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Metformin Improves Carbohydrate Metabolism and Minimizes Walker Tumor Growth in Young Rats

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Abstract

Cancer cachexia occurs in more than 50% of cancer patients and is characterized by body weight loss, particularly lean body mass. Metformin is a drug that is widely prescribed to type 2 diabetes patients, in whom studies have shown a lowered cancer risk. This study was conducted to analyze the effects of Walker 256 tumor cells on muscle glucose uptake and glycogen storage, as well as to examine the modulatory effect of metformin treatment on tumor growth and energy store in tumor-bearing rats. Wistar rats were distributed into 4 groups: control (C), metformin (M), tumor-bearing (W) and tumor-bearing treated with metformin (WM). Metformin was orally administered (33 mg/kg/day by gavage), and all animals were sacrificed when the relative tumor mass reached 10%. Metformin reduced muscle spoliation in the WM group, while the W group exhibited a 10% reduction in lean mass. The tumor mass ratio was markedly decreased in the WM group compared to the W group (23%, p < 0.05). Muscle and liver glycogen storage were significantly reduced in the W group, whereas metformin treatment led to an increase in glycogen storage in the M and WM groups. Liver alkaline phosphatase activity increased in both the tumor-bearing groups, but the WM group had 11% lower activity than the W group. Muscle GLUT4 expression increased in both the metformin-treated groups, and metformin treatment did not affect citrate synthase activity, which was decreased in both the tumor-bearing groups. These results suggest that metformin can attenuate the tumor effects on glucose metabolism and improve the host welfare, which is jeopardized during cancer cachexia.

Key words: Cancer; Walker 256 tumor; Metformin; Glycogen storage; Glucose metabolism.

Introduction

Cachexia has been described as a complex metabolic syndrome that may be associated with other illnesses and is characterized by the loss of muscle, which may or may not be associated with a loss of fat mass [1].

The cachexia process depends on the tumor type, especially in lung and gastrointestinal cancer, and is responsible for up to 20% of all cancer deaths [2]. Therefore, the waste of lean body mass is the most important factor for worse prognosis [3] and is highly correlated with morbidity and mortality in cancer patients [1]. Cancer patients also exhibit metabolic changes in glucose metabolism [4], such as decreased glucose uptake by peripheral tissues, even though glucose utilization by the tumor cells is greatly increased [5].

Metformin (dimethylbiguanide) (M) is currently the most commonly used drug to treat patients with type 2 diabetes mellitus (T2DM), and it has been prescribed to more than 120 million people [6]. T2DM patients treated with metformin take 500-2550 mg daily, and some observational studies have shown that metformin-treated T2DM patients have reduced cancer mortality and morbidity [7]. Although the molecular mechanism by which metformin acts is not exactly known, metformin stimulates cell signaling through insulin receptor (IR), resulting in reduced insulin resistance and decreased serum insulin levels [8].

The Walker 256 (W) tumor has been extensively used as a model to study cachexia [9]. In young rats, Walker neoplastic cells grow extremely fast, causing cachexia in less than 15 days and altering the host glucose metabolism [10]. Although the most important factor in clinical treatment is the maintenance of lean mass, the lipid and carbohydrate stores are also essential.

Thus, the aim of this study was to analyze the modulatory effects of metformin on Walker 256 tumor evolution and to evaluate the muscle and liver tissue responses, focusing on glucose metabolism and its role in the cachexia state, in tumor-bearing rats treated with the same metformin dose typically used in T2DM treatment.

Materials and Methods

Chemicals

All chemical were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise noted.

Animals and tumor implants.

Forty eight male Wistar rats (4 weeks old (100–120 g)) were obtained from the animal facility at CEMIB/State University of Campinas and were housed in collective cages under controlled temperature (22 ± 2°C) and light cycle conditions (12 h light/dark). The animals were allowed a semi-purified control diet (AIN-93G) [11] and water *ad libitum*. Walker 256 cells were originally obtained from Christ Hospital (USA) and were maintained in our laboratory through consecutive subcutaneous or intra peritoneal passages. The Walker cell suspension (1×10^6 cells) was implanted in the subcutaneous right flank. Control rats were injected with 0.9% sodium chloride. The experimental protocol (#895-1) was approved by the Ethical Committee of the State University of Campinas.

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Metformin treatment and experimental procedures

Metformin was dissolved in the drinking water (100 mg/L) and 33 mg/kg body weight was given daily by gavage administration; treatment began at the time of tumor implantation. The metformin concentration was estimated using allometric scaling [12] from human data and using the initial recommended dose for T2DM patients (500 mg/day). Forty eight animals were distributed into 4 groups: control (C), Walker 256 tumor-bearing (W), metformin-treated (M) and tumor-bearing treated with metformin (WM) (n = 12 rats per group). Tumor weight was measured using the formula $TW = -0.079768 + 0.000456 \times L \times W \times T$, where L is the length, W is the width and T is the thickness of the tumor; all parameters were measured using a caliper rule. When the tumor mass reached 10% of the body mass, the animals were subjected to overnight fasting (12 h) and were then sacrificed by cervical dislocation. Blood samples were collected by cardiac puncture to determine serum glucose, lactate and insulin levels. Gastrocnemius muscle and liver samples were collected for glycogen content assay and histochemistry.

In vitro assay for cell viability

W 256 cells isolated from animals bearing Walker tumors were cultured in 199 medium supplemented with 10% fetal bovine serum, 10,000 units/mL penicillin and 10,000 μg/mL streptomycin (LGC Biotecnologia, SP, Brazil) at 37°C in a humid incubator with 5% CO_2. The cells were seeded at a density of 1×10^4 cells per well in 96-well plates and treated with 0 (control), 10, 10^2, 10^3, 10^4 or 10^5 μM metformin for 24, 48 or 72 h. Cell viability was determined using an MTT assay [13] and quantified using a spectrophotometer with a 570 nm filter after solubilization of the formazan with DMSO.

Glucose tolerance test (GTT)

Before sacrifice (2 h), the overnight fasted animals were subjected to an intragastric gavage with 2 g/kg glucose solution, and blood samples were collected by caudal vena bleeding. A serum glucose assay was performed with samples taken at 0 min, 30 min, 60 min, 90 min, and 120 min after glucose intake. The glucose concentration was quantified by enzymatic reaction based on glucose-oxidase [14].

Periodic acid-Schiff (PAS) stain

Muscle and liver samples were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (10.9 g/L, Na_2HPO_4; 3.2 g/L, $NaHPO_4$, pH 7.4) for 24 h and embedded in paraffin wax. The paraffin wax-embedded samples were cut into 5 μm sections for histochemistry analysis using PAS stain. The integrated optical density (IOD) of the PAS positive images was determined by analyzing 20 fields (300 μm² each) from one slide; at least 3 animals were quantified per group using the Image J software (National Institutes of Health, USA).

Citrate synthase activity

Samples of gastrocnemius muscle were collected and immediately frozen in liquid nitrogen. The samples were kept at -80°C until homogenization in homogenizing buffer (2 mM EDTA in 175 mM KCl, pH 7.4). The samples were centrifuged at 16,000 g for 20 min and the citrate synthase activity was accessed using a colorimetric method [15]. All samples were tested for linearity up to 4 min of reaction and the values were normalized to total muscle protein concentration [16].

Lactate concentration

Blood samples were clarified by centrifugation at 1,000 g for 10 min and analyzed for lactate concentration using an enzymatic ultra-violet based assay [17] (Bioclin, MG, Brazil).

Alkaline phosphatase activity

Liver samples were collected, immediately frozen in liquid nitrogen and kept at -80°C until homogenization in cold phosphate-buffered saline (pH 7.4) supplemented with 0.1% Triton X-100. The homogenates were centrifuged at 16,000 g for 20 min and the alkaline phosphatase activity was measured using a colorimetric assay [18] the values were normalized to total liver protein content.

Immunoblotting

Gastrocnemius samples were homogenized in 100 mM Tris, 10 mM $Na_4P_2O_7$, 10 mM FNa, 1 mM Na_3VO_4, 10 mM EDTA, 2 mM PMSF, 0.1 mg/mL aprotinin, 0.1% Triton-X100, pH 7.4, and were centrifuged at 16,000 g for 20 min. Protein concentration was measured using a colorimetric method [16] and the sample was added to 2X loading buffer. The protein sample (40 μg) was resolved by SDS-PAGE in a 10% resolving gel and electroblotted onto a PVDF membrane (EMD Millipore, Billerica, MA, USA). The proteins were revealed using primary antibodies against GAPDH (1:1000) and GLUT4 (1:500) and secondary anti-rabbit (1:10000) and anti-goat (1:10000) antibodies (Santa Cruz biotechnology, CA, USA) after reaction with a chemiluminescent reagent (Thermo Fisher Scientific, Rockford, IL USA). The band intensity was quantified using the Image J software (National Institutes of Health, USA).

Insulin concentration

The serum insulin concentrations of the blood samples were evaluated using an ELISA kit (# EZRMI-13K) according to the manufacturer's instructions (EMD Millipore, Billerica, MA, USA).

Statistical analysis

The results were expressed as the mean + SEM. Comparisons within control and tumour-bearing groups were performed using one-way ANOVA [15] followed by Bonferroni's multiple comparison post-hoc test (Graph Pad Prism software, v3.00 for Windows 98, USA). A nonparametric t test was used when two groups were compared (W group versus WM group). Data were considered statistically significant when the P value was less than 5%.

Results

Effects of tumor growth

Table 1 shows that the tumor-bearing animals developed the cachectic state. Body weight was decreased by 10% in the W animals compared to the C group, whereas the WM group exhibited a non-significant reduction in body weight. Body mass gain was decreased in both the tumor-bearing groups W (34%) and the WM (30%) group, as was the carcass mass (18% in W and 17% in WM). In the *in vitro* experiments with W 256 cells, metformin treatment resulted in a reduction in cell viability when added at higher concentrations (from 10^3 to 10^5 μM); after 72 h exposure, treatment with 10^3 μM metformin led to a 40% decrease in cell viability (Figure 1A). The relative tumor mass (tumor mass to body weight ratio) was decreased by 23% in animals treated with metformin, compared to the W group (Figure 1B). The relative liver mass was increased in both tumor-bearing groups (W and WM = 17%) compared to the C group. The relative adrenal mass was increased in both tumor-bearing groups (W and WM = 22%) compared to the C group. Gastrocnemius mass decreased by 21% in

the W group compared to C, whereas WM exhibited a 10% reduction in muscle Compared to the M group; however, the muscle protein content did not vary among the groups. Serum glucose was highly decreased in both tumor-bearing groups (W = 48%; WM = 41%) (Table 1). The serum insulin level was reduced by 18% in the tumor-bearing group compared to the C group; however, the metformin treatment also decreased the insulin content by 32% and 48% in the M and WM groups, respectively. The serum lactate concentration increased 31% in the W group, but increased only 14% in the WM animals compared to the control group (Table 1).

Tumor growth decreased blood glucose levels

GTT analysis showed that the area under curve decreased in both tumor-bearing groups (W = 36%; WM = 28%) (Table 1). Serum glucose levels decreased in both the tumor-bearing groups (W = 48%; WM = 47% C); conversely, the GTT results showed that metformin was not able to minimize the difference in glycemia between the WM and C groups (Table 1). Serum glucose was decreased by 10% in the M group compared to the C group, demonstrating that metformin was able to stimulate glucose uptake in peripheral tissues (Table 1); however, the area under the curve from the GTT analysis showed similar values for both the M and C groups.

Tumor growth negatively affects liver glycogen storage while metformin treatment modulates this phenotype

Glycogen storage, as assessed by PAS staining, decreased by 28% in the W group compared to C, whereas it was similar in WM and increased by 26% in M (Figure 2A and 2B). Glycogen storage increased by 44% in WM (Figure 2A and 2B) as compared to the W group. The alkaline phosphatase activity in the liver increased in both tumor-bearing groups as compared to the C group; however, this enzyme activity was further reduced in the WM group than in the W group (W = 40%; WM = 25%) (Figure 2C). When comparisons were made between the two tumor-bearing groups, the alkaline phosphatase activity in the WM group was 11% lower than in the W group and was statistical similar to C (Figure 2C).

Tumor growth affects the gastrocnemius muscle and metformin treatment improves its energy storage

The muscle glycogen storage was decreased by 40% in the W animals compared to the C animals, and this reduction was not statistically

significant. On the other hand, in both metformin-treated groups, glycogen storage was greatly increased (2.3-fold in M; 1.1-fold in WM) when compared with C (Figure 3A and 3B). Comparison between the tumor-bearing groups showed that glycogen storage was increased 2.5 times in the WM group compared to the W group. GLUT4 expression was similar in the W and C groups, whereas it increased in the metformin-treated groups, by 13% in M and 17% in WM (Figure 3C). Comparison between the tumor-bearing groups showed that GLUT4 expression in the WM group was 14% higher than in the W group (Figure 3C). Citrate synthase activity in both tumor-bearing groups decreased approximately 23% when compared with C (Figure 3D).

Discussion

We showed that metformin treatment could improve the host cachectic state, enhance the liver and muscle responses, and minimize Walker tumor evolution. We also showed that Walker tumor growth led to a loss of body mass associated with other metabolic changes in the tumor-bearing rats, demonstrating that this type of tumor can lead to damaging effects on the host. The most important point of this paper is that we treated the tumor-bearing animals with metformin at the same dose used to treat T2DM patients, showing that at even a common dose, this treatment could improve the host responses (carbohydrate metabolism) and also reduce the relative tumor mass.

Most of the tumor cells use glucose anaerobically, even under normal oxygen conditions. This feature was previously described by Otto Warburg, who demonstrated that neoplastic cells had greater glycolytic activity than normal cells, even in normoxia [19]. The increasing anaerobic use of glucose is not due to just one reason but can be associated with mutations in mitochondrial genes, increased expression of low Km hexokinase isoforms, oncogenic signals such as *ras* and src and expression of hypoxia-inducible factor (HIF) [20]. The increased glucose consumption provides the neoplastic cells with precursors for DNA, protein and lipid synthesis, as well as increases the serum lactate concentration. Solid and fast growing tumors, such as Walker 256 carcinoma, need to overcome the absence of vascularization to keep growing, and, for this reason, the use of glucose anaerobically can provide the ATP necessary to maintain metabolic reaction, as well as to convert pyruvate into lactate to regenerate NAD+.

Lactate produced by neoplastic cells is responsible for the decrease in the pH of the tumor microenvironment, which plays a crucial role in the activation of HIF. This activation is responsible for the stimulation

	C (n = 12)	W (n = 12)	M (n = 12)	WM (n = 12)
Initial body mass (g)	108.3 ± 4.4	105.8 ± 4.0	112.0 ± 4.1	108.4 ± 3.9
Final body mass (g)	199.6 ± 5.2	179.2 ± 5.6*	204.8 ± 4.3	184.7 ± 3.7
Mass gain (%)	85.7± 3.1	56.8± 3.7*	84.2± 3.4	58.7± 4.2*
Carcass mass (g)	165.5 ± 5.5	135.0 ± 4.1*	164.5 ± 5.2	136.1 ± 3.2*
Relative liver mass (%)	5.2± 0.2	6.1 ± 0.1*	5.2± 0.2	6.1± 0.2*
Relative muscle mass (%)	0.66± 0.04	0.52 ± 0.01*	0.60± 0.02	0.54± 0.01*
Muscle protein (µg/µL)	6.7 ± 0.37	5.8 ± 0.32	6.9 ± 0.46	6.7 ± 0.60
Relative adrenal mass (%)	0.027± 0.002	0.033± 0.002*	0.027± 0.001	0.034± 0.003*
Liver protein (µg/µL)	5.25± 0.16	5.09± 0.03	5.01± 0.02	5.01± 0.03
Serum glucose (mg/dL)	89.8± 4.8	46.6± 4.8*	81.1± 5.1	47.5± 3.9*
GTT -area under curve (mg/dL x 90 min)	7336± 336	4417± 393*	7397± 468	5341± 483*
Serum insulin (ng/mL)	0.563± 0.036	0.463± 0.069	0.384± 0.082	0.287± 0.057*
Serum lactate (mg/dL)	19.69± 0.96	25.83± 0.57*	19.84± 0.42	22.42± 0.51**

Legend: C: control; W: Walker 256 tumor-bearing; M: metformin-treated; WM: Walker 256 tumor-bearing and treated with metformin. The results are expressed as the mean ± SEM. *P < 0.05 versus the C group; ** P<0.05 versus the W group.

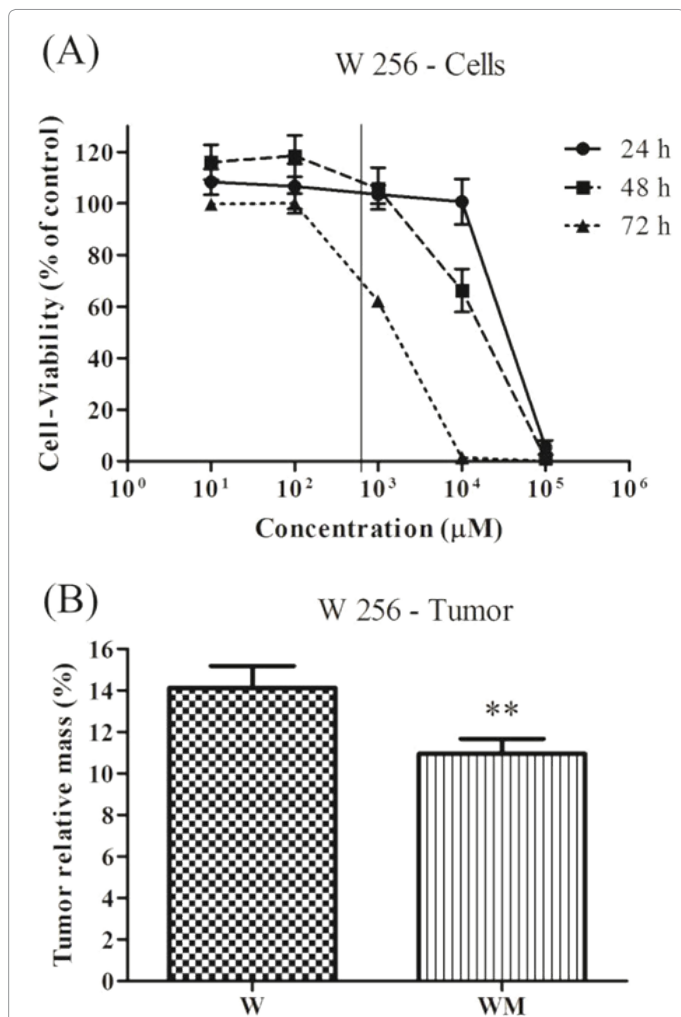**Table 1:** Tumor growth effects on body and organ growth

Figure 1: Effects of metformin on cell viability and tumor growth. (A) Dose-response curve for metformin treatment of W256 cells cultured in vitro for 24, 48 and 72 h. The vertical line shows the same metformin concentration used in the in vivo experiments (600 μM). The results represent the mean ± SEM of three independent experiments. (B) Relative tumor mass in the W and WM groups. Legend: W - tumor-bearing group; WM - tumor-bearing rats treated with metformin. The minimum number of animals used per group was 8. The columns represent the mean ± SEM. ** P<0.05 versus the W group.

tumor-bearing rats than the non-administered tumor-bearing ones. Meanwhile, we also found that metformin inhibited tumor proliferation and cell viability based on the cell culture data, confirming the lower tumor mass to body weight ratio. The reduced tumor mass most likely led to a decrease in the serum lactate concentration in the WM group, which should result in less substrate for the Cori cycle, thus attenuating the tumor growth effects in host tissues, such as liver and muscle. Many studies have confirmed that neoplastic cells produce high lactate levels and consume large amounts of glucose, as confirmed by measuring the lactate dehydrogenase activity and glucose uptake [23]. In this work, we did not measure the activity of this enzyme; however, in our previous work, we showed that this enzyme had increased activity in the tumor-bearing rats. These findings suggest that metformin likely altered the lactate dehydrogenase activity of the tumor cells, and, once the Walker tumor induces host cachexia, reducing the tumor activity most likely benefited the host carcass. It is known that Walker 256 induces cachexia likely through cachexia mediators, which are released by the tumor itself or the host, such as IL-6, TNF-α and proteolysis-inducing factor (PIF) (2); in our previous work, we have observed higher levels of these factors in Walker tumor-bearing animals [24,25]. It is possible that the decreased tumor growth caused by metformin treatment was associated with a decreased release of these cachexia mediators, thus attenuating the tumor effects on the host carcass. A recent study shows that high doses of metformin (500 mg/kg/day) can lead to an efficient decrease in tumor growth in vivo and *in vitro* [26]. In our previous experiment, we also found reduced Walker tumor growth following treatment with 500 mg/kg/day metformin (data not shown). In this study, we demonstrated that even low doses (similar dose given in T2DM patient, corresponding to 600 μM) can be effective at attenuating the tumor growth rate and its effects in the host body *in vitro* and in vivo.

Additionally, glycogen storage increased in the muscle and liver tissue in the tumor-bearing metformin-treated animals. We suggest that the increased GLUT4 expression stimulated by metformin could be responsible for its increased translocation to the cellular membrane,
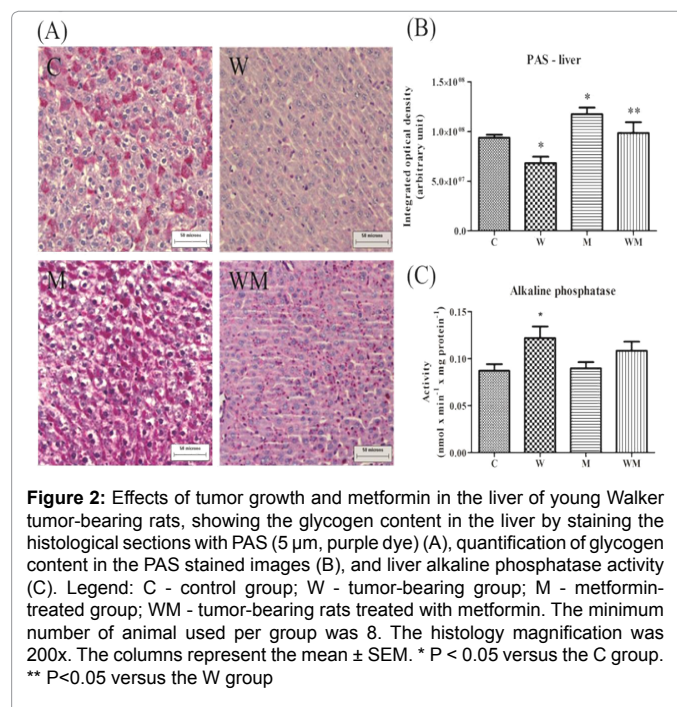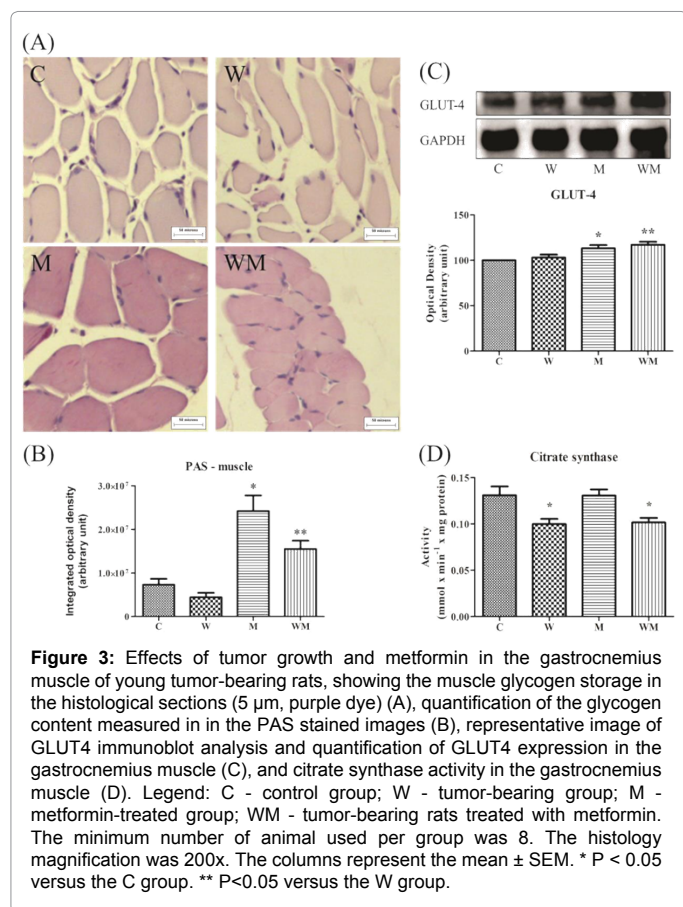
of angiogenesis, glucose uptake, glycolysis, growth factor signaling, apoptosis, invasion and metastasis [21]. As a matter of fact, our data could suggest that metformin treatment was able to attenuate HIF activation, leading to a reduced tumor mass in relation to the carcass weight. In parallel, tumor evolution results in increased adrenal gland activity, as adrenocorticotrophic hormone (ACTH) is enhanced in these tumor-bearing groups; the increase in ACTH leads to increases in both corticosterone and catecholamine hormones, which results in disposable serum glucose (energy mobilization). Although, metformin treatment improved glycogen storage, in this case, the metformin treatment was not able to reverse the increase in ACTH (1.9-fold increase in W group and 1.7-fold increase in WM). Tumor growth with increased Cori cycle activity can result in energy expenditure [22] and contributes, at least in part, to cachexia. The Cori cycle is a pathway in the liver that converts lactate into glucose, resulting in a net loss of 4 ATP molecules for each glucose molecule. Metformin was able to reduce the relative tumor mass, as it was significantly lower in the metformin administered

Figure 2: Effects of tumor growth and metformin in the liver of young Walker tumor-bearing rats, showing the glycogen content in the liver by staining the histological sections with PAS (5 μm, purple dye) (A), quantification of glycogen content in the PAS stained images (B), and liver alkaline phosphatase activity (C). Legend: C - control group; W - tumor-bearing group; M - metformin-treated group; WM - tumor-bearing rats treated with metformin. The minimum number of animal used per group was 8. The histology magnification was 200x. The columns represent the mean ± SEM. * P < 0.05 versus the C group. ** P<0.05 versus the W group

Figure 3: Effects of tumor growth and metformin in the gastrocnemius muscle of young tumor-bearing rats, showing the muscle glycogen storage in the histological sections (5 μm, purple dye) (A), quantification of the glycogen content measured in in the PAS stained images (B), representative image of GLUT4 immunoblot analysis and quantification of GLUT4 expression in the gastrocnemius muscle (C), and citrate synthase activity in the gastrocnemius muscle (D). Legend: C - control group; W - tumor-bearing group; M - metformin-treated group; WM - tumor-bearing rats treated with metformin. The minimum number of animal used per group was 8. The histology magnification was 200x. The columns represent the mean ± SEM. * P < 0.05 versus the C group. ** P<0.05 versus the W group.

leading to increased glucose uptake by the muscle, even under conditions of low insulin. Studies have shown that metformin is able to stimulate the AMP-activated protein kinase (AMPK) [27]. Although the mechanisms remain unclear, one of the roles of AMPK is to stimulate glucose transport through the cellular membrane by stimulating GLUT4 translocation in muscle and lipid cells [28]. Our study is in accordance with the literature, as both metformin-treated groups demonstrated an increase in GLUT4 expression, possibly leading to higher glucose transport in the muscle, which most likely enhanced the muscle glycogen storage. Conversely, other studies reported no changes in glycogen content and synthesis in the muscle because AMPK is activated by metformin. The increased glycogen content in the muscle of both metformin-treated animals may be beneficial to save energy, especially under the catabolic conditions established in the tumor-bearing rats. We verified that there was low citrate synthase activity in both tumor bearing groups, and we suggested the catabolic state found in the host could be associated with a low oxidative capacity of skeletal muscle to minimize energy wasting.

Metformin also increased glucose storage as glycogen in the liver, although the levels did not reach the levels observed in the gastrocnemius muscle. Metformin was able to decrease hepatic gluconeogenesis [27], allowing the glucose that was taken up by hepatocytes to be stored as glycogen. Glycogen storage increased significantly in both metformin-treated groups when compared to their respective controls, suggesting that metformin treatment could preserve the energy store that is important in this catabolic state. Alkaline phosphatase activity can be used to test liver function [29], which could be impaired during this catabolic state due to tumor development, as we observed an increase

in liver weight in both tumor-bearing rats. During cancer evolution, the increased liver weight is the first phenotype that was observed, and this may be a consequence of higher activity cell and synthesis, particularly of C-reactive protein [20], as our previous work showed that tumor-bearing animals had high levels of C-reactive protein. The decreased tumor growth caused by metformin treatment can be responsible for the decreased alkaline phosphatase activity in hepatic cells, indicating the tumor effects on the host were as pronounced as in tumor-bearing group without metformin. This fact suggests that even a common metformin dosage (the same dose used to treat T2DM patients) could result in benefits, as metformin improved energy storage, as well as the tissue response of both the liver and muscle, and also enhanced the muscle glucose transporter, which was associated with decreased tumor growth. Further studies are underway in our laboratory to determine how metformin could counteract the tumor effects on carbohydrate metabolism in parallel with body mass wasting during cancer-induced cachexia. In conclusion, this study shows that low-dose metformin treatment minimized tumor development, as a possible antitumoral agent, and was beneficial to the cachectic state in tumor-bearing rats by improving carbohydrate metabolism.

Acknowledgments

Disclosure statement

The authors declare that they have no competing interests.

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