untreated with metformin, were grafted with Walker 256 carcinoma cells. Tumor weight was evaluated 14 days after cancer cell inoculation. The blood insulin, glucose levels and glucose-induced insulin secretion were evaluated. Results: Chronic metformin treatment improved the glycemic homeostasis in pre-diabetic MSG-rats, glucose intolerance, tissue insulin resistance, hyperinsulinemia and decreased the fat tissue accretion. Meanwhile, the metformin treatment did not interfere with the glucose insulinoetric effect on isolated pancreatic islets. Chronic treatment with metformin was able to decrease the Walker 256 tumor weight by 37% in control and MSG rats. The data demonstrated that the anticancer effect of metformin is not related to its role in correcting metabolism imbalances, such as hyperinsulinemia. However, in morphological assay to apoptosis, metformin treatment increased programmed cell death. Conclusion: Metformin may have a direct effect on cancer growth, and it may programs the rat organism to attenuate the growth of Walker 256 carcinoma.

C.C. da Silva Franco and R.A. Miranda contributed equally to this work.
**Introduction**

An epidemic of overweight is growing in developed countries, reaching a prevalence of more than 50% in countries such as the United States. Furthermore, it is alarming that the incidence of overweight in developing countries is also very high [1, 2]. Worldwide, diabetes one of the most prevalent co-morbidities associated with obesity, which is also related with several types of cancer, including tumors of the breast, colon, endothelium, and rectum, while there is a lower risk associated with other types of tumors, such as prostate cancer [3-5]. Several studies have suggested that diabetes is a weak prognostic factor in cancer patients [3, 5]. The role of metabolic dysfunction, such as hyperinsulinemia, in the increased risk for cancer remains to be clarified. For more than 4 decades, a biguanide oral antihyperglycemic, metformin, has been used to treat diabetes patients throughout the world. Metformin treatment improves hyperglycemia in diabetic patients, ameliorating their health condition [6, 7]. Overall, metformin increases tissue insulin sensitivity, which reduces glycemia, and it also decreases the insulin demand [8]. It has been suggested that decreased insulinemia, indirectly stimulated by metformin, reduces the activity of cellular proliferative pathways via protein kinases, such as AMP-activated kinase (AMPK) [9]. It has also been suggested that, through the AMPK pathway, metformin inhibits the mammalian target of rapamycin (mTOR) gene, which is involved in regulating protein synthesis and cell proliferation [9, 10]. Epidemiological studies and some short-term clinical trials have indicated that metformin reduces the risk of cancer in diabetic and non-diabetic populations [11-15]. However, experimental works investigating the long-term anticancer effects of metformin treatment are lacking.

The present work investigate whether, prior the Walker 256 tumor cells graft, chronic metformin treatment is able to reduce tumor growth dependent or independent of metabolism improvement.

**Materials and Methods**

**Animals**

Newborn male Wistar rats were treated once a day with monosodium L-glutamate [MSG, 4 mg/g body weight (bw); Sigma-Aldrich, Germany] during the first 5 days after birth, MSG was injected subcutaneously into the cervical area of Wistar rat pups. Control animals were injected with an equimolar saline solution. The pups were weaned at 21 days of age [16].

During all experimental protocols, rats were kept at a temperature of 23±2 °C, a light/dark cycle of 12 h and given free access to water and a standard rodent chow diet (Nuvital®; Curitiba, PR, Brazil). The protocols for the experiments were approved by the Ethics Committee for Animal Experimentation of the State University of Maringá.

**Metformin treatment**

Just after weaning, one batch of animals from Control and MSG groups was treated with metformin (Metformin hydrochloride, Medley, Brazil), dissolved in water, at a dose of 250 mg/kg of bw, by gavage once a day, from 21 to 100 days old (Fig. 1). Another batch of animals from Control and MSG groups received water. The metformin treatment was performed by 80 days. Compared with other studies the metformin dose used in the present work have no detectable toxicity to experimental animal [17, 18]. After the metformin treatment was finished, some of the animals of both groups Control and MSG were used in the subsequent experimental proceedings, and some were used to inoculate tumor cells.

**Assessment of obesity**

At 101-day-old, a batch of animals from each experimental group, Control and MSG, treated or untreated with metformin, after 12-h fasting was weighed, anesthetized (thiopental 45 mg/kg bw), and the length was measured as the nasal anal length (NAL) to calculate the Lee index or body mass index using the following equation: Lee index = (bw [g]1/3/NAL [cm]) × 1000, which was used to obesity assessed [19]. After
the last measurements, the animals were decapitated so that the mesenteric fat pad could be removed and weighed.

**Intravenous glucose tolerance test (ivGTT)**

Under ketamine and xylazine anesthesia (3 and 0.6 mg/100 g bw, respectively), a silicone cannula was implanted into the right jugular vein of animals from both groups, Control and MSG treated or untreated with metformin and stabilized on the dorsal region of the neck. The cannula was previously treated with heparinized saline [50 IU heparin/ml 0.9% of saline solution] to avoid blood clots. After 24-h of the surgery and 12-h fast (19:00-07:00 h), without any anesthesia, a glucose load (1 g/kg bw) was infused into the animals’ vein through the cannula. Blood samples were collected immediately before the glucose load (zero time) and at 5, 15, 30 and 45 min. Plasma obtained from blood samples was stored at -20 °C for subsequent determination of the glucose concentration by the glucose oxidase method [20] using a commercial kit (Gold Analisa®, Belo Horizonte, MG, Brazil) and for the determination of insulin levels using a radioimmunoassay (RIA) [21].

**Insulin sensitivity**

Another batch of rats from both Control and MSG groups, treated or untreated with metformin, underwent a surgery to cannula implantation. After a 6-h fast and without any anesthesia the rats were submitted to an intraperitoneal insulin tolerance test (ipITT; 1 U/kg bw of insulin). Samples for blood glucose measurements were collected at zero (basal), 5, 15, 30 and 45 min after insulin injection, as described for the ivGTT. Thereafter, the glucose tissue uptake rate or the rate constant for plasma glucose disappearance (K_itt) was calculated using the formula 0.693/(t ½), as indicated in a previous report [22]. The plasma glucose t1/2 was calculated from the slope of the least square analysis of the plasma glucose concentrations during the linear phase of decline.

**Pancreatic islets isolation**

Pancreatic islets were isolated using the collagenase technique as previously described [23] with some adaptations. At 101 days of age, rats from Control and MSG groups treated or untreated with metformin were anesthetized with thiopental (45 mg/kg of bw) and decapitated, and the abdominal wall was cut and open. Then, 8 ml of Hanks buffered saline solution [HBSS, (mmol l⁻¹): NaCl, 136.9; KCl, 5.4; MgSO₄·7H₂O, 0.81; Na₂HPO₄·0.34; KH₂PO₄·0.44; CaCl₂·2H₂O, 1.26; NaHCO₃, 4.16; glucose, 0.06; BSA (bovine serum albumin) 15 and (O₂, 95% + CO₂, 5% mixed)/10 min, pH 7.4] containing (collagenase type XI, 0.1% plus BSA, 5% and HEPES [N-(2-hydroxyethyl-piperazine)-N’-(2-ethanesulphonic acid)], 0.6%, Sigma-Aldrich®, St. Louis, MO, USA) was injected into the common bile duct. The pancreas was swollen with the collagenase solution and quickly excised and incubated in a glass beaker for 17-18 min at 37 °C. The suspension was then discarded and washed with HBSS in 3 continuous washings. The islets were collected with the aid of a stereomicroscope. At least 3 rats from each of 3 different litters were used for each experimental procedure with each animal group.

**Insulin secretion stimulation**

To adapt the isolated islets to a baseline glucose concentration (5.6 mmol l⁻¹), the cells were pre-incubated for 60 min in 1 ml of normal Krebs-Ringer solution [(mmol l⁻¹): NaCl, 115; NaHCO₃, 24; KCl, 1.6; MgCl₂·6H₂O, 1; CaCl₂·2H₂O, 1; BSA, 15] at pH 7.4 containing 5.6 mmol l⁻¹ glucose. This solution was gassed with (O₂, 95% + CO₂, 5% mixed) to maintain a pH of 7.4. To study insulinotropic effect of glucose, after pre-incubation, the islets were incubated for an additional 60 min in Krebs-Ringer solution containing glucose at different concentrations: 5.6; 8.3; 11.1; 16.7; 20.0 and 24.0 mmol l⁻¹. The insulin secretion was determined by its concentration in samples from the supernatant at the end of incubations; the insulin levels were measured by RIA [21].

**Inoculation of Walker 256 cells**

The tumor Walker 256 is a spontaneous carcinosarcoma from rat mammary gland, that present aggressive biological behavior, locally invasive, with high capacity of metastasis via lymphatic and hematogenous systems [24]. The Walker 256 cells were maintained intraperitoneally in rats. After one week the cells were collected, centrifuged, resuspended in phosphate buffered saline (PBS; 16.5 mmol l⁻¹ of phosphate, 137 mmol l⁻¹ of NaCl and 2.7 mmol l⁻¹ of KCl), pH 7.4, and the cell viability was evaluated using the
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method of trypan blue exclusion. A batch of Control and MSG rats, at 101 days old, treated or untreated with metformin were inoculated subcutaneously with $8.0 \times 10^7$ viable tumor cells/animal into their right rear flanks [25]. The inoculation of the Walker 256 tumor cells was performed 24-h after metformin treatment stopped, as shown in Fig. 1.

Tumor evaluation and Characterization of cachexia

After 14 days of inoculation, 12-h fasting rats were anesthetized (thiopental 45 mg/kg bw) and sacrificed by decapitation and blood samples were collected. Their tumor mass were carefully dissected and weighed to calculate the percentage of body weight loss.

The loss of bw was calculated as follows: $LBW (%) = 100 \times \frac{(bwi - bwf + tm + gbw)}{(bwi + gbw)}$; where $LBW$: loss of body weight; $bwi$: initial body weight (g), and $bwf$: final body weight (g) of rats with tumor; $tm$: tumor mass (g); and $gbw$: body weight gain (g) of rat without tumor during the 14 days the experiment. The rats were considered cachectic when the $LBW$ was higher than 10% [25].

Hematoxylin-eosin staining (H & E) and TUNEL assay

The samples of tumor Walker 256 from each experimental group were collected and fixed in 4% paraformaldehyde, dehydrated with a graded alcohol series, diaphanized, bathed in xylene and embedded in paraffin. The 6 µm thick sections were cut with a rotary microtome, mounted on slides and stained with H & E, to confirm apoptosis in morphological criteria. The slides were analyzed with an optical microscope for the overall assessment of tumor tissue.

The presence of apoptotic cells was investigated by TUNEL assay using the ApopTag Plus Peroxidase In situ Apoptosis Detection kit (Chemicon, Cat no: S7101, USA) according to the manufacturer’s instructions. Walker 256 tumor tissues were deparaffinized using xylene dehydrate with a series of alcohol rinses and washed with PBS. The sections were incubated with a 20 µg/mL of proteinase K for 30 min for protein digestion. Then, tissues were incubated with 3% hydrogen peroxide for 5 min to prevent endogenous peroxidase activity. After washing with PBS, the sections were incubated with Equilibration Buffer. The slides were treated with TdT Enzyme at 37 °C for 60 min. After that, the sections were incubated in Stop/wash Buffer for 10 min and incubated in Anti-Digoxigenin-Peroxidase for 30 min. Apoptotic cells were observed using the Diaminobenzidine (DAB) substrate. Subsequently, the sections were counterstained with Mayer’s hematoxylin, and then mounted. Reaction buffer instead of TDT enzyme was used in negative control tissue preparations. Slides were observed and evaluated using an optical microscope.

Apoptosis quantification

Apoptosis in the Walker 256 tumor tissues was quantified by morphometric analysis as previously described [26] with minor modifications. Summarized, the area of TUNEL-positive cells was measured in five images of randomly selected fields at 400x magnification from 2 or 3 rats, using software for morphometric analysis (Image-Pro Plus software v. 4.5, Media Cybernetics, Silver Spring, MD). The percentage of apoptotic area was calculated from the areas of tumor tissues: % apoptotic area (Apoptotic index: AI) = (apoptotic cells area / non-apoptotic cells area) X 100.

Fig. 1. Schematic diagram of the experimental protocol.
Statistical analyses

The results are given as the mean±SEM and were submitted to a Student’s t test or one-way analyses of variance (one-way ANOVA), followed by a Bonferroni t test. p<0.05 was considered statistically significant. The tests were performed using GraphPad Prism version 5.0 for Windows (GraphPad Software Inc., San Diego, CA, USA).

Results

Characterization of MSG-obesity and metabolism

Table 1 shows that neonatal MSG treatment provoked a 23% decrease in bw when compared to control rats, P<0.0001. Whereas chronic metformin treatment in the control animals did not cause any change, the bw was decreased 19% in the MSG animals, P<0.0001. The body length was reduced by 14% due to neonatal treatment with MSG, P<0.0001. Chronic metformin treatment did not affect the body length in both groups control and MSG. MSG treatment induced a 6% increase in the Lee index, P<0.0001, while chronic metformin treatment caused a 6% reduction in the MSG rats, P<0.0001, without causing any changes in the control rats.
The neonatal treatment with MSG provoked a 20% increase in the area under curve (AUC) of glycemia during ivGTT compared to Control rats, \( P < 0.0001 \), as shown in the insert of Fig. 2A. While chronic metformin treatment reduced glycemia of Control rats by 14%, in the MSG rats it was decreased by 17% (\( P < 0.0001 \); Fig. 2A).

As shown in the insert of Fig. 2B, the ivGTT also revealed that MSG treatment caused a hyperinsulinemia of 68% in the AUC of insulinemia when compared to the Control rats, \( P < 0.0001 \). However chronic metformin treatments decrease 44% and 34% of the total insulinemia of MSG and Control animals, respectively, \( P < 0.0001 \).

Fig. 3 shows data from ipITT. Neonatal treatment with MSG caused a 4-fold decrease in \( K_{\text{itt}} \), \( P < 0.0001 \). While chronic metformin treatment did not change \( K_{\text{itt}} \) in control rats, the MSG rats metformin treated presented a 4-fold increase, \( P < 0.0001 \).

Different glucose concentrations induced insulin secretion in a concentration-dependent manner in rat pancreatic islets from both Control and MSG experimental groups, treated or untreated with metformin, in Fig. 4A, 4B and 4C. Whereas islets from MSG rats displayed greater insulin secretion than islets from Control rats, as shown in Fig. 4A, shifting...
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The neonatal MSG treatment induced a 2-fold increase in fat tissue accumulation compared with control rats, $P<0.0001$, as shown in Fig. 5A; however, chronic administration of metformin reduced fat accretion by 35.4% in MSG rats ($P<0.0001$), while it did not change the fat pad mass in control rats.

**Walker 256 tumor growth and metformin protect effects**

Tumor growth did not alter fat tissue accumulation in both Control and MSG groups, $P<0.0001$, as shown in Fig. 5B. Fig. 6 presented the fasting glycemia and insulinemia for all experimental groups. There was no difference in glycemia among the control group treated the curve to left, $P<0.05$, chronic metformin treatment did not change the glucose-induced insulin secretion curve of Control and MSG animals.

![Fig. 5. The effect of chronic metformin treatment on fat pad stores of rats. The bars represent the mean±SEM of mesenteric fat pad: A) Animals without tumor and B) Animals with tumor. The letters over the bars represent statistical significant differences based on one-way ANOVA ($P<0.0001$) among the groups.](image)

![Fig. 6. Effect of chronic metformin treatment on fasting glycemia and insulinemia in rats inoculated with Walker 256 tumor cells. The bars represent the mean±SEM of the fasting plasma glucose concentration in A) Control rats and B) MSG rats and fasting plasma insulin levels from C) control rats and D) MSG rats. The letters over the bars represent statistical significant differences using one-way ANOVA ($P<0.0001$) among the groups. The symbols (-) and (+) indicate the absence and presence of the tumor, respectively.](image)
or untreated with metformin and inoculated or not with the Walker 256 tumor (Fig. 6A). The tumor-bearing MSG rats showed a 20% decrease in the plasma glucose concentration compared to the MSG rats that were not grafted with tumor cells, $P<0.0005$, as shown in Fig. 6B. The fasting glycemia was reduced in both tumor grafted MSG animals, regardless of the metformin treatment.

Fasting insulinemia was increased 3-fold in the MSG animals compared with the control animals, $P<0.0001$, as shown in Fig. 6C and 6D. While chronic metformin treatment did not change fasting insulinemia in the control animals, there was a 61% decrease in the MSG rats. Tumor transplantation decreased the fasting plasma insulin levels in both control and MSG groups; however, there was a 10-fold reduction in the control rats and a 3-fold in the MSG rats. Tumor-grafted control animals treated with metformin showed a decrease of 5-fold in the fasting insulinemia ($P<0.0001$); while it was not changed in the MSG rats treated with metformin (Fig. 6C and D).

As shown in Fig. 7, the tumor growth was attenuated by 37% in rats, with a prior chronically treatment with metformin, to both groups, $P<0.0001$.

Tumor growth caused a higher bw loss in Control rats (48.8±4.8 g) than in MSG rats (11.7±3.5 g), $P<0.0001$; whereas, chronic metformin treatment reduced the bw loss significantly in control group ($P<0.0001$), it was not observed in the MSG rats, as shown in Fig. 8.

The cachexia was 19.6% and 12.7% in rats from control and MSG groups, respectively; while chronic metformin treatment decreased it by 12.6% and 10.0% in both control and MSG groups, $P<0.0001$; as presented in Fig. 9.
Microscopically histological staining with H & E shows, that Walker tumor cells displayed similar features in all groups (Fig. 10A and 10D). However, the area of zonal necrosis was more prominent in rats treated with metformin, as show in Fig. 10B and 10D.

The TUNEL assay visually evaluated using sections from tumor Walker 256. Tumor cells revealed strong, brown, positive staining in their nuclei and some of them showed fragmented nuclei in both control and MSG group (Fig. 11A and 11C). The positive staining reaction was remarkably increased in tumor cells from rats treated with metformin (Fig. 11B and 11D). AI was similar to lean and obese-MSG rats pre-treated with metformin, increasing 38.9% and 41.8% respectively (Fig. 12).

Discussion

As expected, in the present study we confirm previous reports regarding impaired metabolic hallmarks that are well recognized in the MSG model of pre-diabetic obese rats that display massive adipose tissue accumulation, peripheral insulin resistance associated to high glucose-induced insulin secretion [16]. In addition, metformin treatment improved the
metabolism of MSG prediabetic rats: normalizing the tissue insulin sensitivity and glucose tolerance, reducing fat tissue accretion and suppressing the fasting hyperinsulinemia, as previously reported [27]. By the other hand, metformin treatment did not alter the metabolism of control animals, MSG-untreated rats: insulin sensitivity, glucose tolerance, fasting insulin and glucose levels and fat tissue storage.

Independently of metformin metabolic effect, tumor growth was reduced in both lean- and MSG-rats. In human, rodents and isolated cell cultures metformin treatment inhibited tumor growth [9, 28]. For the first time the present results show that metformin chronic treatment is able to protect both control and MSG animals against tumor growth, even though when the metformin treatment was halted before tumor cells grafts. In contrast, these results are different of short-term metformin treatment effects, which decrease Walker 256 tumor growth, only in obese MSG rats, even though that metformin treatment was initiated with tumor cells graft [29]. Our data suggest that the anticancer effect of metformin is not related to its effects that improve metabolism imbalances, such as hyperinsulinemia, glucose intolerance and fat accretion.

In the current study, during the 14 days of tumor growth, the rats did not receive any metformin doses. Tumor cells were grafted 24 h after the last dose of metformin treatment, which imply that no traces of the antihyperglycemic agent could be found in the blood stream of the rats. It has been reported that metformin plasma elimination half-life, after oral multiple dosages administration, is around 4-9 h [30, 31]; which indicates that metformin might modulate the organism to attenuate tumor growth.

It has been demonstrated that Walker 256 tumor-bearing rats show low levels of plasma insulin, and it decreases glucose-induced insulin secretion in pancreatic beta-cells [32, 33]. Indeed, Walker 256 tumor growth reduced insulin levels 10-fold in control rats, while in MSG animals the decrease in the insulin levels was only 3-fold. Based on this we can suggest that the Walker 256 tumor interferes with insulin levels as much as a low plasma insulin concentration in metformin-untreated animals, which is in agreement with some previously findings [32, 33].

Rats grafted with Walker 256 tumor cells showed reduced cancer growth when they were treated with exogenous insulin [32, 34]. Unfortunately, the capacity of exogenous insulin to reduce Walker 256 tumor cells growth in MSG-rats was not tested in the current work; although, it has been shown that MSG-rats show severe insulin resistance and high plasma insulin levels [35].

Moreover, metformin did not reduce the glucose insulinotropic effect in isolated pancreatic islets from control and MSG rats. It has been documented that a major effect of metformin on the metabolic improvement is the increase of peripheral tissue insulin sensitivity; however, while reducing insulin resistance in white adipose, muscle and liver tissues, metformin treatment also reduces insulin demand, which may provoke a reduction of blood insulin levels [9].

Tumor growth was the same magnitude in MSG and control rats, the insulin decrease provoked by metformin chronic treatment did not change the cancer aggressiveness. These
results support the theory that metformin exert a direct inhibitory effect on tumor growth [36, 37]. At the same line of idea, our data show that Walker 256 tumor cells either in control or MSG-rats treated with metformin presented increased Tunel positive tumor cells, confirming that the antihyperglycemic drug might exert a direct effect on the Walker 256 tumor cells growth.

Although during 14 days there was no treatment with metformin, the drug-treated animals exhibited inhibition of the tumor growth. It allows us to speculate that metformin could protect the rat organism against tumor growth. According to Pearce and colleagues, animals immunized with a vaccine created with the objective of increasing the development of CD8 T lymphocytes, which are responsible by humoral immunological defense, treated with metformin for 3 weeks, showed less mortality after tumor cells inoculation when compared to animals that did not received metformin treatment; even though the drug treatment was halted 24 hours before the tumor grafts, suggesting that treatment with metformin improves the generation of memory T cells, resulting in enhanced antitumoral protective immunity [38].

It is also possible that metformin reduces the neoangiogenesis, which decreases the capacity of tumor to receive nutrients to the growth. One hallmark of neoangiogenesis is the increased vascular endothelial growth factor (VEGF) levels. In an ovarian tumor treated with metformin, the neoangiogenesis was inhibited showing low levels of VEGF [39, 40].

Keeping in mind that the data presented in the current work were obtained using rats and that only one type of tumor was tested, our results are consistent to conclude that early chronic metformin treatment, prior to the tumor cell grafts and/or their growth, induced an imprint effect that attenuated the cancer growth independent of the positive effects of the metformin on metabolism, which indicates a direct effect of metformin on the Walker 256 tumor growth. With respect to the rapid increase of the incidence of cancer around the world, it is important to consider the possible use of metformin to attenuate the suffering and mortality caused by cancer; perhaps it could be used as prophylactic anticancer substance in patients with a predisposition to develop tumors.

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Disclosure Statement

The authors declare that there is no conflict of interest associated with this manuscript.

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