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Effects of Ouabain in Ehrlich Tumor Development in vitro and in vivo

Amanda Costa Ayres Salmeron[1], Maria Beatriz Calado[2], Mateus Da Silva Matias Antunes[3,*], Gabriel Rodrigues da Silva[4], Deyse Cristina Madruga Carvalho[4], Beatriz Fernandes de Souza[4], Márcia Regina Piuvezam[5], Sandra Rodrigues Mascarenhas[4]

[1]Edmond and Lily Safra International Institute of Neuroscience, Santos Dumont Institute. Macaíba, Rio Grande do Norte, Brazil;
[2]Laboratory of Immunopathology Keizo Asami, Federal University of Pernambuco, Recife Pernambuco, Brazil;
[3]School of Pharmaceutical Sciences, University of São Paulo. Ribeirão Preto, São Paulo, Brazil;
[4]Biotechnology Center, Federal University of Paraíba, João Pessoa, Paraíba, Brazil;
[5]Health Sciences Center, Federal University of Paraíba, João Pessoa, Paraíba, Brazil.

Corresponding author*
mateusmatias08@gmail.com

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Abstract

Ouabain (OUA) is a cardiotonic steroid with an immunomodulatory and anti-inflammatory role in different experimental models. Currently, the potential antineoplastic effect of OUA has been studied, however, research is needed to better understand OUA role during tumor development. Therefore, our aim was to investigate the OUA effects on Ehrlich tumor (ET) development in vitro and in vivo. To evaluate the cytotoxic effects of OUA on ET in vitro the cells were incubated with different concentrations of OUA during 24h and 48h and our results showed that only the [1000 μM] decreased the number and viability of ET cells in the two analyzed times. To study the OUA effects on ET in vivo, Swiss mice were pretreated with 0.56 mg/kg of OUA intraperitoneally (i.p.) for three consecutive days. To develop ET in the solid form, one hour after the last day of pretreatment, ET cells were inoculated subcutaneously into the footpad and the animals were monitored for 13 days. To develop the ascitic form, ET cells were inoculated (i.p.) and the animals were monitored for 3 days. OUA was able to reduce the thickness and weight of the tumor paw, in addition to reduce the weight of the popliteal lymph node. In the ascitic tumor, OUA reduced the number of neutrophils and macrophages and increased the lymphocytes in the peritoneum. Thus, we demonstrated that OUA affects ET development both in vitro and in vivo. Our results suggest a new perspective in the anti-inflammatory, immunomodulatory and a possible anti-cancer role of ouabain and brings new concepts about the pathophysiological role of this substance.

Keywords: Ehrlich tumor; inflammatory microenvironment; ouabain; solid tumor; tumor development.

INTRODUCTION

Cancer is characterized by disordered cell growth with the ability to metastasize and interfere with organs function (López-Lázaro, 2018). The tumor development follows several complex steps, in which the cells gradually acquire a neoplastic phenotype (Arneth, 2019). Many tumors can alter and control the microenvironment, as a survival strategy, to interfere with the host immune response. One of the cancer hallmarks is the inflammatory microenvironment induced by tumor development (Hanahan & Weinberg, 2011). A better understanding of the inflammation role during tumor evolution and looking for new substances that can alter inflammatory parameters, such as cell migration and number of viable cells, can be a way to delay tumor development, allowing a longer time window of treatment and resulting in a better prognosis. (Kimiz-Gebologlu et al., 2018; Wellenstein et al., 2019).

Ehrlich's transplantable tumor originated from a spontaneous mammary gland carcinoma in female mice and has been used widely for several studies on experimental oncology (Havelek et al., 2017; Safwat et al., 2020). Like other transplantable tumors, this model is very useful since it is possible to determine and adjust the concentration of neoplastic cells to be inoculated and to quantify the growth and regression of the tumor. Ehrlich tumor cells develop ascetically when the cells are injected intraperitoneally (Fernandes et al., 2015) or solidly when the cells are injected subcutaneously or in the footpad (Bahr et al., 2015). This tumor corresponds to an aggressive carcinoma with fast growth, which induces myelosuppression and affects the inflammatory response (Brozovic et al., 2009), being a good experimental model for studying the interaction of different substances and the immune system in the tumor development (da Mota et al., 2018; Machado et al., 2017; Moura et al., 2018).

To search for new substances that can modulate inflammation and the immune system, and possibly delay the tumor development, ouabain (OUA) has shown broader effects (Cavalcante-Silva et al., 2017; Galvão et

al., 2017; Jacob et al., 2013; Leite et al., 2015; Takada et al., 2009). Different in vitro and in vivo models have demonstrated the modulation of cytokines by OUA, establishing this molecule as a striking immunomodulator. However, the ability to interfere in immunologic functions is not restricted to the expression of cytokines, because OUA also modulates the migration of inflammatory cells, inhibits the pathway of inflammatory mediators, and decreases edema (Cavalcante-Silva et al., 2017; Rodrigues-Mascarenhas et al., 2011). In addition, OUA has been described as a substance capable of inhibiting neuroinflammation. Moreover, in vitro studies have shown the antineoplastic potential of OUA in different human tumor cell lines as lung A549 cancer cells, breast MCF7 cancer cells, prostate DU 145 cancer cells, osteosarcoma U-2 OS cells, melanoma A375 and SK-Mel-28 cells, and glioma U-87MG cells (Chang et al., 2019; Chou et al., 2018; Wang et al., 2021; Xiao et al., 2017; Yang et al., 2018).

Modulating the immune system has proven to be one of the most important strategies to fight against tumors. While OUA exerts a meaningful role on inflammation, these effects during tumor development have not been properly explored. Therefore, to gain more insights about new compounds to control tumor growth, we investigate the effects of OUA on Ehrlich tumor cells in vitro and on animal experimental models of solid and ascitic tumors.

MATERIALS AND METHODS

Animals

Female Swiss mice aged between six and eight weeks, weighing between 25 and 30 g, were used. The animals were kept in polypropylene cages at a temperature of 25 ± 1ºC. They were subjected to light/dark cycles of 12 hours and with free access to water and food throughout the experimentation period. The experimental protocols were submitted to the Animal Research Ethics Committee of the Pharmaceuticals and Medicines Research Institute/UFPB and were approved under number 093/2016. Euthanasia of the animals in all experiments was done with ketamine/xylazine solution (ketamine 100 mg/kg and xylazine 10 mg/kg) injected intramuscularly (i.m.) followed by cervical dislocation.

Ehrlich tumor cells and in vitro culture

Cryopreserved samples of Ehrlich tumor cells diluted in 1 mL of fetal bovine serum (FBS) + Dimethyl Sulfoxide 10% (DMSO) were thawed and grown in 14 mL Dulbecco's Modified Eagle's Medium High Glucose (DMEM; Sigma D1152), supplemented 2,0 g/L sodium bicarbonate, 1 mM/L sodium pyruvate (0,11g/L), 1% Penicillin-Streptomycin (Gibco, USA) + 10% FBS. For experiments a concentration of 5x105 viable cells/mL were used. Cell viability was determined by Neubauer chamber counting using the trypan blue 0.4% (Gibco, USA) exclusion method and an optical microscope (200x increase).

After thawing, the cells were maintained in culture for 7 days and subcultures were performed every 2 days, in a 1:3 ratio. After the initial 7 days of cell maintenance and stabilization, the contents of the culture flask was centrifuged at 235 xg at 37°C for 4 minutes (Hermle Z 326 K centrifuge), the supernatant was discarded and the cells resuspended in 1 mL of DMEM + 10% FBS. After, Ehrlich tumor cells were seeded in 96-well plate at a concentration of 5x104 viable cells/well together with OUA at the concentrations: 1 nM, 10 nM, 100 nM, 1 μM, 10 μM, 100 μM and 1000μM based on studies that describe the concentrations at which digitalis are capable of inhibiting the Na+/K+ ATPase pump (Aizman et al., 2001; Bortner et al., 1997; Fontana et al., 2013; Goto et al, 1992). For the control group phosphate-buffered saline solution (PBS) was used. The plates were incubated in a humid incubator at 37°C and 5% CO2, protected from light. All tests were performed in triplicate and two different times were analyzed: 24 hours and 48 hours.

Procedures

- ***Cell viability evaluation and basal cytokine production***

After OUA incubation, cell viability was evaluated by Neubauer chamber counting using the trypan blue exclusion method and MTT assay.

To perform MTT assay, the 96-well plates were centrifuged at 100 xg at 4°C for 6 minutes (Hermle Z 326 K centrifuge). The supernatant was discarded and 100 μL of DMEM supplemented with 10% FBS + 10% MTT reagent (PBS + 0.5 g/mL MTT) was added to the wells. Then the plate was incubated for 4 hours in a humid incubator at 37°C and 5% CO2, protected from light. After the incubation time, 100 μL of SDS 10% was added to the wells. The plates were under agitation, and protected from light, overnight. Subsequently, the plates were analyzed on a spectrophotometer at a wavelength of 570 nm and the absorbance values of each sample were obtained.

To measure the production of pro-inflammatory cytokines in vitro, the plates were centrifuged at 100 xg at 4°C for 6 minutes (Hermle Z 326 K centrifuge). The supernatant was collected, and the cytokine production was evaluated by sandwich enzyme-linked immunosorbent assay (ELISA). The cytokines IL-6, TNF-α and IL-1β were quantified using the kit and protocol indicated by the manufacturer (Bioscience, USA). The analysis was performed on a spectrophotometer, at a wavelength of 450 nm, where the absorbance values of each sample were obtained.

- ***Induction of Ehrlich's tumor in vivo***

Cryopreserved samples of Ehrlich tumor cells were thawed, diluted in PBS and inoculated intraperitoneal

(i.p.) at the concentration of 1x106 cells per animal in a volume of 300 µL, this process is called zero passage. After five days the animals were euthanized and collected the peritoneal lavage and performed the first passage, as described previously. After two passages in vivo, the cells were inoculated into the experimental animals. For experiments, 1x105 cells in a volume of 300 µL was inoculated via i.p. in mice for ascitic tumor model and 1x105 cells in a volume of 50 µL was inoculated subcutaneously in the footpad of the left paw for solid tumor model. In the solid Ehrlich tumor model, the contralateral paw was used as a control for the group itself.

- ***Animal pretreatment protocol***

All the animals were pre-treated with PBS or OUA at concentration 0.56 mg/kg i.p. for three consecutive days (Jacob et al., 2013; Leite et al., 2015; Rodrigues-Mascarenhas et al., 2006). This dose of OUA was chosen because this concentration corresponds to the levels found physiologically in the human organism (Blaustein, 1993).

Moreover, for solid Ehrlich tumor model, on the third day of pretreatment, 1 hour after the last pretreatment, 50 µL of PBS or Ehrlich tumor cells were inoculated in the footpad of the left paw and 50 µL of PBS in the right paw. For ascitic Ehrlich tumor model, 1 hour after the last pretreatment, 300 µL of PBS or Ehrlich tumor cells were inoculated in the animal peritoneum.

- ***Solid Ehrlich tumor model***

Mice were randomly separated into three different groups: G1, G2 and G3. G1 group (n = 24) was the positive control and was pre-treated with 200 µL of PBS and Ehrlich tumor cells were inoculated in the footpad of the left paw. G2 group (n = 24) was the experimental group pre-treated with 200 µL of OUA and Ehrlich tumor cells were inoculated in the footpad of the left paw. At least, G3 group (n = 20) was the negative control pre-treated with 200 µL of OUA and PBS were inoculated in the footpad of the left paw.

Tumor mass thickness was measured using a digital micrometer (Digimess, São Paulo, Brazil) one hour after inoculation, 24 hours later and thereafter every four days until the 13th day, when the animals were euthanized.

After euthanasia, the popliteal lymph nodes of the right and left paw were removed using surgical forceps and the organs were weighed. The right and left foot pads were removed by dislocating the feet at the tibio-tarsal junction and were also weighed. Subsequently, the lymph nodes were macerated in 6 mL of PBS. This suspension was centrifuged (200 xg, 4°C, 5 min), the supernatant discarded, and the pellet formed resuspended in 1 mL of PBS solution and cell count was performed using trypan blue exclusion method.

For differential counting, 50 µL of cell suspension was centrifuged in a Cytospin Centrifuge Serocite® FANEM MOD2400 - Crosshead Ref.: 248.059.600 (1500 rpm for 10 min). Then, the slides were stained with a Panotic Kit. After 24 hours of drying, the slides were analyzed using an optical microscope using immersion oil (1,000x increase). The slides were run in the tail region until the count of 100 cells.

- ***Ascitic Ehrlich tumor model***

Mice were randomly separated into three different groups: G1, G2 and G3. G1 group (n = 6) was the negative control and was pre-treated with 200 µL of saline and 1 hour after the last pretreatment, saline was inoculated. G2 group (n = 6) was the positive control group pre-treated with 200 µL of saline and 1 hour after the last pretreatment, Ehrlich tumor cells were inoculated. At least, G3 group (n = 14) was the experimental group pre-treated with 200 µL of OUA and 1 hour after the last pretreatment, Ehrlich tumor cells were inoculated.

The animals were daily weighed before, during the pretreatment, and after the inoculation of Ehrlich cells. After 72 hours of tumor cells inoculation, all animals were euthanized, and the abdominal circumference was measured and calculated the tumor volume. After, using a 5 mL syringe, 3 mL of cold PBS was introduced into the peritoneum and the volume of ascitic fluid was aspirated. The samples were centrifuged at 200 xg 4°C for 5 minutes (Hermle Z 326 K centrifuge), the supernatant was discarded, and the cell pellet formed was resuspended in 1 mL of PBS and used for the perform total cell count and differential leukocyte count. Differential cell counting was performed as described previously.

Data analysis

Statistical analyses were performed using PRISM® 6.0 software (GraphPad, USA). Agostino-Pearson and Shapiro Wilk tests were performed to verify the Gaussian distribution. When necessary, the data were normalized using the formula: Y = [(Y * 100 / 1.647)]. All results were analyzed using the one-way ANOVA method for non-parametric data with Tukey's post-test. Values of $p < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

OUA decreases the number and viability of Ehrlich tumor cells after 24 and 48 hours at 1000 µM

To understand whether the OUA has an influence on Ehrlich's tumor cells growth, in vitro assays were performed. At 1000 µM of OUA, Ehrlich tumor cells reduced cell viability in 39.6% and 49.3% at both 24 and 48 hours, respectively, when assessed by the MTT test (Figure 1A-B). Moreover, by trypan blue exclusion method, OUA has also been shown to be able to reduce the number of total cells in culture (Figure 1C-D) and cell viability of at 1000 µM concentration after 24 and 48 hours, reducing viability in 15.8% and 28.1%, respectively (Figure 1E-F).

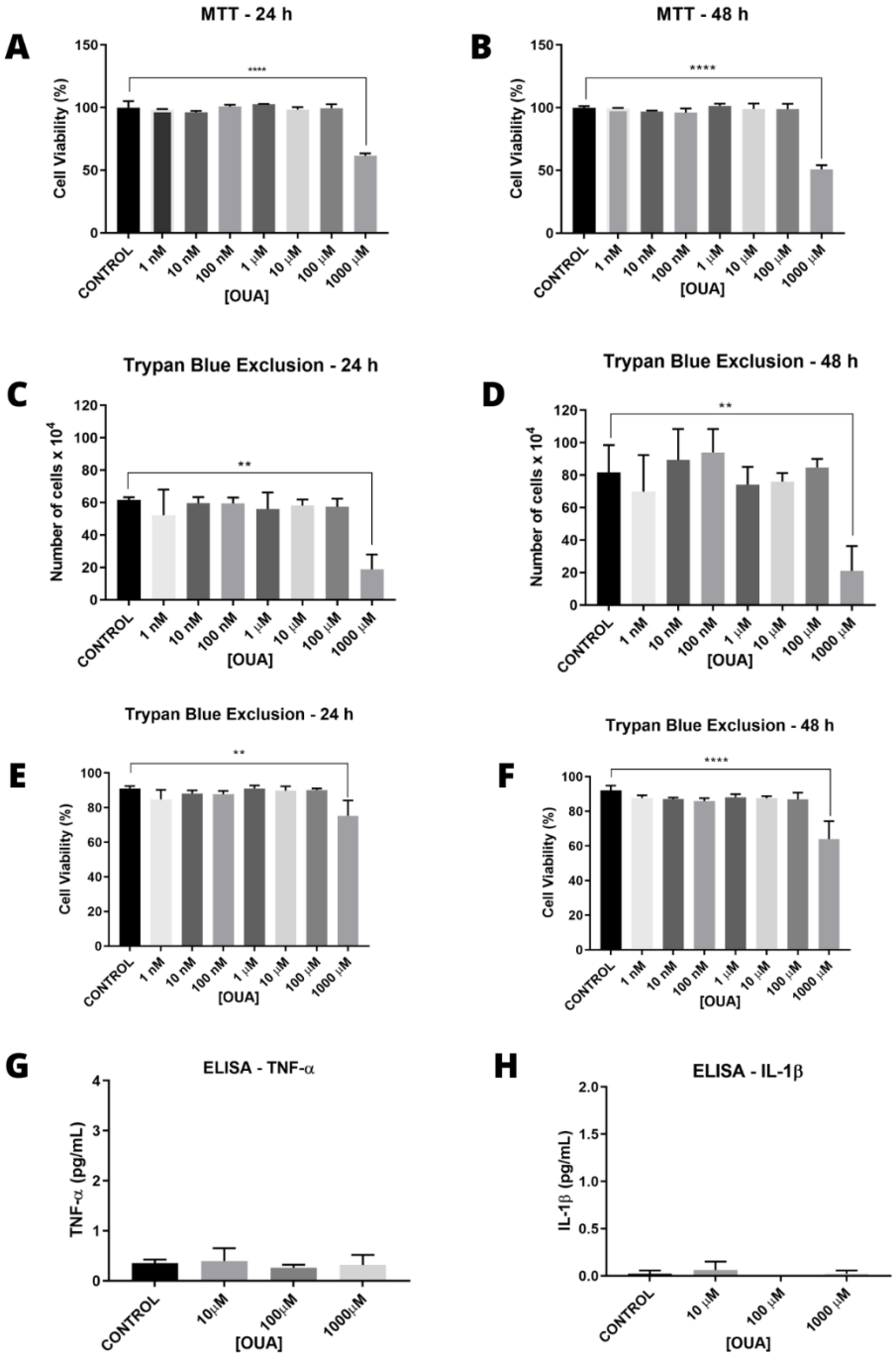

Figure 1. Effect of ouabain on number and viability of Ehrlich tumor cells in vitro. Cell viability was tested by MTT after (A) 24 hours and (B) 48 hours of treatment with OUA. Cell viability was tested by trypan blue exclusion after (C) 24 hours and (D) 48 hours of treatment with OUA. The total cell numbers were analyzed by trypan blue after (E) 24 hours and (F) 48 hours of treatment with OUA. Although, OUA did not alter the basal production of TNF-α (G) and IL-1β (H). The graphs A and B were normalized and plotted as the mean ± SEM. The other graphs are plotted as the mean ± SEM. All graphs were analyzed using the one-way ANOVA for non-parametric data with Tukey's multiple comparison post-test. ** $p < 0.01$ and **** $p < 0.0001$.

OUA does not alter basal cytokine production in Ehrlich cells

Our results showed that Ehrlich tumor cells produces a low level of basal cytokines TNF-α and IL-1β. Although, OUA does not alter the basal production of the evaluated cytokines (Figure 1G-H). Moreover, our results did not detect the presence of IL-6 in any group (data not shown).

OUA delay solid tumor development in mice footpad

On the fifth day, it was a significant increase in paw thickness between G1 and G3. On the 9th and 13th day, G2 group showed a reduction in the thickness of the paw with the solid tumor in relation to G1 (Figure 2A).

Moreover, in G2 group, OUA reduced the weight of the tumor paw (Figure 2B) and increased the weight of the left popliteal lymph node (lymph node closest to the tumor site) in relation to G1 group (Figure 2C). Regarding the total cell count in the left popliteal lymph node, it was possible to observe that there was no significant difference between groups G1 and G2 (Figure 2D). Furthermore, the differential cell count indicated that was no significant difference in the pattern of cell migration between the groups analyzed (data not shown).

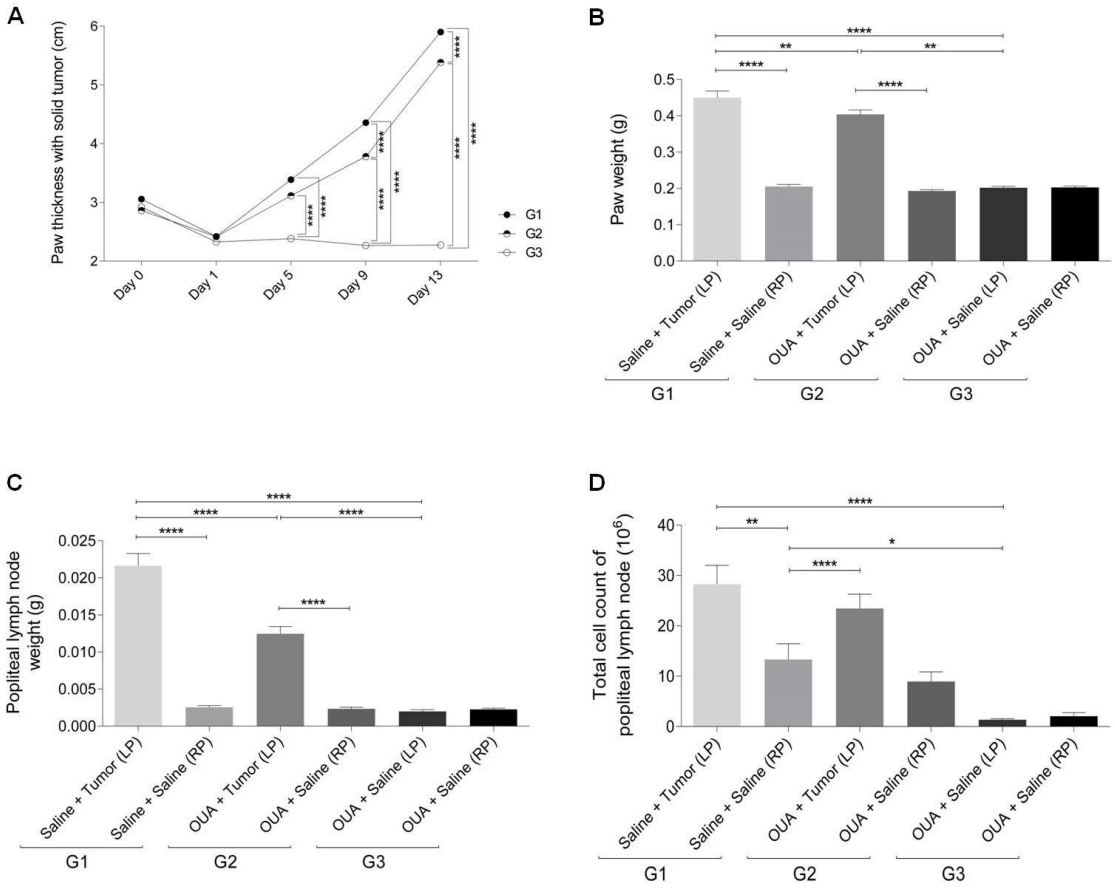

Figure 2. Effect of ouabain pretreatment on tumor development of the solid Ehrlich tumor in the footpad of mice. Paw thickness with the tumor was measured on the day of Ehrlich cell inoculation and 3, 5, 9 and 13 days after tumor inoculation (A). On the 13th day the animals were euthanized. The (B) paws and (C) popliteal lymph nodes on the right and left side were weighed. The total cell counting of lymph node maceration was also performed (D). The graphs are plotted as the mean ± SEM. All graphs were analyzed using the one-way ANOVA for non-parametric data with Tukey's multiple comparison post-test. * $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$.

OUA decreases neutrophils and macrophages migration to the tumor microenvironment and increases the migration of lymphocytes

Our results showed that there was no significant difference between G2 and G3 in relation to the gain or reduction in animal weight (Figure 3A), abdominal circumference (Figure 3B) and the ascitic fluid volume (Figure 3C) of the animals.

Regarding the total cell count, there was no significant difference between G1, G2 and G3 (Figure 3D), however, the cell profile of the peritoneal lavage was different between the groups. The G2 showed a 42.72% increase in the number of neutrophils, a 40.9% reduction in the number of lymphocytes and a 23.3% increase in the number of macrophages when compared to G1. It was observed that OUA pretreatment reduced cell migration to the tumor microenvironment. The G3 had a reduction of 81% in the number of neutrophils and 55% in the number of macrophages when compared to the G2. On the other hand, the OUA increased the quantity of lymphocytes by 182.17% also in relation to the G2 (Figure 3E).

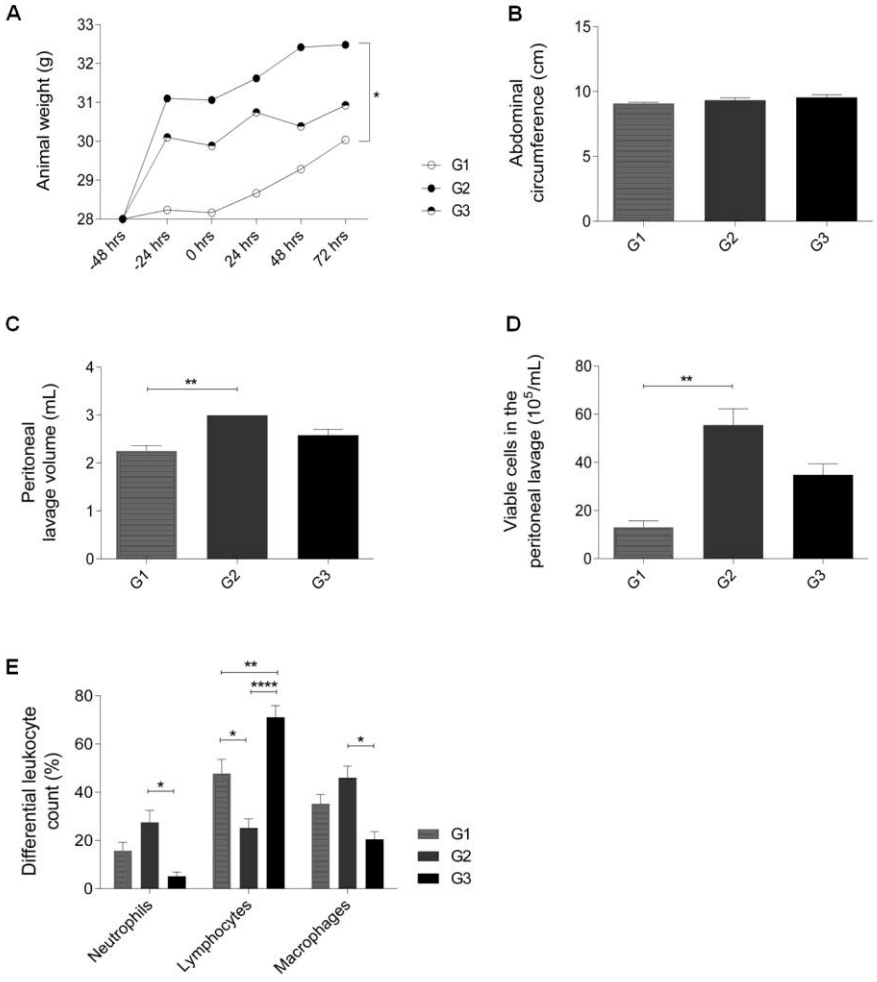

Figure 3. Effect of ouabain pretreatment on tumor development of the ascitic Ehrlich tumor. (A) The animals were monitored and weighed during the 3 pretreatments (-48, -24 and -1h) and 72 hours after the inoculation of Ehrlich tumor cells intraperitoneally. After 3 days of tumor growth, the animals were euthanized. (B) The abdominal circumference was measured and the peritoneal lavage (C) was analyzed by tumor volume, (D) cell viability and (E) differential leukocyte count. The graphs are plotted as the mean ± SEM. All graphs were analyzed using the one-way ANOVA for non-parametric data with Tukey's multiple comparison post-test. * $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$.

Discussion

The development of malignant tumors and the inflammatory process are closely linked events. Previous studies have shown that chronic inflammation can make individuals more susceptible to the progression of different tumors (Diakos et al., 2014; Korniluk et al., 2017). Moreover, the tumor development interferes with the innate immune response altering production and secretion of signaling molecules, such as cytokines and chemokines, capable of promoting alterations in tissue structure, formation of new vessels or other events that assist in tumor progression (Misra et al., 2018; Taniguchi & Karin, 2018; Velloso et al., 2019). There are different strategies to try eliminating or delay tumor growth, thus, one of the areas of experimental oncology has been looking for, is substances that can control the inflammatory microenvironment during tumor development such as OUA.

First, to analyze the effects of OUA treatment on Ehrlich tumor cells, we evaluated cytotoxicity and proinflammatory cytokine basal production in vitro. The results of cell viability tests showed that only the highest concentration tested (1000 μM) was able to decrease cell viability of Ehrlich tumor cells in both times analyzed, suggesting a dose-dependent response. This dose-dependent response was also observed by other researchers who evaluated the cytotoxicity of five glycosides, including ouabain, in several human tumor cell lines (Johansson et al., 2001). Furthermore, at both times tested, the viable cell concentration was significantly lower in the 1000 μM concentration suggesting that OUA has a cytotoxic effect on cells only at this concentration.

Regarding the mechanism of action that triggered the reduction of cell viability and concentration of cells in the 1000 μM group, we hypothesize two possibilities: the

first involves classic inhibition of the Na+/K+ ATPase pump, which can results in a reduction in the concentrations of K+ in the cytoplasm concomitant with higher Ca2+ concentrations in the cell cytoplasm, triggering cell death by apoptosis, through the activation of Caspases-3 (Diakos et al., 2014); and the second possibility involves inhibiting the Na+/K+ ATPase pump, or altering its kinetics, increasing Na+ concentrations along with a reduction in K+ concentration in the cell cytoplasm, favoring the polarization of the cytoplasmic membrane of the tumor cell. Thus, cells with lower levels of glutathione, commonly seen in tumor cells, inhibit the depolarization of the cytoplasm membrane causing an increase of tyrosine kinase expression and Ras protein, in addition to stimulating the production of superoxide ions, inducing the cell to apoptosis (Valente et al., 2003).

Second, to assess the effects in vivo of OUA pretreatment on the development of the Ehrlich's solid tumor, our data showed a reduction in the thickness of the paw with the solid tumor in OUA pretreated animals. We observed a weight loss of the tumor paw and the left popliteal lymph node. Other studies have already shown that OUA can reduce vascular permeability and edema (Leite et al., 2015; Rodrigues-Mascarenhas et al., 2009, 2011). Thus, to investigate whether these observed reductions could be associated with alterations in the lymphatic organ drainage, we evaluated the total and differential cell count of the popliteal lymph node. It is interesting to note that although a reduction in the weight of the left lymph node is observed, whose paw received Ehrlich cells together with the pretreatment with OUA, there was no statistically significant change in the cell count and cell profile, although the graph shows a trend.

The increase in the volume of lymph nodes is known as lymphedema and occurs when there is a functional overload of the lymphatic system, either due to hereditary causes or acquired through obstruction or injury caused by infectious processes, diseases, surgery, obesity or as a consequence of malignancies (Grada & Phillips, 2017; Petrek et al., 2000). Therefore, it is possible that the reduction in the lymph node weight of the animal pretreated with OUA occurred due to the reduction of fluid leakage in the inflamed site, where the Ehrlich cells were inoculated, and not due to the reduction in the number of cells in the popliteal lymph node. This is a hypothesis supported by previous data from our group that report the OUA ability to reduce plasma exudate leakage in an experimental model of edema in mice (Rodrigues-Mascarenhas et al., 2011).

Regarding the ascitic tumor model, our study showed that pretreatment with the OUA reduced the migration of cells from the innate immune system to the tumor microenvironment and it is one of the OUA anti-inflammatory mechanisms of the action. Our group has already demonstrated the reduction of eosinophils migration in the experimental model of allergic pulmonary inflammation (Galvão et al., 2017) and neutrophils in the experimental model of peritonitis (Leite et al., 2015). In addition, the reduction of inflammatory cell migration may indirectly, delaying tumor progression (Tsutsui et al., 2005). Moreover, a study carried out by our group already demonstrated that OUA can inhibit the activation of the NF-κB and p-38 proteins (Mascarenhas et al., 2014), both playing an important role in the carcinogenic process, especially in the early stages (Agrawal et al., 2001).

Furthermore, the immune system has a dual role in tumor development may help in the process of carcinogenesis, through the release of proinflammatory cytokines and stimulation of angiogenesis that favors metastases, but it may also be involved in the process to eliminate the tumor with the expression of tumor-associated antigens (De Visser et al., 2006). The level of lymphocytes can be a prognostic marker of tumor aggressiveness. It was observed that lower levels of lymphocytes were associated with a greater degree of tumor development and metastasis formation (Coffelt et al., 2015). The M1 macrophages are associated with the secretion of pro-inflammatory cytokines, such as interleukin-1β (IL-1β) and TNF-α; and M2 have an anti-inflammatory profile, with greater secretion of interleukin-10 (IL-10) (Najafi et al., 2019). Additionally, N1 neutrophils can express more immune-activating cytokines and chemokines, lower levels of arginase and greater ability to kill tumor cells. The N2 phenotype is related to the depletion of polymorphs in experimental models to reduce the number of metastases, without the aid of the primary neoplastic focus (Giese et al., 2019; Park et al., 2016; Sagiv et al., 2015).

Although the OUA reduced the tumor volume in the Ehrlich experimental models in vivo, this reduction is probably not associated with the cytotoxicity of the glycoside in Ehrlich cells. Mathematically, the OUA concentration used in our in vivo experiments approaches 96 μM/dose and, at that concentration, the OUA did not demonstrate cytotoxicity to Ehrlich cells in the tests conducted in vitro. Most likely, the OUA has negatively modulated the development of the tumor due to its anti-inflammatory activity. This does not exclude the fact that the OUA has previously reported antitumor activity. However, the OUA antitumor activity has been demonstrated only in human tumor cell lines as A549 cells, MCF7 cells, DU 145 cells, U-2 OS cells, A375 cells, SK-Mel-28 cells and U-87MG cells (Chang et al., 2019; Chou et al., 2018; Wang et al., 2021; Xiao et al., 2017; Yang et al., 2018). Here we evaluated the effect of OUA on murine cells that are about 1000 times more resistant to the cytotoxic effects of OUA compared to human cells due to a difference in the isoform of the α1 subunit of the Na+/K+ ATPase pump that makes murine cells less sensitive to the OUA (Akimova et al., 2015).

The pathophysiological role of ouabain on tumor development is still little explored, however, here we demonstrate that OUA can play an immunomodulatory role in both ascitic and solid Ehrlich tumors and delay

tumor development. It is known that at a concentration of 1000 μM OUA acts by inhibiting Na+/K+ ATPase, consequently reversing the kinetics of the Na+/Ca2+ exchanger, resulting in an increase in intracellular Ca2+ that triggers apoptosis via activation of Caspase-3. However, in the in vivo experiments the doses administered to the animals (96 μM/dose) was much lower, precisely to understand the role of the OUA in hormonal levels. Therefore, our hypothesis is that ouabain in hormonal concentrations acts by downregulating inflammation and thereby interfering with the migration of inflammatory cells to the ascitic tumor and decreasing the extravasation of plasma exudate reducing the tumor mass in mice with solid tumor (Rodrigues-Mascarenhas et al., 2011). This could be related to the capacity of ouabain to inhibit the nuclear factor NF-kB in low concentrations (Leite et al., 2015). This work presents new perspectives on the anti-inflammatory potential of OUA and brings new ideas about the pathophysiological role of this hormone.

CONCLUSIONS

OUA in higher doses can decrease the number and viability of Ehrlich tumor cells in vitro. In addition, animals that were pretreated with the physiological concentration of ouabain showed a delay in the development of solid and ascitic tumors. Pretreatment with ouabain reduced the paw thickness and weight in animals with a solid tumor, in addition to reducing the weight of the lymph node closest to the paw with tumor. In animals with ascitic tumor, pretreatment with ouabain altered the migration profile of lymphocytes, neutrophils and macrophages to the microenvironment of tumor development. Taken together, our work demonstrates that ouabain can delay tumor development both in vitro and in vivo, this expands the anti-inflammatory, immunomodulatory and a possible anti-cancer role of ouabain.

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Authors' Contributions: Sandra Rodrigues Mascarenhas & Deyse Cristina Madruga Carvalho designed the study. Amanda Costa Ayres Salmeron, Maria Beatriz Calado & Mateus Da Silva Matias Antunes carried out the laboratory work, collected and analyzed the data, and wrote the manuscript. Gabriel Rodrigues da Silva & Beatriz Fernandes de Souza helped in the treatment of animals. Márcia Regina Piuvezam kindly provided the place where the experiments were carried out. All authors read and approved the final version of the manuscript

Competing Interests: The authors declare that there are no competing interests.

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