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Carboplatin effect on canine benign mixed tumour-derived cells cultured under three-dimension system: apoptosis, cell viability and mitochondrial dysfunction

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Abstract. Canine mammary carcinomas represent an important pathology in small animal clinic, and benign mixed type carcinomas (MC-BMT) are one of the most diagnosed worldwide. The use of chemotherapeutic carboplatin has been one of the new protocols for the treatment of BMT. In this respect, three-dimensional cell culture (3D) represents an alternative in the evaluation of drugs by simulating what occurs *in vivo*. The present study aimed to verify the effect of carboplatin on 3D culture of cells derived from TMB, in addition to possible changes in cell viability, ballpoint size, apoptosis and Bcl-2/Bax ratio. For this, tumours samples were collected during mastectomy surgery procedure in private veterinary clinics, which were submitted to in vitro culture and part to histopathological analysis. After 28 days of 3D culture, spheroids were documented in both groups (treated and control) and sizes and morphology were compared. The carboplatin interfered in the cell viability by affecting their division and promoting apoptotic events. In the treated group, a higher transcription of Bax and caspase 3 was observed, in addition to low levels of caspases 2, 8 and 9, which was not observed in the control group. We thus suggest that mitochondrial dysfunction plays a critical role in cancer progression and that targeting mitochondrial alterations and mitochondrial retrograde signalling might be a promising strategy for the development of selective anticancer therapy. Thus, it was possible to demonstrate that the results achieved may contribute to the establishment of a new chemotherapy therapy in female dogs with MC-BMT.

Keywords: Apoptosis, carboplatin, celular culture 3D, MC-TMB

Efeito da carboplatina em células derivadas de tumor misto benigno canino cultivadas em sistema tridimensional: apoptose, viabilidade celular e disfunção mitocondrial

Resumo. Os carcinomas mamários caninos representam importante ocorrência na clínica de pequenos animais, sendo os do tipo misto benigno (MC-TMB) um dos mais diagnosticados em todo o mundo. A utilização do quimioterápico carboplatina tem sido um dos novos protocolos para o tratamento de TMB. Neste sentido, a cultura celular tridimensional (3D) representa uma alternativa na avaliação de fármacos por simular o que ocorre *in vivo*. O presente estudo teve por objetivo verificar a influência da carboplatina em cultivo 3D de células provenientes de TMB, além de possíveis alterações em viabilidade

celular, tamanho de esferoides, apoptose e razão Bcl-2/Bax. Para isto, amostras de neoplasias mamárias foram coletadas durante procedimento de mastectomia em clínicas veterinárias particulares, as quais foram submetidas ao cultivo *in vitro*, e parte dos fragmentos obtidos à análise histopatológica. Após 28 dias de cultivo 3D, a formação de esferoides foi documentada em ambos os grupos (tratado e controle) e o tamanho e a morfologia foram comparados. A carboplatina interferiu na viabilidade celular ao afetar a divisão das mesmas e promover eventos apoptóticos. No grupo tratado, foi observada maior transcrição de Bax e de caspase 3, além de baixos níveis de caspases 2, 8 e 9, o que não foi observado no grupo controle. Sugerimos, portanto, que a disfunção mitocondrial desempenha um papel crítico na progressão do câncer e que o direcionamento de alterações mitocondriais e sinalização retrógrada mitocondrial pode ser uma estratégia promissora para o desenvolvimento de terapia anticancerígena seletiva. Desta forma, foi possível demonstrar que os resultados alcançados podem contribuir no estabelecimento de uma nova terapia quimioterápica em fêmeas caninas com MC-TMB.

Palavras-chave: Apoptose, carboplatina, cultivo celular 3D, MC-TMB

Introduction

Canine mammary tumours are common pathology in small animal clinics (Cassali 2013; Ferreira et al., 2003; Salas et al., 2015). Female dogs mostly present mammary carcinoma classified as benign mixed tumour (MC-BMT) worldwide (Frengki et al., 2021). The MC-BMT originates from the epithelial malignant transformation observed as *in situ* growth or with infiltrating characteristics, as evidenced by stroma invasion performed by cancer cells or complete replacement of the pre-existing benign lesion (Cassali et al., 2013; Estrela-Lima et al., 2010; Owen, 1980).

In order to control and/or eliminate MC-BMT efforts to test new adjuvant therapies is under investigation (Sousa et al., 2014; Ulukaya et al., 2004). Primarily, veterinary clinicians are testing new treatment protocols that have higher cure rates, longevity and better quality of life (Damasceno et al., 2012; Estrela-Lima et al., 2010). In this respect, carboplatin classified as platinum-derived chemotherapy agent. Its application in veterinary oncology medicine was as an alternative treatment in cats, who cannot receive cisplatin (Rangel et al., 2009). Recent studies evaluate the Cox-2-associated carboplatin (piroxican) efficacy to treat mammary gland carcinoma (Lavalle et al., 2012; Sousa et al., 2014).

The three-dimension (3D) culture system capacity and is to simulate a tissue microenvironment similar to the "in vivo" conditions (Cardoso et al., 2017). This important technique verifies the efficacy of several drugs, and associate the behaviour of cellular interactions. The dog mammary tumours treatment, chemotherapy resistance is a note of concern similar to human therapy (Alfarouk et al., 2015; Bertolini et al., 2015; Cassali et al., 2013; Ferreira et al., 2003;). In order to overcome this obstacle, new studies are necessary to understand tumours “in vitro” behaviour.

The Bcl-2 family belongs to a group of anti-apoptotic proteins that have four homologues’ domains BH (Bcl-2, Bcl-X_L, Bcl-w, Mcl-l e Bfl-l (Bcl-2A1) and proapoptótics, Bax, Bak e Bok, divided into three families BH (BH1, BH2 e BH3). The multiplex domains of pro-apoptotic protein as Bax is frequently recruited to apoptosis activation apoptosis. The Bcl-2/Bax ratios represent an important parameter to a prognostic of various tumours type. Substantial amount of evidence suggests that mitochondria play a major role in inducing apoptosis in cancer cells (Chia-Chi et al., 2016). Mechanisms of cell death response to carboplatin therapy is dependent on different dose and cell types (Sousa et al., 2012). While carboplatin can have effect in canine breast cancer cells the effect of the treatment on mitochondrial bioenergetics remains unknown.

This study aimed to verify the carboplatin influence on MC-BMT cultivated under 3D conditions. In addition, cell viability, spheroids size, apoptosis as well BcL-2/Bax ratios and mitochondrial function were investigated in two groups: MC-BMT treated and MC-BMT untreated. Finally, the results obtained may contribute greatly to the advancement of veterinary oncology.

Material and methods

Tumours cell culture

Breast neoplasms samples of approximately 1-cm^2 under routine asepsis and antisepsis care at the hospital routine of private veterinary clinic, during the previously determined radical mastectomy surgical procedures. After the collection, the samples, under refrigeration, were transported to the Veterinary Virology Laboratory in conical aseptic tubes (Falcon®, Becton Dickinson, Franklin Lopes, NJ, USA) and immediately prepared for *in vitro* cultivation. Furthermore, other fragments of the same samples fixed in 10% formaldehyde solution during 48 hours and processed according to the paraffin inclusion technique. Finally, for the pathological diagnosis using histopathological examination, according to the World Health Organization - Institute of Pathology of the American Armed Forces was proceeded (Misdorp, 2002).

Chemotherapy assay under in vitro 3D culture

After 28 days of 3D cultivation, the group of BMT samples, in duplicate, were submitted to chemotherapy treatment with Carboplatin at a concentration of 10mg/ml (27μM) (Vancel®, Pierre Fabre laboratories of Brazil, RJ) and at the dose of (1.25μM1). The spheroids formed in 3D cultivation after 28 days were documented in both two groups, treated and control. After the chemotherapy was applied in the 6 wells and again made the photographic record with 3 days after treatment for further analysis. After examining the chemotherapy cytotoxic effect, area (μm^3) of each spheroid was measured before and after treatment, respectively. The photographs were performed by Olympus IX 70 inverted microscope (Tokyo, Japan). The size and number of spheroids measured using AxioVision™ 4.8 software (Carl Zeiss). From each well were measured an average of 8-10 fields used for these measurements and spheroid samples were subjected to cell viability. In the end, both groups were destined for histopathological processing for hematoxylin and eosin staining standard procedure.

Cell viability

Cell viability was measured using alamarBlue® (Invitrogen®) assay from treatment and no treatment spheroids cultured in 3D system. The assay is based on the conversion of a non-fluorescent dye to the red fluorescent dye resorufin in response to chemical reduction of growth medium resulting from cell growth. Briefly, 10% alamarBlue® corresponded to 2 ml were added per well and incubated during 1h. After this time, supernatant of each well was submitted to fluorescence intensity measurement at 530-590 nm (Biophotometer™, GE, Uppsala, SW).

RNA extraction and real time PCR analysis and quantification of gene expression

In order to observe the cellular damage without and after chemotherapy treatment under 3D culture, molecular analysis of relative quantification of apoptotic genes was made through the real time polymerase chain reaction. To perform this stage of the experiment, part of the 3D cultivation samples was separated and frozen in a freezer at -80º C, until the moment, they were processed. Total RNA was extracted from spheroids using TRIzol LS Reagent (Ambion, cat#10296010) following the manufacturer`s instructions. Freshly, prepared total RNA (1 μg) was used as a template for synthesis of first-strand cDNA with commercial random hexamer primers using ThermoScript™ RT-PCR system following the manufacturer's instructions. The cDNA products were used as templates for single tube real-time quantitative PCR in TaqMan™ customers probes: Cf02741602_m1 (BCL-2), Cf02622185_g1 (Bax), Cf02707196_m1 (Caspase 2), Cf02622231-m1 (caspase 3), Cf02707196_m1 (caspase 8), Cf02627332_m1 (caspase 9), Cf03034055_u1 (beta-actin). The beta-actin gene was used in order to normalize the real time PCR reaction (Livak & Schmittgen, 2001).

Assay for mitochondrial membrane potential (ΔΨm)

To verify mitochondria damage induced by carboplatin we examined the integrity of the inner membrane JC-1 (mitochondrial membrane potential kit (MAK 160, Sigma - Aldrich Inc., St. Louis, MO, USA) is a fluorescent probe used to measure the polarization of mitochondrial membrane. When the electrochemical gradient across the mitochondrial membrane collapses in damaged cells, the reagent

does not accumulate in the mitochondria and no aggregates form. Cells were treated as described above, followed by trypsinization and staining with 10g/mL JC-1 for 15 min in a tissue culture incubator. Cells were then rinsed with HMME and then cultured in a tissue culture incubator for 60 min, followed by analysis using a FACSCalibur flow cytometer (Becton—Dickinson). The mitochondrial membrane potential measurement was carried out on a flow cytometer with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. A total of 10.000 cells per sample were analyzed.

Statistical analysis

Data are presented as mean ± SD. Analysis of variance (ANOVA) with LSD test post hoc was used for testing significance using GraphPad software, version 9.2. $P < 0.05$ was taken as being statistically significant $P < 0.05$.

Results

The 3D cultures presented cell morphology and integrity differences in two group studied. In the control group there were intact cells with well-delimited cytoplasm, hyperchromatic and visible and sometimes multiple nucleolus. However, in the treated group, it was possible to observe intact cell clusters with neoplastic characteristics, as well as cell death, such as apoptotic bodies, cellular debris and no more viable cells (Figures 1 A and B, respectively). The sizes were considered statistically different ($P < 0.05$).

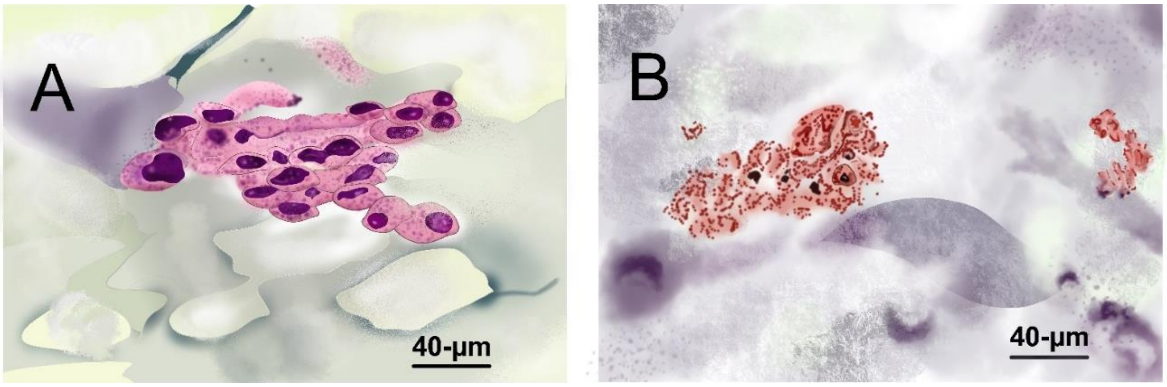

Figure 1. Photomicrography of spheroids obtained from 3D culture and submitted to hematoxylin-eosin staining. **A**) Spheroids from control group. **B**) Spheroids from treated group showing the carboplatin effect (magnification 400x) obtained the picture under light microscopy and AxionVision™ software.

The viability of cells revealed that carboplatin affects cell division and induced higher loss of viable cells (Figure 2, $P < 0.05$).

The spheroids size was statistically different when treated and control groups were compared. The size average of treated group was 30-50µm^3 and control group presented 190-200 µm^3 (Figure 3).

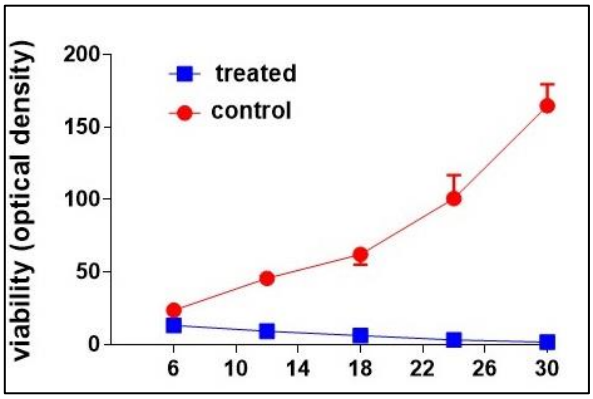

Figure 2. Viability comparison between treated and control group caused by carboplatin effect showing statistically different at all studied points ($P < 0.05$).

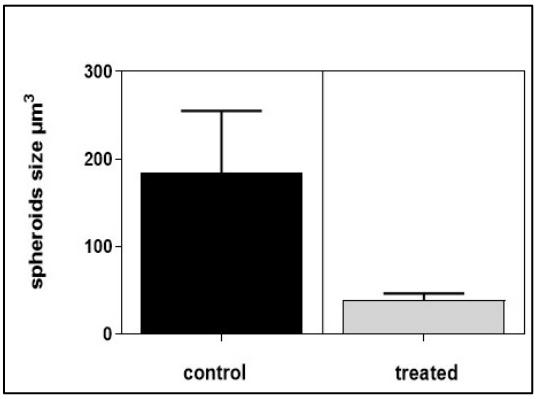

Figure 3. Bar graph demonstrating statistical differences between control and treated groups related to carboplatin effect on spheroids size ($P < 0.05$).

The control group presented no transcription of caspase 8, caspase 9, Bax and BCl-2 genes (Figure 4). However, when exposed to carboplatin the treated cell presented higher expression of caspase 3 and low levels of caspase 2, 8 and 9 transcripts (Figure 4). In order to determine BCl-2/Bax ratios, treated group revealed Bax highest transcription, an apoptotic factor related to intrinsic pathway (Figure 4).

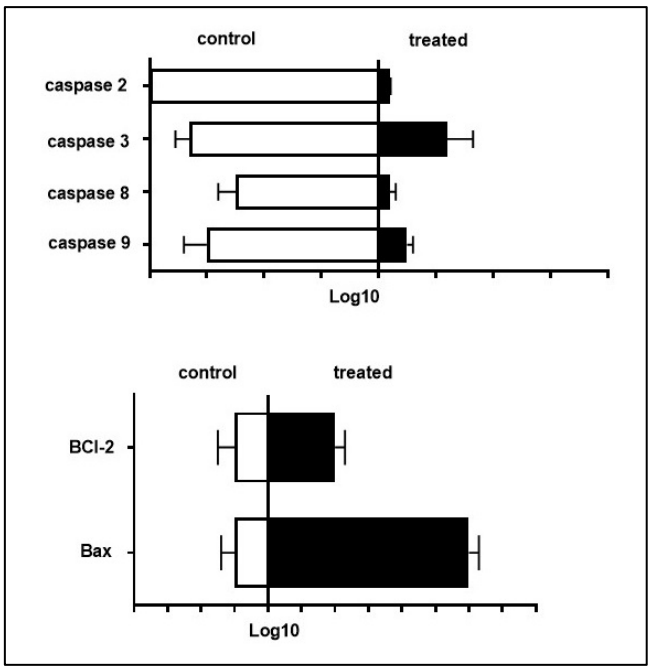

Figure 4. Transcription of apoptotic genes between control and treated groups. Tha data are presented as Log_{10} of media (triplicates) C*t* values obtained from StepOne™ real time software. Graph in the top, white bars represent negative results (not expressed) and black represent positive results. Caspase 3 transcription was higher when compared to 2, 8 and 9 under effect of carboplatin ($p<0.05$). Graph in the down under carboplatin effect Bax transcription was considered statistically higher than BCl-2 expression ($P < 0.005$).

Mitochondria are intracellular organelles in eukaryotic cells that participate in bioenergetic metabolism and cellular homeostasis, including the generation of ATP through electron transport and oxidative phosphorylation. In this study, the carboplatin induced a failure on electron transport by depolarization of mitochondria membrane measured by JC-1 excitation (Figure 5).

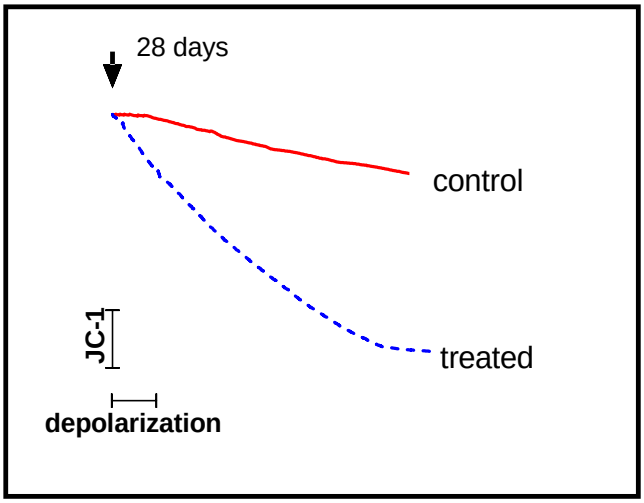

Figure 5. The effect of carboplatin on mitochondrial transmembrane potential (ΔΨm). Mitochondrial membrane potential was determined using the potential-sensitive fluorescent dye JC-1. The collapse was indicated by a reduction of transmembrane polarization. The percentage in each profile represents the percentage of cells with ΔΨm.

Discussion

The cancer is the main causes of human death worldwide and clinical therapy resistance is considered an obstacle nowadays (Jin et al., 2020). Besides, can be found in all kinds of cancer all possible treatments, such as molecular targeted therapy, immunotherapy, and chemotherapy (Fisusi et al., 2019; Nowak-Sliwinska et al., 2019). Normally, multidrug cancer treatments are used to increase efficacy and reducing the high drug doses that most of the times present adverse symptoms (Narayan et al., 2020). Consequently, targeting several pathways to inhibit tumour growth and enhance survival is the future reducing the death rate from cancer (Wang et al., 2020). This study demonstrated cytotoxic activity of carboplatin, in tumour benign mixed canine cells cultured under three-dimensional system and apoptosis induced.

The Bcl-2, as an antiapoptotic protein, prevents cell death when it occurs by chemotherapy stimuli and irradiation. The pro-apoptotic protein Bax, also a member of the Bcl-2 family, induces cell death (Vasan et al., 2019). Bax activation leads to apoptosis that is related to caspases 8, 9 and efector caspase 3, the latter degrading most cellular proteins (Chee et al., 2013). These two proteins, Bax and Bcl-2, promote balance by determining cell death or cell survival and logevidadede according to the stimuli presented (Uwano et al., 2012). Regarding the results found, we found that the Bax gene was expressed at highest level in all cells under treatment, and caspase 2, 3, 8 and caspase 9, indicating that these cells were entering apoptosis through the intrinsic mitochondrial pathway (Frengki et al., 2021). Caspase 2 showed same level as caspase 3 expression in treated cells. This results suggesting that carboplatin induces apoptosis of tumour cells by binding in cell death receptors and mitochondrial pathway (Wang et al., 2020). The pro-apoptotic activity of chemotherapeutic agents such as carboplatin occurs regardless of the interference of death receptors in the cell membrane, leaving the action of chemotherapy by mitochondrial route (Chia-Chi et al., 2016).

Most cancer drugs act inducing apoptosis and many neoplasms can develop mechanisms to escape the cellular death pathways by expression of anti-apoptotic proteins such as BCl-2 (Wang et al., 2020). Thus, high expression of BCl-2 among canine neoplasms accelerates cell expansion, inhibiting therapeutic action of anti-cancer drugs.

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Conflicts of interest

The authors declare no conflicts of interest.

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