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Caffeine induces metformin anticancer effect on fibrosarcoma in hamsters

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Abstract. – OBJECTIVE: We investigated the effect of metformin and caffeine on fibrosarcoma in hamsters.

MATERIALS AND METHODS: 32 Syrian golden hamsters of both sexes, weighing approximately 100 g, were randomly allocated to 3 experimental and 2 control groups, with a minimum of 6 animals per group. 2 x 10^6 BHK-21/C13 cells in 1 ml were injected subcutaneously into the animals' back in 4 groups. The first experimental group started peroral treatment with metformin 500 mg/kg daily, the second with caffeine 100 mg/kg daily and the third with a combination of metformin 500 mg/kg and caffeine 100 mg/kg daily, via a gastric probe 3 days before tumor inoculation. After 2 weeks, when the tumors were approximately 2 cm in the control group, all animals were sacrificed. The blood was collected for glucose and other analyses. The tumors were excised and weighed and their diameters were measured. The tumor samples were pathohistologically (HE) and immunohistochemically (Ki-67, CD 31, COX IV, GLUT-1, iNOS) assessed and the main organs toxicologically analyzed, including the control animals that had received metformin and caffeine. Tumor volume was determined using the formula $LxS^2/2$, where L was the longest and S the shortest diameter. Ki-67-positive cells in the tumor samples were quantified. Images were taken and processed by software UTHSCSA Image Tools for Windows Version 3.00. Statistical significances were determined by the Student's *t*-test.

RESULTS: The combination of metformin and caffeine inhibited fibrosarcoma growth in hamsters without toxicity.

CONCLUSIONS: Administration of metformin with caffeine might be an effective and safe approach in novel nontoxic adjuvant anticancer treatment.

Key Words:
Metformin, Caffeine, Hamsters, BHK-21/C13, Fibrosarcoma.

Introduction

Metformin decreases glycolytic capacity, mitochondrial respiration and energetic efficacy in lymphocytic leukemia cells *in vitro*[1]. It also causes ATP depletion, accumulation of AMP and phosphorylation of AMPK (AMP-activated protein kinase)[1]. AMPK is a negative regulator of the Warburg effect. The activation of AMPK and reduction of glucose metabolism (inhibition of oxidative phosphorylation) by metformin (mitochondrial poison) opposes tumor progression. Glucose-starvation or glycolysis inhibition potentiates this effect[1].

AMPK reduces mTOR (mammalian target of rapamycin) complex 1 (mTOR C1) signaling and S6K1 (ribosomal protein S6 kinase 1) phosphorylation implicated in protein synthesis and cancer cell proliferation[2]. The participation of mTOR in the genesis of sarcoma is related to the IGF (Insulin-like Growth Factor) system in these tumors through the upregulation of insulin/IGF-1 receptor signaling pathway[2]. The inhibition of mTOR and mTOR C1-mediated signal pathway results in a direct antiangiogenic effect[2]. This makes mTOR inhibitors a natural choice to test in sarcomas[2].

Metformin inhibits the matrix metalloproteinase-9 (MMP-9) activity (independently of AMPK), as well as the invasion and migration of human fibrosarcoma cells *in vitro* via Ca-dependent signaling pathways. Therefore, metformin has the potential to be an antisarcoma drug[3].

Metformin can function as an antifolate that induces the alteration of carbon flow through folate-related one-carbon metabolic pathways, which secondarily induces the ATM (ataxia teleangiectasia mutated) kinase and downstream AMPK in

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breast cancer cells *in vitro*[4]. Metformin acts in a manner similar to an antifolate chemotherapeutic agent, inhibiting DNA replication and cell proliferation[4]. This action does not imply direct inhibition of the folic acid cycle enzymes, targeted by methotrexate, and thereby induces significantly fewer side effects[4]. A few studies have addressed vitamin B12 metabolism alteration behind metformin´s anticancer activity[4,5].

Metformin *in vitro* caused the transcriptional regulation of unfolded protein response in breast cancer cells[6]. Metformin can inhibit the PLCε gene expression, Notch1/Hes and androgen receptor signaling pathways, as well as proliferation, invasion and apoptosis in the castration resistant prostate cancer cell culture[7]. Moreover, metformin was demonstrated to influence autophagy, cell migration (metastatic state), senescence, cancer stem cells and cell immunity in various cancer cell lines[8,9].

An appropriate combination may improve metformin's efficacy. The combination with 2-deoxyglycose, a competitive inhibitor of glycolysis (widely used in PET/CT scanning), was more effective *in vitro* than either compound alone against human carcinoma (gastric, esophageal, breast, prostate)[10,11] and in eight sarcoma cell lines[12]. Sarcoma cells in cultures were 2 to 5-fold more sensitive to the combination than normal cells. The synergistic anticancer action of metformin and natural antioxidant caffeic acid is exhibited through the regulation of mitochondrial metabolism in cervical carcinoma cells[13].

Like metformin, caffeine can induce apoptosis in various human cancer cell lines, such as neuroblastoma[14], lung[15] and pancreatic[16] adenocarcinomas, leukemia cells[17] and non-small lung carcinoma[18]. Furthermore, caffeine enhances the toxicity of radiation and sensitivity of cancer cells to chemotherapy[19].

The presence of caffeine increases the cisplatin-induced lung carcinoma cell killing *in vitro* by inducing ATM activation and changing the activity of ATR, two important protein kinases involved in DNA damage-induced cell cycle arrest and apoptosis[20].

An important part of our research motivation was the finding that caffeine enhanced pemetrexed's antifolate activity in the four studied mesothelioma cell lines[21]. An increase in pemetrexed-induced phosphorylation of ATM and a delay in cell cycle progression by caffeine[21] are consistent with an earlier report of superactivation of ATM/ATR in cells exposed to caffeine[22].

Based on the above-mentioned separate *in vitro* evidence of metformin's[4] and caffeine's[21] antifolate activity by ATM induction, we perceived a possible synergistic anticancer effect of these drugs. There are no published *in vitro* or *in vivo* experiments about the anticancer effects of the combination of these two drugs. Furthermore, host metabolism may be indirectly anticancer prepared with metformin by the reduction of gluconeogenesis[10], circulating insulin[10] and total serum bile acids[23]. Additionally, caffeine promotes an antitumor immune response by the stimulation of adenosine A receptors[24]. There are a few contradictory studies on metformin's anticancer effect *in vivo* on solid tumors, including sarcomas, in mice[10, 25-28]. On the other hand, caffeine can inhibit UVB-induced skin cancer in mice[29]. Caffeine also reduced the rate of 3-methylcholanthrene intramuscularly induced fibrosarcomas and inhibited tumor growth after fibrosarcoma cell inoculation in mice[24]. In addition, the antisarcoma effect of these two drugs has not been investigated in hamsters so far. Therefore, the aim of this study is to answer whether metformin, caffeine and their combination can suppress solid tumor growth in an experimental animal model such as fibrosarcoma in hamsters.

Materials and Methods

Animal Model

The study was carried out on 32 adult Syrian golden hamsters aged between 12 and 20 weeks (weighing approximately 100 g) of both sexes, after obtaining the positive opinion of the Ethics Committee of the University of Novi Sad, Faculty of Medicine, Republic of Serbia, under approved Institutional Experimental Animal Care and Use of Protocols.

The animals were randomized into 3 experimental and 2 control groups (with a minimum of 6 hamsters per group). The treatment with metformin, caffeine (Galenika, Belgrade, Serbia) and their combination in experimental groups was initiated 3 days before subcutaneous inoculation of BHK-21/C13 cells[30] (2×10^6 in 1 ml) into the animals' back, for the production of the s.c. tumor (BHK fibrosarcoma[31]). It was continued for 14 days after inoculation (when the tumors were approximately 2 cm in the untreated control group). At the end of the experiment, all animals were sacrificed, including those treated for 14 days in the control group that had received metformin and caffeine without tumor inoculation.

For the treated groups, metformin was dissolved in physiological saline and administered once daily via a gastric probe in a dose of 500 mg/kg (in 1 ml per100 g weight), equivalent to a human dose of 40 mg/kg (by normalization to surface area), which is the maximum dose used in diabetic patients. Caffeine 100 mg/kg was prepared and administered in the same way. The control group with tumors received isovolemic vehicle only (1 ml/100 g animal weight).

At sacrifice, the tumors were excided, tumor weight and size were measured and tumor volume was calculated as $L \times S^2/2$ (L was the longest, and S the shortest diameter). Tumor slices were assessed pathohistologically and immunohistochemically for verification of tumor growth and angiogenesis. Blood samples were collected for glucose and other conventional blood tests (erythrocytes, leucocytes, lymphocytes, monocytes, granulocytes, platelets, hemoglobin, hematocrit, MCV, MCH, MCHC, serum proteins, albumins, sedimentation, partial thromboplastin time). Because the dosage of metformin used in our study (500 mg/kg/d) was higher than 850 mg/d usually used in diabetic patients, the weight of hamsters was measured to evaluate possible side effects caused by metformin and/or caffeine. The main animal organs were analyzed toxicologically.

Immunohistochemistry

Besides the principal HE staining, immunohistochemical Ki-67, CD 31, COX IV, GLUT-1 and i NOS staining (Sigma-Aldrich, St. Louis, MO, USA) were performed for the determination of tumor proliferation, penetration, vasculature and necrosis.

For Ki-67 staining, tumor slices were fixed in cold methanol, blocked with 4% bovine serum albumin/phosphate buffered saline (BSA/PBS) for 1 hour at room temperature and incubated with anti-Ki-67 pAb (1:50, Sigma-Aldrich, St. Louis, MO, USA). Polyclonal anti-rabbit FITC (1:80, Sigma-Aldrich, St. Louis, MO, USA) was used as a secondary antibody. Nuclei were counterstained with Hoechst 33256 (Sigma-Aldrich, St. Louis, MO, USA). Images were taken using Leica MC190HD camera (Leica Camera AG, Wetzlar, Germany) and processed by software UTHSCSA Image Tools for Windows Version 3.00[32]. In each sample image, the number of Ki-67-positive cells was obtained. The mean numbers of Ki-67-positive cells (20 tumor images of each animal) were compared between the groups.

Statistical Analysis

The differences between the groups in tumor weight, volume, mean number of Ki-67-positive cells marked on images and other measured parameters were determined using the Student´s *t*-test. The results were considered to be statistically significant at $p<0.05$.

Results

Subcutaneous inoculation of BHK-21/C13 cells into hamsters resulted in fibrosarcoma formation at the site of injection in all inoculated animals. Peroral treatment with a metformin and caffeine combination significantly inhibited tumor growth. This was verified by significantly decreased tumor weight and volume and by reduced proliferation status of tumor cells as shown by Ki-67 staining on hamster tumor sections (Table I.). Note that in this study only the combination of metformin and caffeine was related to a statistically significant antitumor effect.

Pathohistological and immunohistochemical evaluation revealed a decrease in tissue penetration, an expansion of necrosis and hemorrhagic areas and a reduction of vasculature in all analyzed slices of tumors treated with the combination of metformin and caffeine, when compared with controls (Figure 1).

The treatments had no significant effect on the body weight of the animals during the course of the study (Table I).

Metformin caused a slight decrease in the fasting blood glucose levels in the hamsters, as shown in Table I. Also, the experimental and control groups were statistically compared in terms of red and white blood cells, platelet number, hemoglobin levels, hematocrit, serum proteins, sedimentation, activated partial tromboplastin time and other examined blood laboratory values, but no significant difference was observed between the groups. Examination of the main organs revealed no pathological or toxicological changes in the experimental and control groups.

Discussion

The combination of metformin 250 mg/kg/d and 2-deoxyglucose 500 mg/kg/d administered intraperitonealy significantly inhibited subcutaneous tumor growth in mouse xenograft models

Table I. Characteristics of animals and tumors in control and groups treated with metformin and caffeine.

Hamster No	Weight (g)		Sex	Tumor			Serum glucose mmol/l
	Beginning	End		Weight mg	Volume cm^3	Ki-67 $\bar{x}$ for 20	
Control group with inoculated tumor, without treatment							
1	91	95	F	2060	1.50000	17	4.8
2	105	117	F	6880	5.00000	23	3.9
3	93	94	F	260	0.18750	14	3.3
4	94	110	F	1720	1.00000	20	5.1
5	105	101	M	2910	2.11981	18	4.5
6	97	95	M	1400	1.25025	19	4.2
$\bar{x}$	97.5	102		2538	1.84293	18.5	4.3
±SD	6.12	9.51		2297	1.67114	3.02	0.65
Control group without tumor inoculation, treated with metformin 500 mg/kg and caffeine 100 mg/kg							
1	103	110	M	0	0	0	2.2
2	92	98	M	0	0	0	2.7
3	108	110	F	0	0	0	4.0
4	80	91	M	0	0	0	4.0
5	89	98	F	0	0	0	7.2
6	101	105	F	0	0	0	3.8
$\bar{x}$	95.5	102					4.0
±SD	10.37	7.62					1.74
Treated with metformin 500 mg/kg							
1	91	89	F	10	0.0240	6	6.9
2	90	93	M	450	0.01250	14	3.9
3	107	120	F	1880	0.7955	16	2.9
4	87	92	M	1950	1.680	25	3.7
5	65	72	F	1390	0.870	16	4.1
6	88	95	M	1410	0.690	15	3.3
7	77	79	F	1500	0.710	16	4.6
8	72	75	F	190	0.042	7	3.4
$\bar{x}$	84.63	89.37		1097.5	0.6171	14.38	4.1
±SD	13.04	15.15		766.23	0.5561	5.93	1.24
p (*t*-test)				> 0.05	> 0.05	> 0.05	> 0.05
Treated with caffeine 100 mg/kg							
1	88	100	F	1500	1.2091	18	10.2
2	76	89	F	1300	1.2567	12	3.0
3	90	105	M	3000	2.2180	15	3.4
4	92	90	F	820	0.6981	10	2.9
5	115	120	M	3100	2.8790	20	6.2
6	78	85	M	4200	3.5221	22	3.9
$\bar{x}$	89.83	98.17		2320	1.9638	16.17	4.9
±SD	13.95	13.04		1309	1.0956	4.67	2.85
p (*t*-test)				> 0.05	> 0.05	> 0.05	> 0.05
Treated with metformin 500 mg/kg and caffeine 100 mg/kg							
1	94	74	M	350	0.180	16	3.5
2	67	76	M	340	0.216	13	3.3
3	77	78	F	300	0.245	12	3.2
4	105	92	F	1050	0.648	18	3.8
5	91	90	M	160	0.080	8	3.8
6	110	118	F	310	0.198	13	4.0
$\bar{x}$	90.67	88		418	0.261	13.33	3.60
±SD	11.31	31.11		317	0.198	3.44	0.32
p (*t*-test)				< 0.05	< 0.05	= 0.02	< 0.05

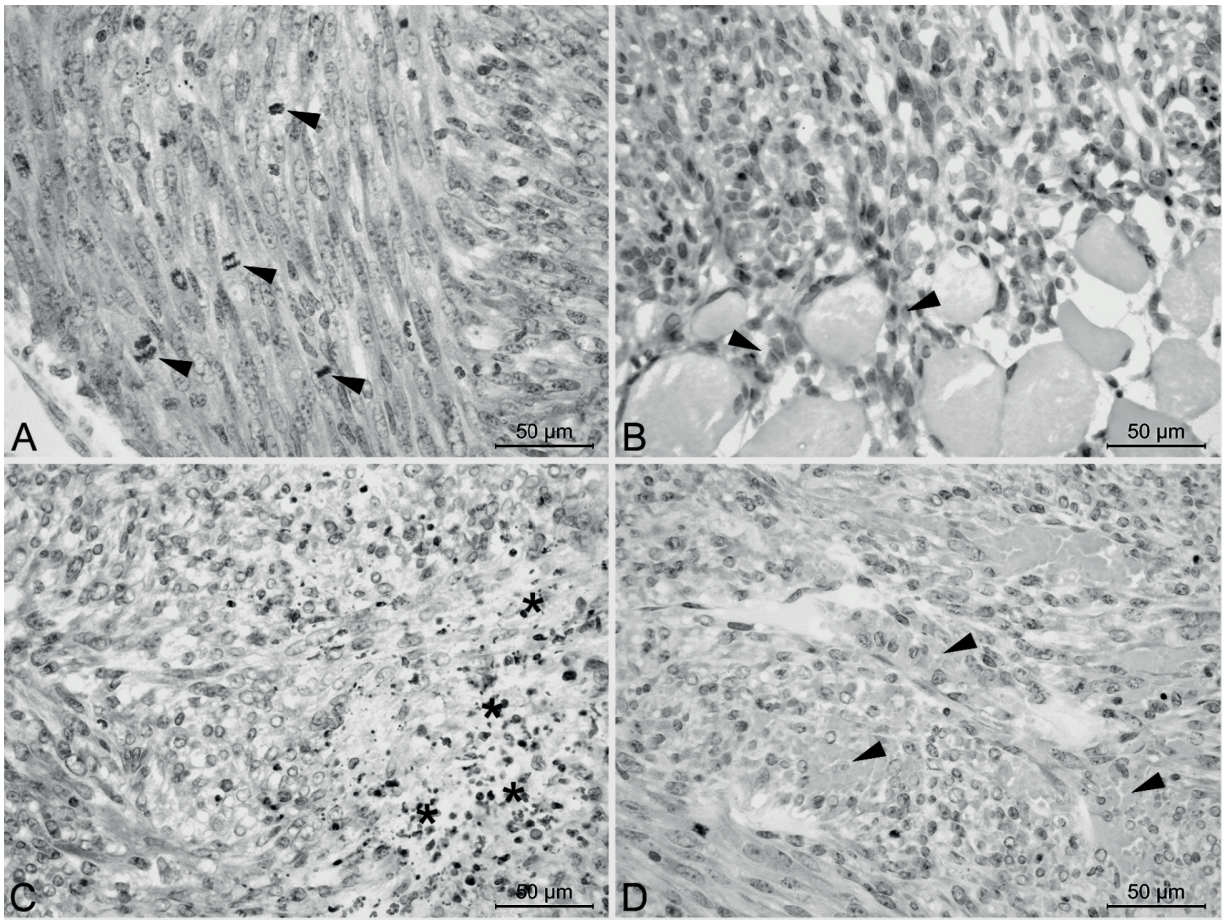

Figure 1. BHK fibrosarcoma: ***A)*** mitoses; ***B)*** penetration into skeletal striated muscle; ***C)*** multiple areas of tumor necrosis; ***D)*** tumor vasculature and hemorrhagic areas.

after carcinoma (breast, esophagogastric) inoculation. This, however, was not the case with either component alone[10].

In the human myeloma xenograft mouse model, the animals that had received intraperitoneal metformin treatment at a dose of 200 mg/kg/d had a lower tumor burden and prolonged survival when compared with the control group[25].

Intraperitoneal metformin treatment of 2-4 mg/kg/d significantly inhibited of B- and T-cell lymphoma growth after subcutaneous inoculation into nude mice[26].

At a dose of 250 mg/kg/d administered p.o. intragastrically, metformin inhibited tumor growth and angiogenesis in an oesophageal squamous cell carcinoma xenograft mouse model only when the treatment started 7 days before implantation[27]. Rapid cell proliferation and relatively weak angiogenesis in these tumors caused hypoxia and ischemia, leading to necrosis.

The efficacy of 200-500 mg/kg/d of metformin administered p.o. or intraperitonealy was not confirmed against Ewing sarcoma xenografts in athymic nude mice[28]. It was explained by hypoxia, a common feature of solid tumors.

Previous studies have shown that caffeine inhibits carcinogenesis in mouse models[24,29-33]. Of particular relevance to our study was the finding that caffeine treatment reduces fibrosarcoma growth in a mouse model[24]. This is in accordance with our results in hamsters regarding combined caffeine and metformin fibrosarcoma treatment.

Caffeine induced human umbilical vein endothelial cell culture apoptosis and inhibited the formation of new blood vessels in chick embryonic chorioallantoic membrane *in vivo*[33].

The activation of ATM with the exhibition of the antifolate activity on cell cultures and inhibition of the angiogenesis were observed separa-

tely in earlier studies for metformin[4,28] and caffeine[21,33]. The influence on these targets may be responsible for the synergistic anticancer effect of the drug combination shown in our study.

Metformin levels in the colorectal cancer cells of xenograft-bearing mice (9-215 μM) treated perorally and intraperitoneally corresponded to the plasma concentrations[34]. This indicates consistent delivery of the drug to tumor tissue. We used the same order of magnitude in oral hamster doses. The maximum metformin dose in diabetic patients, up to 3 g/d, is equivalent to 500 mg/kg/d of our hamster doses, normalized to body surface. The anticancer activities of our metformin and caffeine doses during simultaneous application in hamsters, and the possibility of achieving comparably high nontoxic metformin levels in humans, combined with nontoxic caffeine doses, suggest the prospect of realizing effective nontoxic oncological therapy with this drug combination. Necessary clinical trials will elucidate whether the combination of metformin and caffeine has the potential to become an adjuvant treatment in current anticancer and especially antisarcoma therapies.

Conclusions

Since nontoxic metformin p.o. doses with nontoxic caffeine doses significantly inhibited sarcoma growth in hamsters, this combination may be a safe novel adjuvant human sarcoma therapy.

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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