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Inhibition of bioenergetic metabolism by the combination of metformin and 2-deoxyglucose highly decreases viability of feline mammary carcinoma cells

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ABSTRACT

Feline mammary carcinoma (FMC) is a highly aggressive pathology that has been proposed as an interesting model of breast cancer disease, especially for the hormone refractory subgroup. Recently, cancer cell metabolism has been described as a hallmark of cancer cells. Here, we investigate the effects and mechanism of metabolic modulation by metformin (MET, anti-diabetic drug), 2-deoxyglucose (2DG, hexokinase inhibitor) or a combination of both drugs, MET/2DG on two established FMC cells lines: *AlRB* (HER2 (3 +) and Ki67 < 5%) and *AlRATN* (HER2 (−) and Ki67 > 15%). We found that treatments significantly decreased both FMC cells viability by up to 80%. *AlRB* resulted more sensitive to 2DG than *AlRATN* (IC50: 3.15 *vs* 6.32 mM, respectively). The combination of MET/2DG potentiated the effects of the individually added drugs on FMC cells. In addition, MET/2DG caused an increased in intracellular oxidants, autophagic vesicles and completely inhibited colony formation. Conversely, only MET significantly altered plasma membrane integrity, presented late apoptotic/necrotic cells and increased both glucose consumption and lactate concentration. Our results support further studies to investigate the potential use of this metabolic modulation approach in a clinical veterinary setting.

1. Introduction

Feline mammary carcinomas (FMC) are highly frequent and have been proposed as spontaneous models of specific human breast cancers, particularly the hormone refractory subtypes (De Maria et al., 2005; Santos et al., 2012). FMC biological behavior is characterized by a rapid growth, high proliferation rates, and the ability to metastasize to regional lymph nodes and distant organs (Millanta et al., 2005). As it was observed in humans, the expression of the HER2 oncogene in feline mammary tissues correlates with the clinical course of the disease (Millanta et al., 2005). In this context, we have recently established and characterized different cell lines derived from a spontaneous FMC tumor as an *in vitro* model for studying this and others pathologies (Villaverde et al., 2016). Here, we compare two different variants, *AlRB*, with low proliferative Ki67 index (< 5%; doubling time (DT): 25 h) and *AlRATN*, with high proliferative Ki67 index (> 15%, DT: 16 h). These lines also differ in HER2 status, being *AlRB* positive (3 +) and *AlRATN* negative (−). In addition, both cell lines are negative for estrogen (ER) and progesterone receptor (PR).

Most cancer cells display increased glucose uptake, even in the presence of adequate oxygen levels. This phenomenon is known as the Warburg effect and suggests a dependency on glycolysis, especially in rapidly growing tumors (Warburg et al., 1924). Thus, cancer cell bioenergetic metabolism appears as an attractive area of clinical and pre-clinical therapy developments as an “Achille's heel” of tumors (Kroemer and Poyssegur, 2008; Granja et al., 2015). Metformin (MET) is a biguanide, clinically known as an oral well tolerated anti-diabetic drug. Recent studies show that MET decreases cancer cell viability and tumor growth in different xenograft models (Akinyeke et al., 2013; Nakamura et al., 2014; Kourelis and Siegel, 2012). Furthermore, retrospective epidemiological studies have revealed a decrease in the incidence of cancer in diabetic patients treated with MET (Chlebowski

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et al., 2012). Apparently, MET modulates cell metabolism at different cell levels by increasing glycolysis, inhibiting respiratory chain complex I and ultimately inhibiting mTOR. This leads to growth arrest and apoptosis (Ochoa-Gonzalez et al., 2016; Guo et al., 2015). However, the molecular mechanisms underlying the MET antitumor effects still remain unclear.

On the other hand, 2-deoxyglucose (2DG) is a reversible inhibitor of hexokinase, the first and rate-limiting enzyme of glycolysis (Zhang et al., 2014). The inhibition of glycolysis decreases the production of glycolytic intermediates, which are the precursors of nucleic acids and phospholipids. In addition, depletion of glucose-6-phosphate also decreases the pentose phosphate pathway (PPP) and consequently the antioxidant defenses of cancer cells. At present, different studies explored the combination of 2DG with chemotherapy as sensitizer (Maschek et al., 2004).

The aim of the present work was to investigate for the first time the potential antitumor effect of modulating bioenergetic pathways by a combination of MET and 2DG on feline mammary carcinoma cell lines and the mechanism involved in the effectiveness of this dual combination, as a preclinical model of both veterinary and human mammary disease.

2. Materials and methods

2.1. Cell culture

AlRB and *AlRATN* cells were established from an infiltrating mammary adenocarcinoma as has been previously described (Villaverde et al., 2016). Briefly, a mammary tumor from a 10-year-old female Siamese cat was resected. Tumor cells were mechanically disrupted. The resulting cell suspension was centrifuged, washed with PBS and cultured as monolayers at 37 °C in a humidified atmosphere of 95% air and 5% CO2 with DMEM/F12 medium (Invitrogen, Carlsbad, CA, USA) containing 10% FBS (Invitrogen), 10 mM HEPES (pH 7.4) and antibiotics. Serial passages of subconfluent monolayers were made by trypsinization (0.25% trypsin and 0.02% EDTA in PBS). Passages from 20 to 30 were arbitrarily named *AlRB* and identify as PR- and ER-negative and highly positive (3 +) for the HER2 oncogene. Passages from 100 to 120 were arbitrarily named *AlRATN* thus referring to its negative immunoreactivity for HER2.

2.2. Viability

2.2.1. Acidic phosphatase assay (APH)

Intracellular acid phosphatase in viable cells hydrolyzes *p*-nitrophenyl phosphate to *p*-nitrophenol. Its absorption at 405 nm is directly proportional to the cell number (Friedrich et al., 2007). After treatments, 96 wells plates were washed with PBS and fresh PBS was added to a final volume of 100 µL. Then, 100 µL of the assay buffer (0.1 M sodium acetate, 0.1% Triton-X-100, supplemented with *p*-nitrophenyl phosphate) was added per well and incubated for 90 min at 37 °C. Following incubation, 10 µL of 1 N NaOH was supplemented to each well, and absorption at 405 nm was measured within 10 min on a Microplate Analyzer. The percentage of cell viability was calculated from the absorbances ratio between treated and untreated (100%) control cells.

2.3. Colony formation

AlRATN cells were seeded (5×10^3 cells/well) in a 6 well plate and treated with 2DG (0.5 mM), MET (1 mM) or a combination of both MET/2DG for 10 days. After that, the wells were washed with PBS, fixed with acetate:ethanol (3:1) for 10 min and stained with Cristal Violet 0.5% for 5 min. The colonies were visualized and counted with the simple sight. This assay was not performed for *AlRB* cells because they cannot generate colonies (Villaverde et al., 2016).

2.4. Flow cytometry

Control and treated cells were harvested after 48 h, washed with PBS and incubated with: 5 mg/mL propidium iodide (Sigma, PI, Loss of membrane integrity) for 5 min, or 0.5 µM 2′,7′-dichlorodihydrofluorescein diacetate (Invitrogen, DCF, Intracellular oxidants) for 20 min, or 10 µg/mL of acridine orange (Sigma, AO, Acidic vesicles) for 15 min, or 50 µM 6-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (Sigma, 6-NBDG, Glucose uptake) for 20 min. Then, cells were subjected to single-channel flow cytometry and the cellular fluorescence intensity was measure with a BD FACScalibur Flow Cytometer.

2.5. Glucose and lactate content in cell culture media

After 48 h of treatments, 5 µL of each supernatant was transferred to a new 96 wells plate. Then, concentration of glucose and lactate was determined colorimetrically by specific commercial kits (Weiner Lab., and Cobas/Roche respectively).

2.6. Autophagy analyses by fluorescence microscopy of RFP-LC3

2.6.1. LC3 is a protein involved in autophagy

AlRB cells grown as subconfluent monolayers were transfected with a plasmid construct of RFP-LC3. Positive cells were selected by geneticine (750 µg/mL). Then, *AlRB* RFP-LC3 cells were treated with 2DG (0.5 mM), MET (1 mM) or a combination of both MET/2DG. After 48 h of treatments, red LC3 cytoplasm punctation were evaluated by fluorescence microscopy.

2.7. Late apoptotic/necrotic events

AlRB and *AlRATN* cells grown over glass were treated with 2DG (0.5 mM), MET (1 mM) or a combination of both MET/2DG for 48 h. After treatment, non-fixed cells were incubated with acridine orange (10 µg/mL, green) and ethidium bromide (10 µg/mL, red) for 1 min and the stained was evaluated by fluorescent microscopy. The co-localization of both stains indicates late apoptotic or necrotic events (orange/red).

2.8. Statistics

Differences between groups were analyzed with one- or two-way ANOVA followed by multiple comparisons Tukey's test. $P < 0.05$ values were considered statistically significant. Analyses were made using INFOSTAT free edition and GraphPad Prism 6 software (GraphPad Software Inc., USA).

3. Results

3.1. The combination of metformin and 2-deoxyglucose increased individual cytotoxic effects on feline mammary carcinoma cells

To determine the effects of bioenergetic modulation on FMC cell viability, *AlRB* and *AlRATN* cells were treated with 0.1–10 mM MET or 0.5–10 mM 2DG. After five days, cell viability was determined by the acidic phosphatase (APH) assay as described in Materials and methods. We found that MET and 2DG decreased the viability of both cell lines in a concentration- dependent manner. The lowly proliferative and HER2 positive *AlRB*, was significantly more sensitive to 2DG than the highly proliferative and HER2 negative, *AlRATN* (IC50s: 3.15 *vs.* 6.32 mM respectively, $p < 0.01$) (Fig. 1A). On the other hand, MET treatment did not show differences between the two cell lines (Fig. 1B).

As a synergistic effect of combining MET and 2DG has been previously reported (Ben Sahra et al., 2010; Levesley et al., 2013 and Cheong et al., 2011), we studied this possibility on FMC cells.

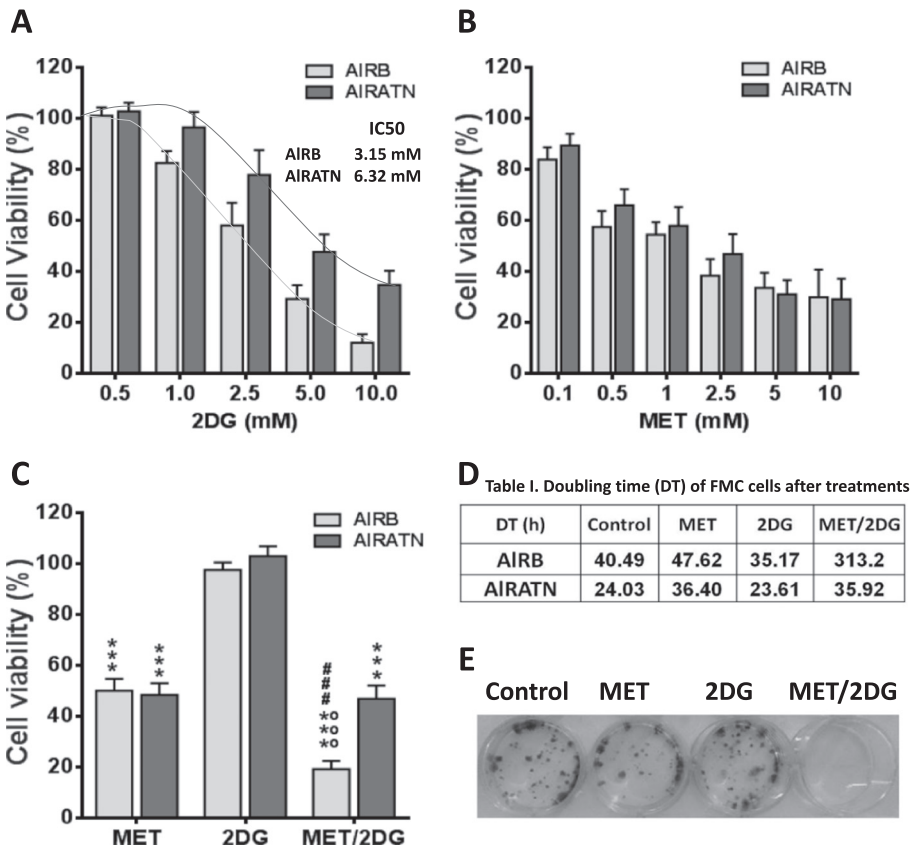


Fig. 1. Cytotoxic effects of 2-deoxyglucose and metformin on FMC cells. *AlRB* and *AlRATN* cells were treated with (A) 2 deoxyglucose (2DG, 0.5–10 mM), (B) metformin (MET, 0.1–10 mM) or (C) MET (1 mM), 2DG (0.5 mM) or a combination of both MET/2DG (1 mM/0.5 mM). Control and treated cells were subjected to APH assay (after 5 days of treatment (A–C) or counted by using a Neubauer chamber, every other day for 5 days (D) and the doubling time (DT) was calculated. Results are expressed as percentage of viable cells with respect to control cells as means ± s.e.m. of $n > 4$ independent experiments. $^{***}p < 0.001$, respect to each control; $^{°°°}p < 0.001$, respect to *AlRB*-MET; $^{###}p < 0.001$ respect to *AlRATN*-MET/2DG. (E) *AlRATN* colonies after 14 d of treatment with MET, 2DG or a combination of both MET/2DG (n = 5).

DT (h)	Control	MET	2DG	MET/2DG
AIRB	40.49	47.62	35.17	313.2
AIRATN	24.03	36.40	23.61	35.92

Table 1
Doubling time (DT) of FMC cells after treatments.

Therefore, *AlRB* and *AlRATN* cells were treated with 1 mM MET, 0.5 mM 2DG or a combination of both. Control and treated cells were subjected to APH assay (after 5 days of treatment, Fig. 1C) or counted by using a Neubauer chamber, every other day for 5 days (Fig. 1D). While the combination MET/2DG showed a synergistic cytotoxic effect on *AlRB*, 2DG did not potentiate the cytotoxic effect of MET as single agent on *AlRATN* cells. The interactions between MET and 2DG presented in *AlRB* were analyzed by the Chou and Talalay method (Chou and Talalay, 1984). Thus confirming that was a synergistic effect ($I < 1$). In addition, we found that MET/2DG strongly inhibited the proliferation of *AlRB* compared to control (doubling time: 313.2 h *vs* 40.5 h, respectively; Fig. 1D, Table 1) whereas MET, as single agent, did not affect the proliferation rate of this cell line. On the other hand, *AlRATN* doubling time (24.0 h) was mildly affected by both, MET (36.4 h) and MET/2DG (35.9 h).

In this case, non-synergistic effect was observed (Fig. 1D, Table 1). We did find a synergistic effect between MET and 2DG on *AlRATN* cells when we performed a colony formation assay. While control *AlRATN* developed colonies from singles cells, control *AlRB* cells did not, so this FMC cell line was excluded off from the experiment.

3.2. Less sensitive AlRATN showed higher uptake of glucose and lactate production than AlRB

Since MET inhibits the mitochondrial complex I (Ochoa-Gonzalez et al., 2016), it is expected to stimulate glycolytic rates and to increase lactate production. In agreement with this, we found that MET treatment increased glucose consumption in both FMC cell lines, *AlRB* and *AlRATN* ($p < 0.05$ and $p < 0.01$ respectively, Fig. 2A).

However, only sensitive *AlRB* increased lactate production after MET treatment ($p < 0.05$, Fig. 2B). It is worth to point out that, *AlRATN* control cells presented higher glucose consumption and lactate production than *AlRB* control cells ($p < 0.01$, Fig. 2A and B). In addition, we used 6-NBDG (6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-6-deoxyglucose), a nonmetabolizable fluorescent glucose derivative, to estimate glucose uptake. The time-dependent uptake of 6-NBDG in FMC cells is shown in Fig. 2C. Highly proliferative *AlRATN* did not show 6-NBDG uptake over the first 5 min followed by a rapid uptake phase that apparently did not reach a maximum after 30 min. Lowly proliferative *AlRB* lacked of 6-NBDG uptake over the first 10 min (5 min longer than *AlRATN*) followed by a mild uptake, reaching a maximum accumulation and a decay phase after 20 min. We also investigate the effect of 6-aminonicotinamide (6-AN) on the viability of CMF tumor cells. A significant fraction of cancer cells glucose has been shown to be metabolized through the pentose phosphate pathway (PPP; Elf et al., 2016).

The PPP provides NADPH for biosynthetic processes and redox balance, and ribose sugars for nucleotide synthesis, *via* the enzyme glucose 6-phosphate dehydrogenase. We found that *AlRB* cells were sensitive to PPP inhibition by 6-AN ($p < 0.05$) while *AlRATN* were not sensitive under the same conditions (Fig. 2D).

3.3. The synergistic combination of metformin and 2-deoxyglucose induced an autophagic response

The energetic stress may lead to autophagic mechanism activation. Then, in order to explore this possibility, vital staining with acridine orange (AO) was used to visualize the AO-stained red acidic vesicles related with autolysosome (Vucicevic et al., 2011). At first, we analyzed

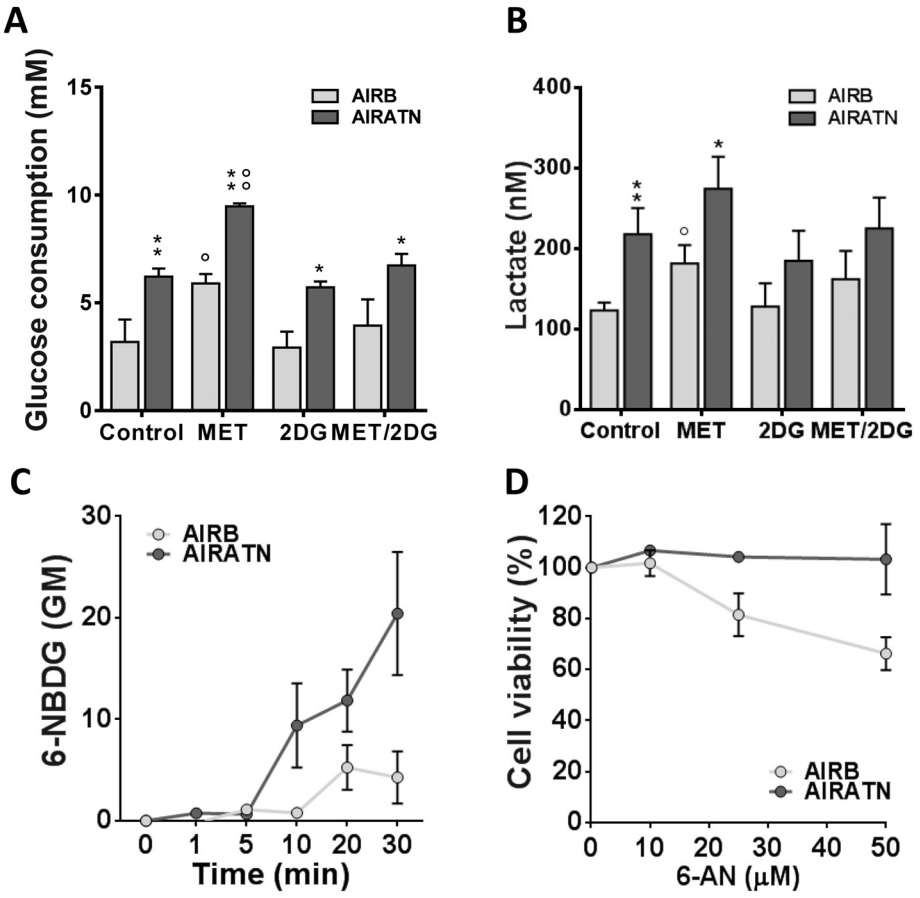


Fig. 2. Glucose consumption and lactate production by FMC cells. *AlRB* and *AlRATN* cells were treated with metformin (MET, 1 mM), 2-deoxyglucose (2DG, 0.5 mM) or a combination of both (MET/2DG). After 48 h of treatment, the concentration of (A) glucose or (B) lactate were evaluated in cells supernatants as described in Materials and Methods. Results are expressed media ± s.e.m.; n > 4. (C) Time course of glucose uptake measured by using the fluorescent glucose analogue 6-NBDG. The final data were analyzed using the Flowing software and the medium intensity of fluorescence was calculated (geometric mean: GM). (D) FMC cells were treated with 6-aminonicotinamide (6-AN, an inhibitor of PPP) for 120 h after that, the viability was measured by APH assay. Results are expressed as means ± s.e.m.; n > 3. $^{*}p < 0.05$ and $^{**}p < 0.01$ respect to *AlRB*; $^{\circ}p < 0.05$ and $^{\circ\circ}p < 0.01$ respect to each control. PPP: pentose phosphate pathway.

by flow cytometry the cell complexity that could be related to autophagic processes by the dispersion of the cell cloud from the density dot plot. *AlRB* and *AlRATN* cells were incubated with acridine orange (AO) for 15 min before being analyzed.

Side-scattered light (SSC) is proportional to cell granularity or internal complexity, whereas forward-scattered light (FSC) indicates cell area or size. *AlRB* control cells presented a higher cell complexity than *AlRATN* control cells as indicated by an increase in the number of cells with higher SSC (Fig. 3A). Independently of the cell line, the combination of MET/2DG but not the single agents increased the proportion of cells with higher complexity. In agreement with this, we found that the combination of MET/2DG induced accumulation of AO-stained red acidic vesicles resulting in an increase in cellular red fluorescence (higher FL3, Fig. 3B and C). In this case, MET as single agent, also caused an increase in the proportion of *AlRATN* with AO-stained red acidic vesicles (Fig. 3B and C).

In order to confirm the autophagic nature of AO positive vesicles, we lipofected AIRB cells with a plasmid construct containing LC3B gene tagged with the red fluorescent protein (RFP). LC3B II is known to bind to the autophagosome, therefore the conversion of LC3B I to LC3B II was considered to be a reliable marker of autophagy. Thus, after 48 h of treatments, we found that MET alone or in combination with 2DG resulted in a cytoplasmic accumulation of LC3B II (red dots, white arrows, Fig. 3D). These cytoplasmic red punctation were also observed after 2DG treatment.

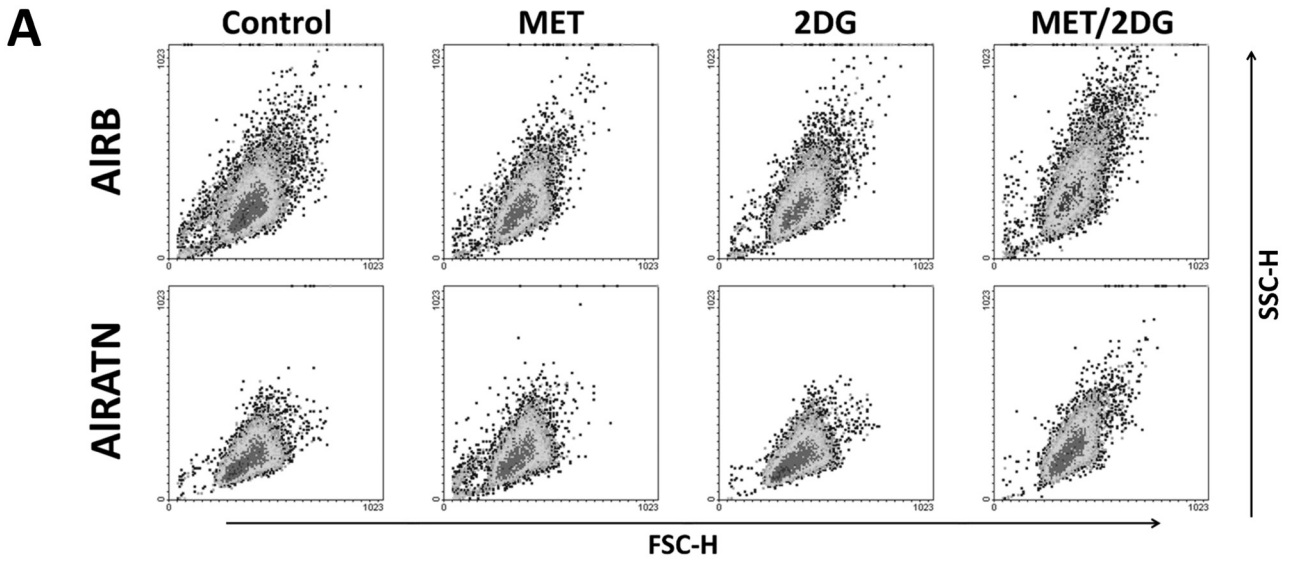
3.4. Metformin, but not the combination of metformin and 2-deoxyglucose caused late apoptotic/necrotic events

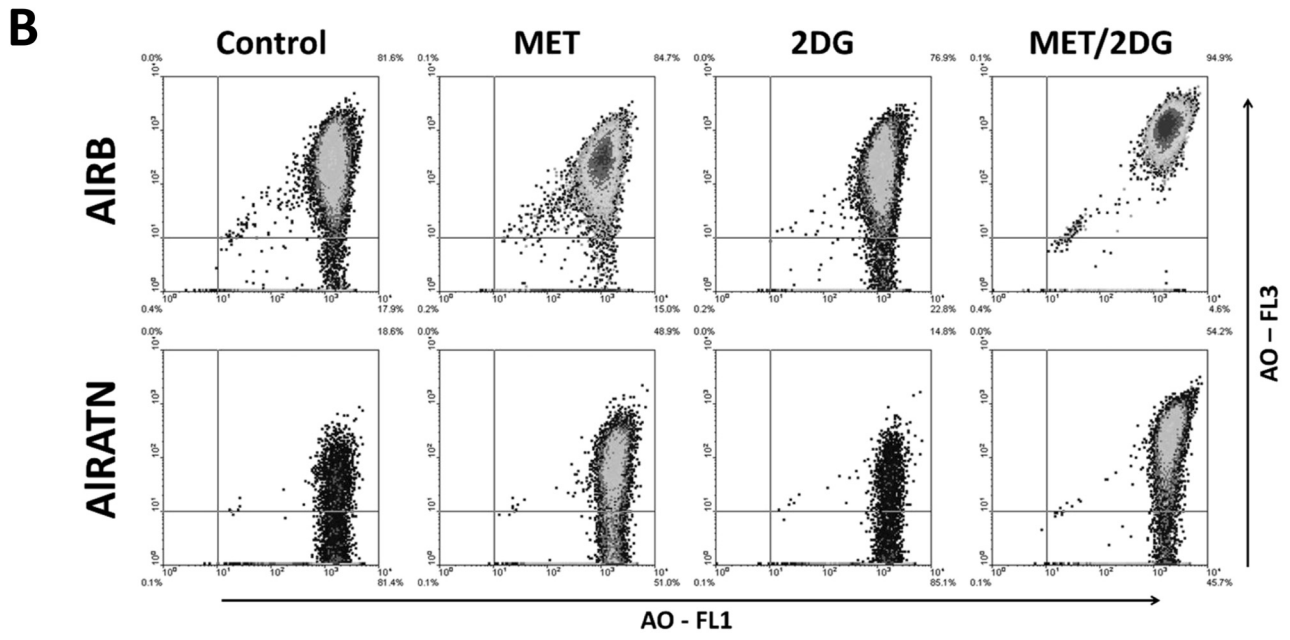
Double staining with acridine orange/ethidium bromide (AO/EB) (Squier and Cohen, 2011) allowed the observation of apoptotic/necrotic events following 48 h of treatments with bioenergetic modulators. Cell membrane is permeable to acridine orange (green) whereas ethidium bromide (red) enters only in late apoptotic or necrotic cells because of the disruption of the cell membrane integrity during these processes. We found that *AlRB* and *AlRATN* control cells, and cells treated with 2DG or MET/2DG mostly appeared as green-stained healthy cells. In contrast, MET treatment as single agent increased the number of late apoptotic/necrotic cells as evidenced by the increase of ethidium bromide (EB) red-stained nuclei in both cell lines after 48 h of treatment (Fig. 4A). In agreement with AO/EB staining, both cell lines exhibited a loss of membrane integrity (increased PI uptake) after 48 h of treatment with MET (but not MET/2DG), as measured by flow cytometry (Fig. 4B and C, $p < 0.05$), suggesting that the effects of MET/2DG previously observed (Fig. 1C and D) might be probably due to a decrease in cellular proliferation.

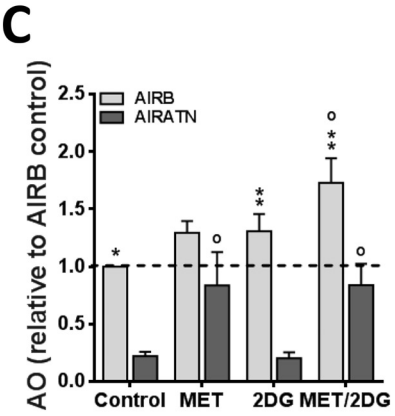
3.5. The combination of metformin and 2-deoxyglucose increased intracellular oxidants

Since bioenergetic blockage may also affect the intracellular redox state, we quantified the intracellular oxidants by cytometry as described in Materials and Methods. Briefly, *AlRB* and *AlRATN* cells were treated with 1 mM MET, 0.5 mM 2DG or a combination of both (MET/2DG). After 48 h of treatments, cells were loaded with the cell permeable non-fluorescent DCFH-DA that after being oxidized became the green fluorescent DCF.

Only MET/2DG, neither MET nor 2DG alone, caused a statistically significant increase in intracellular oxidants content as observed by the right shift on the fluorescence intensity axis (FL1, Fig. 5A) and by the quantification of DCF cell content from at least 4 independent experiments (Fig. 5B). Independently of sensitivities, *AlRB* and *AlRATN* did not show differences in oxidant levels, either in the basal redox state or after treatments.







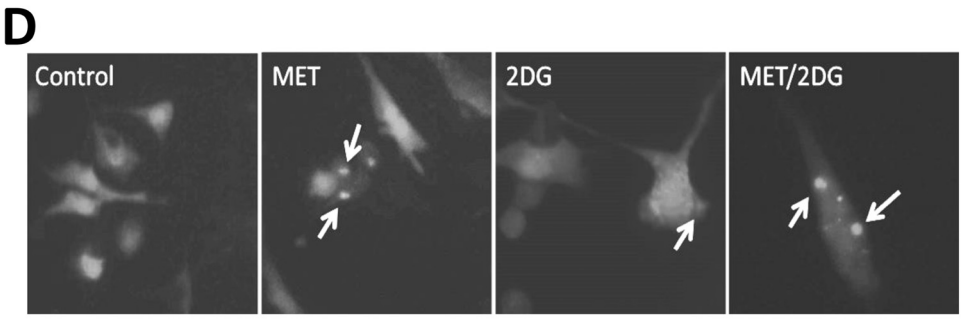


Fig. 3. Autophagy response induced by metformin and 2-deoxyglucose in FCM cells. *AlRB* and *AlRATN* cells were treated with metformin (MET, 1 mM), 2-deoxyglucose (2DG, 0.5 mM) or a combination of both (MET/2DG). Then, cells were incubated for 20 min with AO (acridin orange) and measured by flow cytometry (see Materials and Methods). Intracellular complexity (FSC-H) and cell size (SSC-H) were defined (A). The acidic vesicles were determined (B, up to the right) and the quantification respect to *AlRB*-Control is shown in C. $^{*}p < 0.05$ and $^{**}p < 0.01$, respect to *AlRATN*; $^{\circ}p < 0.05$ and $^{\circ\circ}p < 0.01$, respect to each control. D) *AlRB* cells were transfected with a construct which have a fusion protein RFP-LC3 (a fusion of red fluorescent protein and the protein LC3). The images were taken after 48 h of treatments.

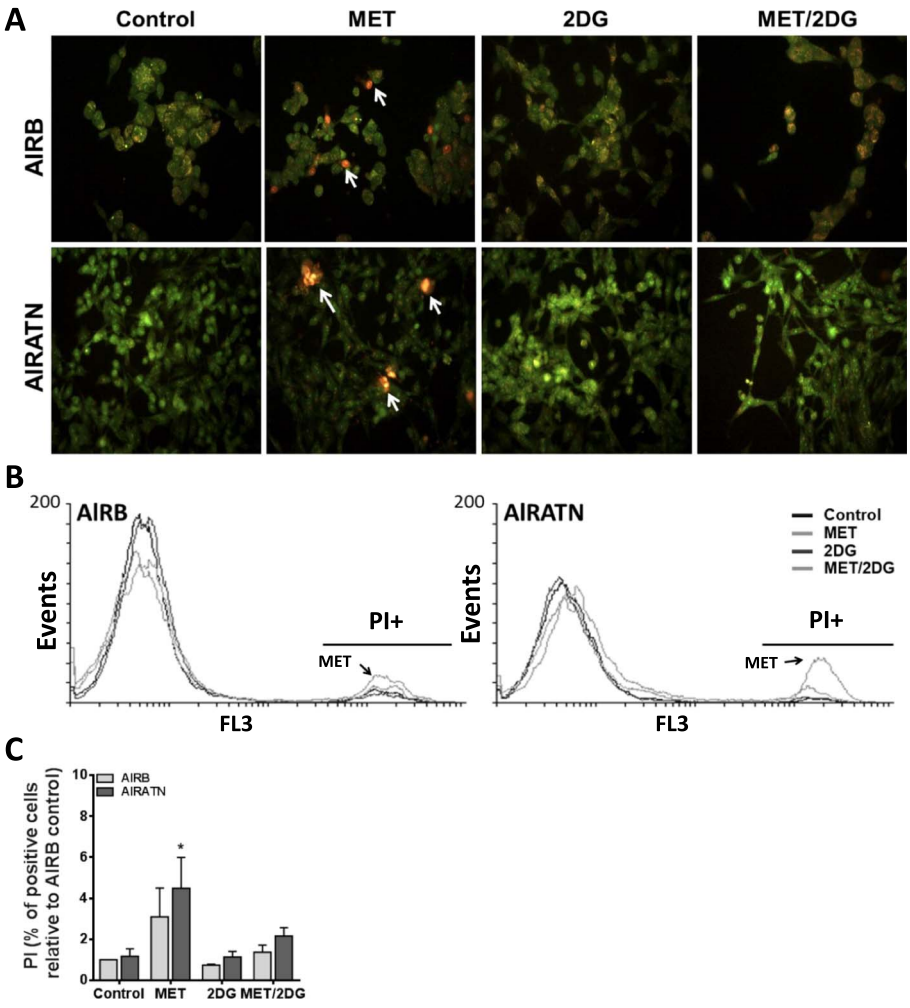


Fig. 4. Apoptosis/necrosis induced by metformin treatment in FMC cell variants. *AIRB* and *AIRATN* cells were treated with metformin (MET, 1 mM), 2-deoxyglucose (2DG, 0.5 mM) or a combination of both (MET/2DG). After treatment, non-fixed cells were incubated with acridine orange/ethidium bromide (AO/EB) and evaluated by microscopy (A) or propidium iodide (PI, 5 μg/mL) and evaluated by flow cytometry (B) as described in Materials and Methods. Results are expressed as means ± s.e.m., n > 4 independent experiments. $^{*}p < 0.05$ respect to control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

In agreement with previous studies (Granja et al., 2015), our results support the idea of bioenergetic modulation as an emerging strategy to treat cancer. As far as we know, this is the first report of metabolic modulators for treating feline mammary carcinoma (FMC), suggesting the use of metformin (MET, as oxidative phosphorylation inhibitor) alone or in combination with 2-deoxyglucose (2DG, as glycolysis inhibitor) in veterinary medicine.

FMC has been proposed as an interesting model of breast cancer, specially the hormone refractory subgroup (De Maria et al., 2005). In the present study, we showed a high antitumor activity by 2DG and

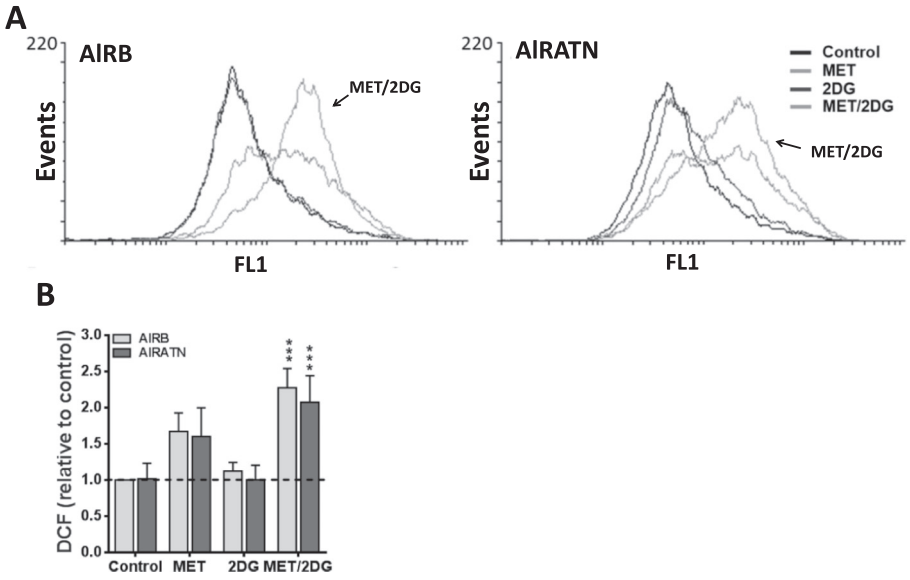


Fig. 5. Oxidative mechanism involved in the effectiveness of the combination treatment of metformin and 2-deoxyglucose on FMC cells. *AIRB* and *AIRATN* cells were treated with metformin (MET, 1 mM), 2-deoxyglucose (2DG, 0.5 mM) or a combination of both (MET/2DG). After treatment, non-fixed cell suspensions were incubated with DCF (diclorofluorescein) for 20 min and evaluated by flow cytometry (A) as described in Materials and Methods. Results are expressed as means ± s.e.m. of n > 2 independent experiments (B). $^{***}p < 0.001$ respect to control.

MET in a concentration dependent manner on both evaluated FMC cell lines, independently of HER2 status (Fig. 1A and B). In this context, it is worth pointing out that FMC has been proposed as an interesting model to study breast cancer, specially the hormone refractory subgroup (De Maria et al., 2005). On the other hand, MET and 2DG are both already FDA approved drugs for human administration. Although, MET and 2DG use in cancer treatment are still under research (Granja et al., 2015). Moreover, our results are consistent with that previously reported in various cancer cell lines and in preclinical cancer models which describe a synergistic effect between MET and 2DG (Ben Sahra et al., 2010) with the advantages of having reduced both drugs concentration compared to those previously reported.

Despite the lack of synergistic effect on viability, the clonogenic assay clearly revealed that the MET/2DG treatment was better inducing cell killing in apparently resistant *AlRATN* cells than single drug additions (Fig. 1E). This complete inhibitory effect on clonogenic survival could be of especial interest during metastasis development.

In response to bioenergetic blockage, cancer cells can activate autophagy in an attempt to restore the energy supply (Klarer et al., 2014). We found that the cytoplasm of MET-, 2DG- and MET/2DG-treated cells presented red punctate (by overexpressing RFP-LC3 protein, Fig. 3D) and increased the FL3 signal of acridine orange probe denoting acidic vesicles related to autophagy (Fig. 3B and C). MET (but not MET/2DG-induced) autophagic vesicles were significantly inhibited by 3-methyl adenine (an autophagy inhibitor, data not shown). Thus, suggesting that not only autophagy but other mechanisms would be involved in the combinatory treatment efficacy. Since MET has been previously reported as an inhibitor of the mitochondrial electron transport chain Complex I, total intracellular oxidants have been evaluated as possible mediators of the cytotoxic effects (Chan and Miskimins, 2012). We found that MET/2DG combinatory therapy showed an increase in intracellular oxidant content significantly higher than each drug as single agent (Fig. 5). One possible explanation could be the fact that HK (hexokinase) inhibition by 2DG indirectly decreases glucose 6 phosphate dehydrogenase (G6PD) activity (the rate limiting enzyme of the pentose phosphate pathway, PPP) as a result of having depleted its substrate glucose 6 phosphate. The PPP is supposed to finally restitute reduced glutathione (GSH), which plays a crucial role at the antioxidants defense system.

In accordance with previous results (Keller et al., 2011), the inhibition of oxidative phosphorylation by MET induced FMC cells to increase glucose consumption and consequently to produce more lactate (Fig. 2A and B). Combination with 2DG (thus inhibiting HK, the first and rate limiting enzyme of glycolysis), did not allow MET-treated cells to increase glucose consumption or lactate production (Fig. 2A and B). Since lactic acidosis has been previously reported as an adverse effect in patient during metformin treatment (Keller et al., 2011), the combination with 2DG which in turn may prevent lactate production could result an advantage.

The mechanism by which MET (but not MET/2DG) modifies plasma membrane integrity and triggers late apoptosis/necrosis has yet to be fully elucidated. A possible mechanism may be through the observed increase in lactate concentration in the supernatant of MET- (but not MET/2DG-) treated *AlRB* cells, since lactate has been related to cause membrane injury (Gong et al., 2016).

Two reports about the use of MET against feline tumors were recently published. In the first one, a pilot study involving tumor bearing cats that were orally treated, a clinically relevant dose of MET displayed mild to moderate side effects and suggested a potential pharmacologic activity (Wypij, 2017). In the second one, MET showed *in vitro* proliferation inhibitory activity on a feline injection site sarcoma cell line (Pierro et al., 2017).

5. Conclusion

Altogether, these findings encourage continuing the study of metabolism as a promising target against feline mammary carcinoma. Additional studies are required to determine whether MET or MET/2DG is able to confer clinical advantages over current available therapies in an oncological veterinary setting.

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Conflicts of interest

None.

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