Veterinary and Comparative Oncology

Original Article

DOI: 10.1111/vco.12220

Cell cycle kinetics, apoptosis rates and gene expressions of *MDR-1*, *TP53*, *BCL-2* and *BAX* in transmissible venereal tumour cells and their association with therapy response

M. M. Flórez[1,2], H. B. Fêo[1], G. N. da Silva[3], R. S. Yamatogi[4], A. J. Aguiar[1], J. P. Araújo Jr.[4] and N. S. Rocha[1]

[1]Department of Veterinary Clinics, Faculty of Veterinary Medicine, São Paulo State University – UNESP, Botucatu, Brazil
[2]Veterinary Pathology Research Group, Faculty of Agricultural Sciences, Universidad de Caldas, Manizales, Colombia
[3]Department of Clinical Analysis. Pharmacy School, Universidade Federal de Ouro Preto, Ouro Preto, Brazil
[4]Department of Microbiology and immunology, Institute of Biosciences of Botucatu (IBB) and Biotechnology Institute (IBTEC), São Pablo State University – UNESP, Botucatu, Brazil

Abstract

Transmissible venereal tumour (TVT) generally presents different degrees of aggressiveness, which makes them unresponsive to conventional treatment protocols. This implies a progressive alteration of their biological profile. This study aimed to evaluate the cytotoxicity, cell survival, apoptosis and cell cycle alterations in TVT cell cultures subjected to treatment with vincristine. Similarly, it assessed possible implications of MDR-1, TP53, BCL-2, and BAX gene expressions in eight TVT primary cultures for both resistance to chemotherapy and biological behaviour. When comparing TVT cells receiving vincristine to those untreated, a statistical difference related to increased cytotoxicity and decreased survival rates, and alterations in G1 and S cell cycle phases were found but without detectable differences in apoptosis. Increased MDR-1 gene expression was observed after treatment. The groups did not differ statistically in relation to the TP53, BAX and BCL-2 genes. Although preliminary, the findings suggest that such augmented expression is related to tumour malignancy and chemotherapy resistance.

Keywords
apoptosis, chemotherapy, cytotoxicity, MDR-1, toxicogenomic

Correspondence address:
Mauricio M. Flórez
Department of Veterinary Clinics
Faculty of Veterinary Medicine
São Paulo State University – UNESP
Botucatu
SP
Brazil
e-mail:
maomontoya53@yahoo.es

Introduction

Despite its uncertain origin, transmissible venereal tumour (TVT) is a malignancy classified morphologically as a round cell neoplasm with plasmacytoid or lymphocytic aspect.[1] The cells with lymphocytic aspect are small and have a regular shape with a round core, which has coarse chromatin and one or two nucleoli. The cytoplasm is sparse and finely granulated, with vacuoles in the cell periphery. The cells with plasmacytoid aspect are bulky and present an irregular contour, with eccentric nucleus and abundant cytoplasm.[1–3]

According to this development, we may observe a nodular and friable tissue with hemorrhagic areas, often slightly marked and presenting ulcerations. This tumour may present in solitary or multiple form, presenting aspect of cauliflower or plates, with the presence of a serosanguineous secretion and possible secondary bacterial infection. Animals can also present itching and show behavioural

changes, often becoming aggressive or apathetic, lethargic and anorexic. In more advanced cases, with perineal tumour progression, urinary retention may be observed.[3,4]

The transplantation occurs when intact host tumour cells lose the ability to express molecules of major histocompatibility complex (MHC) class I and II, enabling tissue transposition to a healthy animal by contact between skin and/or damaged mucous membranes. The characteristics of canine coitus allows prolonged contact abrasions on genital mucous membranes, making the coitus an efficient transmission mode. Once a tumour is established, its proliferation may occur by reaching other locations, which eventually develops into metastases.[2–4]

Spontaneous regression is documented in cases of experimental TVT, but the same is not routinely reported in clinical care.[3,5] The tumour spontaneously regresses in healthy animals, and such regression is associated with the infiltration of lymphocytes and plasma cells as well as necrosis and apoptosis. Despite Higgins reporting spontaneous tumour regression, numerous clinical studies have not registered this event. In addition, the chronic presence of tumours for periods of 4 years opposes to this theory.[3,4]

The therapeutic protocol established for TVT, namely a weekly application of vincristine sulphate as a single agent (four to eight intravenous shots), is the most widely used for tumour regression.[2–4] Despite a description that 90% of dogs responded positively to this treatment, the presence of signs and symptoms suggesting serious side effects leads to a treatment interruption in a high number of cases.[5,6]

One suitable drug against vincristine-resistant tumours is doxorubicin.[7] Vinblastine, prednisone or their combination, is less often applied because of greater side effects.[8,9] Therefore, TVT therapy is currently restricted to a small number of drugs, which are sometimes insufficient because of specific tumour resistances.[5,6,10]

The progressive increase of TVTs with high percentages of aggressiveness and variable response to chemotherapy, including resistance, is partially due to the high expression of p-glycoprotein (Pgp) by tumour cells, which leads to the expulsion of chemotherapeutic.[11–13] Thus, treatment cost increases, as do side effects such as anorexia, nephrotoxicity and myelosuppression.

Similarly, other mechanisms related to therapy resistance and variable biological behaviour of tumours include changes in pro- and anti-apoptotic genes from the family *BCL-2*, and DNA repair systems, which are associated with the family *TP53*. However, this aspect has been scarcely studied in TVT.

Pgp, also known as ABCB1, is produced by the gene *MDR-1*. This protein has a molecular mass of 170 kDa, 12 transmembrane domains and 2 ATP bond sites, and belongs to the group of ABC proteins (ATP-binding cassette). They are energy-dependent channels involved in the transfer of biological molecules through the membrane against concentration gradient, with high expression in tumour tissues previously exposed to drugs.[14,15]

Pgp expresses in different tissues,[16,17] and defends cells from cytotoxic agents under normal conditions.[17,18] However, an overexpression of Pgp not only induces to a multidrug resistance[19] by reducing intracellular drug concentration to non-lethal levels,[15,20,21] but also seems to play a role in preventing early apoptosis in tumour cells.[22] The *TP53* gene is one that often mutates, including in human cancer. Several reports indicate alteration of *TP53* in about the half of studied tumours.[23] From a clinical point of view, *TP53* inactivation or mutation is a usual and severe molecular event for most tumours. There are mutant *TP53* forms displaying longer half-life times, oncogenic potential and negative effects on the unique types, leading to chemoresistance.[24]

Substantial evidence supports the hypothesis that the expression of *MDR-1* (Pgp) is regulated by some mutants of p53 protein, and suggests that the response to chemotherapy or radiation may depend in part on *TP53* status before treatment.[25]

The *TP53* gene is located on the short arm of human chromosome 17 (17p13),[24] that encodes p53 formation a nuclear phosphoprotein of 53 kDa consisting of 393 amino acids, which has implications for the cell cycle, DNA repair synthesis, cell differentiation, genomic plasticity and cell death programming.[26,27]

A *TP53* mutation has been described in dogs.[28] In TVT, Choi and Kim described the first reports about its mutation.[29] Sánchez-Servín *et al.* supported further findings.[30] Nonetheless, in spite of the gene mutation evidence in the tumour, the ability of this feature to generate some changes in the protein function remains unknown.[31]

According to Sánchez-Servín *et al.* determining the role of *TP53* polymorphisms in the pathogenesis and response to chemotherapy is still necessary in relation to TVT.[30] In addition, Stockmann *et al.* and others emphasise the need to assess the effects of expression of the protein and its family members on TVT cells, by relating the findings to prognosis and possible treatment options.[32]

The genes *BAX* and *BCL-2* belong to the same family that comprises about 25 genes. They encode proteins regulating permeability of the outer membrane of mitochondria, and are subdivided according to their domain and function in apoptotic (Bax, Bad, Bak and Bok, etc.), and anti-apoptotic (*BCL-2* itself, *BCL-XL* and *BCL-W*) processes.[14,33,34] Because *BCL-2* overexpression was identified in B cell malignancies, it has been observed in different types of tumours and cell lines in both humans and other animals.[35–37] Its involvement in cancer has also been determined.[14] However, reports on *BCL-2* participation in carcinogenesis are heterogeneous. On the one hand, overexpression confers resistance of many cell types to drugs and radiation.[34,37] On the other hand, overexpression also relates to low and favourable malignant phenotype prognoses.[38]

In TVT, Bcl-2 family protein expression has been identified. According to Stockmann *et al.* Bcl-2 overexpression is independent of the TVT development stage.[32] Previously, Frenzel *et al.* suggested that overexpression of Bcl-2 may promote the acquisition of functions associated with tumour progression and survival.[39] Similarly, Amaral *et al.* emphasise that less aggressive TVT have a high rate of apoptosis, which may lead to better prognosis.[40]

Thus, culture studies have formidably increased the understanding of the pathogenesis of certain cancers, and provided a basis for developing new methods of tumour diagnosis and treatment.[41] As to TVT, the success of culturing has been described by other researchers,[42–47] although few analyses were made in their studies.

It is known that some TVTs have varying degrees of aggressiveness and resistance to chemotherapy. This demonstrates the need for a specific treatment for each type of tumour, which would minimize cost and side effects by avoiding excessive use of chemotherapy.[6,8,11]

Finally, considering the importance of achieving further compression of TVT evolution and pathogenicity, this study aimed to evaluate the cytotoxicity, cell survival, apoptosis and cell cycle alterations in TVT cell cultures subjected to treatment with vincristine. Similarly, it assessed possible implications of *MDR-1*, *TP53, BCL-2* and *BAX* gene expressions for both resistance to chemotherapy and biological behaviour, in TVT primary cultures.

Results

Eight cell tumour cultures were isolated (Fig. 1). After establishing subcultures, cells were characterized by immunocytochemistry, presenting positive values for vimentin, lysozyme, alpha-antitrypsin and negative for CD3 and CD79α. Chromosome analysis revealed numbers ranging from 56 to 70. None of them exhibited the same number as dog somatic cells (data being prepared for publication).

Cytotoxicity and survival in TVT cells

The cytotoxicity test showed greater cytotoxicity and high significance in treated cells at 0.25, 0.5 and 1 μM ($P < 0.01$), when compared with control samples. On the other hand, when comparing treatments, there was greater cytotoxicity at 1 μM than 0.25 μM ($P < 0.05$) (Table 1, Fig. 2).

In relation to survival analysis, treated cells displayed lower levels and higher significance ($P < 0.01$). In addition, no difference among different concentrations was found ($P > 0.05$) (Table 1, Fig. 3).

Apoptosis

In this case, evaluated parameters were viable cells, initial apoptosis, late apoptosis and necrosis. Differences among cells with and without treatment were not observed ($P > 0.05$) (Table 2).

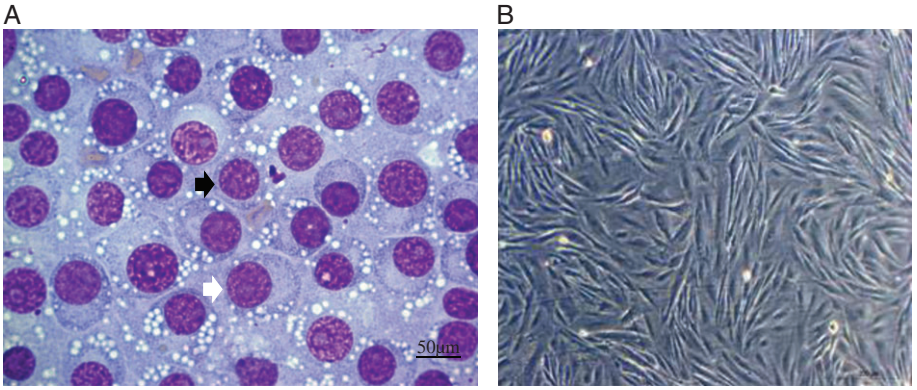

Figure 1. Isolation of TVT cells. (A) Cytology in TVT (×40 obj). Black arrow: lymphocytic standard (round cells, little cytoplasm and high nucleus:cytoplasm ratio). White arrow: plasmacytoid standard (ovoid cells, broad cytoplasm and eccentric nuclei). (B) TVT cell culture, third *passage*, form ranging from spindle to oval, bar 200 µm.

Table 1. Percent viability (cytotoxicity) and TVT cell survival after vincristine treatment

Test	Negative control	0.25 µM	0.5 µM	1 µM
Percentage of viable cells (cytotoxicity)	100^{a}	55.3 ± 14^{b}	$48.1 \pm 18^{b,c}$	39.8 ± 17^{c}
Percentage of survival	100^{a}	40.7 ± 16^{b}	35.8 ± 18^{b}	33.5 ± 17^{b}

Data expressed as average ± of the standard deviation. Different letters indicate statistical differences a,b ($P < 0.01$); b,c ($P < 0.05$).

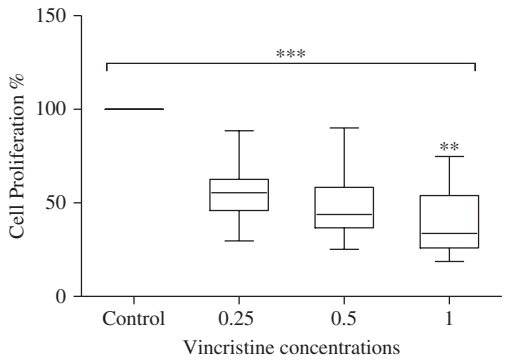

Figure 2. Percentage of TVT cell viability after 24-h vincristine treatment. A statistical difference between controls and treatments can be noted *** ($P < 0.01$), between 0.25 and 0.5 µM ** ($P < 0.05$).

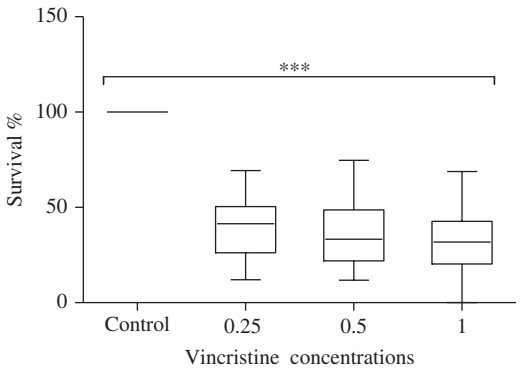

Figure 3. Percentage of TVT cell survival 5 days after vincristine treatment. A statistical difference between control and treatments can be observed ***($P < 0.01$).

Cell cycle

In this analysis, the cell cycle phases G1, G2, S and sub G1 were taken into account. Treated cell analysis showed a significant decrease in G1, for all treatments and in S phases, only in T3 (1 µM), at respective significance levels of $P < 0.01$ and $P < 0.05$. In the phase sub G1, a significant increase ($P < 0.01$) was also found in treated cells. In addition, a difference between T3 compared with T1 ($P < 0.05$) was evident. Similarly, differences in G2 phase remained undetectable in any treatment ($P > 0.05$) (Table 3).

Gene expression

In all samples, integrity of 18S and 28S ribosomal RNA, on agarose gel, was suitable. Analysis of the gene *MDR-1* disclosed variable expressions ranging from high (RQ = 2.19 to 4.48) to low (RQ = 0.006 to 0.1). A statistical difference ($P < 0.05$) was determined by comparing

Table 2. Percentage of viable TVT cells in apoptosis and necrosis after vincristine treatment

Cell state	Control	0.25 μM	0.5 μM	1 μM
Viable	80.96 ± 10.57	72.41 ± 15.41	73.80 ± 16.08	69.60 ± 22.17
Initial apoptosis	12.17 ± 10.02	19.10 ± 15.82	18.83 ± 17.11	18.53 ± 18.54
Late apoptosis	5.37 ± 3.46	6.56 ± 3.85	5.19 ± 2.46	8.26 ± 11.79
Necrosis	1.5 ± 2.32	1.92 ± 2.61	2.17 ± 3.12	3.56 ± 4.83

Data expressed as average ± standard deviation. Statistical comparisons data showed no significant differences ($P > 0.05$).

Table 3. Kinetics of the cell cycle in TVT cells 48 h after vincristine treatment

Cell state	Control	0.25 μM	0.5 μM	1 μM
G1 (%)	63.7 ± 15.17[a]	43.39 ± 15.8[b]	41.94 ± 16.74[b]	39.50 ± 14.69[b]
S(%)	2.9 ± 1.51[a]	2.15 ± 0.69[ab]	2.4 ± 1.07[ab]	1.67 ± 0.6[b]
G2 (%)	19.98 ± 12.08[a]	23.28 ± 11.94[a]	19.07 ± 7.74[a]	16.34 ± 7.59[a]
Sub G1 (%)	13.42 ± 7.91[a]	31.17 ± 13.52[b]	36.59 ± 15.17[bc]	42.49 ± 17.29[c]

Data expressed as average ± standard deviation. Different letters indicate statistical differences a,b ($P < 0.01$); b,c ($P < 0.05$).

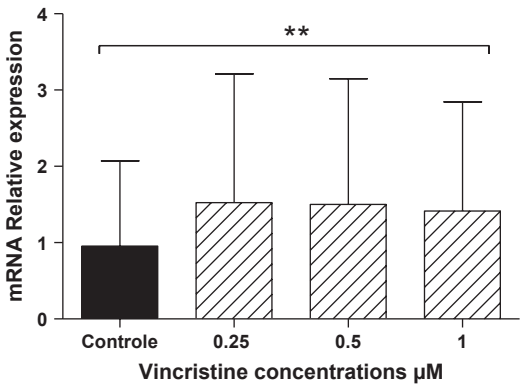

Figure 4. mRNA relative expression *of MDR-1* in TVT cells after vincristine treatment. Endogenous control by RPS5N and RPS19. Evidence of a statistical difference between treated and control cells $^{**}P < 0.05$.

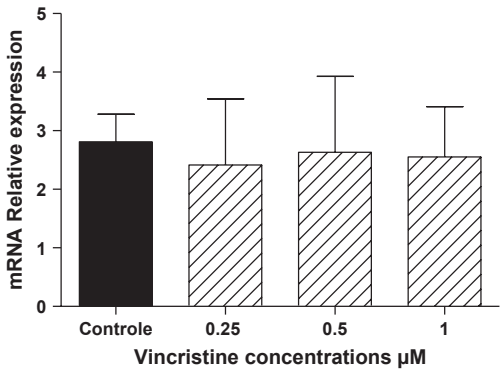

Figure 5. mRNA relative expression of *TP53* in TVT cells after vincristine treatment. Endogenous control by RPS5 and RPS19. No evidence of a statistical difference between treated and control cells was found at $P > 0.05$.

treated and control cells (Fig. 4). As to *TP53*, *BAX* and *BCL-2* gene expressions, statistical analysis revealed no significant differences in groups with and without treatment ($P > 0.05$). Is important to note that the QR value for *TP53* expression was > 2.00 in samples with and without treatment (Fig. 5).

Discussion

A high-level of Pgp (*MDR-1*) expression in tumours is associated with a reduced susceptibility to therapy in dogs,[48–53] and humans.[15,20,21] Therefore, patients with this type of alteration require a greater number of doses or higher drug concentrations during treatment.

In this investigation, cells subjected to vincristine displayed a variable expression of the *MDR-1* gene. These results are important for two reasons. First, this allows demonstration of variability in TVT cells as a response to therapy. Second, results are an indication of an existing modulator effect of vincristine on *MDR-1* gene expression.

Utilizing this rationale, researchers reported on the association between the use of vinca alkaloids and an increased Pgp expression, which develops in parallel drug resistance.[53–56] Similarly, an outcome from a previous research study conducted by the

authors of this work indicates that TVTs with a significantly greater immunoreactivity to the staining with anti-glycoprotein-p antibody was also directly related to a partial response to chemotherapy.[11,12]

Given these findings, in some TVT cultures, *MDR-1* expression may be modulated by vincristine, besides being an important mechanism for the regulation of drug resistance. Such a position is based on the action mechanisms of *MDR-1*. As stated, Pgp (*MDR-1*) carries cytotoxic agents such as vincristine to the outside of cell,[21] which reduces its levels to non-lethal concentrations.[17,18] Consequently, Pgp (*MDR-1*) overexpression is associated with drug resistance.[20] Furthermore, under this condition, a significant influence on the apoptosis mechanisms in the tumour cells can be observed, namely preventing early apoptosis in tumour cells.[22]

On the other hand, a lower *MDR-1* expression has been reported. However, this condition is still controversial given its description as a leading factor for an increased exposure to toxic agents, which eventually raises the risk of developing alterations in the cell genome. This would cause different grades of malignancy,[22] a condition that was also observed in cells through this study, specifically in those untreated. For this reason, further studies are still needed to identify the function of TVT cell conditions.

TVT cells with low levels of Pgp expression may require chemotherapy to increase its expression as in other tumours.[55] Moreover, multiclonality in TVT cells is possible due to its primary origin, including clonal selection when creating cultures. However, further studies are necessary to clarify these aspects because this is the first veterinary oncological study to identify *MDR-1* gene expression in TVT by means of real-time reverse transcription (RT-qPCR) at the *in vitro* level.

The *TP53* expression found in cells was generally high, a condition associated with poor prognosis in lymphomas,[57] and breast cancer.[58] Nevertheless, in other tumours a variable behaviour is described because of their functions. *TP53* induces p53 activation that contributes to apoptosis or autophagy.[59] Furthermore, this protein participates in different phases of the cell cycle such as the G1 phase of transactivation by p21, and the G2/M by blocking cell entry to mitosis.[27]

P53 produced by *TP53* may present a dual mechanism in cells, that is, protector and inducer of apoptosis.[27] Given the results, the occurrence of these mechanisms may also occur in TVT cells. In the first case, this process can cause a longer cell survival, and increase the possibility of malignant transformation.

TP53 overexpression has been considered a marker for the presence of mutations[25]; in TVT, researchers have already described gene mutations.[29–31] Thus, the results of this research regarding *TP53* may be related to what has already been disclosed. Therefore, further investigations should be pursued.

Another apoptotic pathway stimulated by *TP53* is *BAX* gene expression.[34,60] A high expression of this gene in all cells also exhibiting high *TP53* expression was expected during the present investigation, because the latter promotes apoptosis through *BAX* upregulation and *BCL-2* downregulation.[61] However, this condition was not observed. In this case, the presence of *TP53* mutations in TVT cells is possible, because the loss of *TP53* activity in cancer cells has been associated with limited *BAX* production and apoptosis activation by the mitochondrial pathway.[62,63]

Finally, in reference to gene expression of the *BCL-2* family, our results are unprecedented in TVT; mRNA expression of *BCL-2* and *BAX* did not differ between groups. In this tumour, only the expression of Bcl-2 protein has been shown so far. With respect to Stockmann *et al.*, the high expression of Bcl-2 protein was revealed by immunohistochemistry.[38] Nonetheless, identifying the implications of this condition within the tumour is necessary.

In other studies, *BCL-2* overexpression was found by analysing expression of the same genes in other types of tumours in humans and other animals.[14,35–37,63,64] This condition confers to cells resistance to apoptotic stimuli,[34] and prevents cell death induced by drugs or radiation therapy,[37,65] besides accelerating tumour genesis by *MYC* deregulation and other oncogenes. However, this situation is also associated with phenotypes

that present poor malignancy and favourable prognosis.[34] On the basis of this results, such implications are improbable in TVT.

At the end of this research, *in vitro* results in TVT cells also differed from descriptions in the literature of vincristine as an inducer of *BAX* expression in the process of activating cell death mechanisms.[66] Such a divergence should still be investigated to determine whether these interactions have some association with tumour biological behaviour.

The Cell Proliferation Kit II (XTT) test, performed in the analysis, is one of the most commonly used colorimetric indicators of cell viability, being able to assess the mitochondrial cell function in accordance with the enzymatic reduction of the tetrazolium salt by mitochondrial dehydrogenases in viable cells.[67] However, this test does not easily differentiate between cytocidal activity and the cytostatic compound. In relation to the cited work, vincristine used in the tests is a drug of proven cytostatic effect[68,69] that induces apoptosis or cell death after a prolonged arrest of the cell cycle.[69–71]

Despite this evidence about vincristine during the experimental apoptosis analysis, no differences were observed between treated and control cells after 48 h of treatment. However, a detailed cell cycle analysis in samples receiving treatment showed a decrease in the percentage of cells in G1 phase and a significant increase in sub G1 phase, with the latter being dependent on concentration. In other words, an anti-proliferative effect was present. In any case, differences in the number of cells with proliferative capacity (G2 phase) were not found. All results demonstrated the existence of a cell sensitivity to the test compound.

By design, vincristine concentrations in this work were close to the therapeutic dose *in vitro*.[72–75] Similarly, time was spent just for allowing vincristine to develop its cytostatic potential.[66,74,76] Thus, results agreed in relation to cell behaviour when facing antitumour agent.

Taking into account the above data, the description of vincristine antitumour potential varies according to concentration, exposure time,[71] and the number of cells in the mitosis phase during the exposure period.[71,77] Researchers using the drug for solid tumour treatment, and other cell lines of different TVT, also described high percentages of apoptosis.[71,77,78] In addition, Shi *et al.*, when comparing various cancer cell lines observed that death, as a response to antimitotic treatment, varies widely.[79]

In TVT, variable responses to treatment are also described. Hsiao *et al.* reported on TVT apoptosis, both in neoplasias healing spontaneously and in those receiving treatment.[80] However, the two cases associated this condition with the presence of cytotoxic T cells. On the other hand, Stettner *et al.* described the contrary, because apoptosis was observed in tumours with scarce presence of such cells.[81] This raises questions about whether a residual activity of cytotoxic T cells is also sufficient to generate apoptosis.

Accordingly, apoptosis has not been confirmed as the main mechanism of TVT cellular death induced by vincristine. Similarly, new research studies are needed, since the evidence in the present work ties this drug to other death mechanisms. Researchers found that cells undergoing prolonged arrest of mitosis by drugs can follow generally two pathways: going into a typical process of apoptosis from the beginning; or passing to a dropout process of 'slippage', in which cells depart from mitosis and return to G1 phase, maintaining a tetraploid state.[65,82]

Nevertheless, researchers describing such a mechanism differ as to the possible development of these cells. For example, Jordan *et al.* reported that cells undergoing this process die at the next interphase, or after one or more cycles.[74] Other scientists such as Panvichian *et al.* indicate possible death during mitosis.[83] However, Chen and Horwitz disclose a continued and abnormal division of these cells.[84]

Furthermore, Orth *et al.* in treating MCF7 cells resistant to apoptosis, found that after the 'slippage' process cells showed DNA damage, which induced p53 and p21, as well as other types of cycle inhibitors, to deprive cells of a new mitosis.[85] Based on these results, it may be proposed that some types of cells showing partial apoptosis depend on a combination with DNA damage to induce their death.

Considering these different hypotheses, results generated by this research in relation to apoptosis and the cell cycle may be attributable to some

of these mechanisms, which characterizes unprecedented outcomes. Thus, investigating the influence of types of death other than apoptosis in TVT cells may give rise to new therapeutic options.

As the results of G2 phase, investigators, such as Mujagic *et al.* treated sarcomas with vincristine, and described a transient accumulation of cells related to this phase, peaking 4 and 8 h after treatment.[73] After this period, levels decreased, including drug presence. It is reported that even cells are more susceptible to the lethal effects of vincristine as they progress from S phase to G2.[68,73,86] Taking into account the findings of this work, even in the case of another type of tumour, similar events may occur in TVT cells.

Moreover, evaluations of cytotoxicity and survival are essential for providing information about cellular damage.[87] In this study, vincristine was employed to demonstrate how TVT cells behave in an environment devoid of immune system and extracellular matrix. In this case, cells decreased metabolism, and exhibited high cytotoxicity and low survival. To the best of our knowledge, the literature has no reference to *in vitro* results similar to TVT culture. Nonetheless, findings for other cancers concluded that such a situation is related to the action mechanism of vinca alkaloids, which inhibits cell proliferation and causes mitosis blockage and cell metabolism.[71,88] Therefore, it is believed that similar mechanisms must occur in TVT cells, because vincristine is a universal chemotherapy drug.

Authors describe response rates ranging from 8 to 100% when vincristine is used for the treatment of tumours *in vitro*, corroborating this work.[89,90]

Divergences found in this study include a variable cytotoxicity level at concentrations of 0.25 and 1 μM, in which treatment with the highest drug concentration produced the highest cytotoxicity levels, thus establishing an association with antitumour potential of vincristine. In this case, vincristine acts according to its concentration and exposure time.[71,73] Nevertheless, vincristine at a high concentration completely abolishes microtube depolymerization.[66,71,73] Moreover, a high-vincristine concentration provokes lethal cytotoxicity.[71] Thus, in this work, higher doses resulted in lower cell metabolism, a finding that can account for the observed outcomes.

Conclusions

Given the conditions under which this research was carried out, the following conclusion can be established: *MDR-1* gene expression (Pgp) was higher in TVT cells with lower cytotoxicity and higher survival levels after chemotherapy with vincristine.

However, these cells present high expression of the *TP53* gene. In this case, the presence of *TP53* mutations in TVT cells is possible. The *BAX* genes and *BCL-2* showed no significant changes in expression after the treatment. This fact must be clarified in order to identify the association with chemotherapy resistance and tumour malignancy. Cell death in TVT cultures, after treatment with vincristine, may be linked to other mechanisms of cell death such as mitotic catastrophe, beyond the possibility that these cells may possess a mechanism favouring the escape from mitosis.

Finally, the identification of higher expression of *MDR-1* in TVT cells could help to improve the therapy in animals presenting this type of tumours, which would minimize cost and side effects by avoiding excessive use of vincristine and/or other treatments.

Methods

Tumour collection

This study was submitted to the Ethics Committee on Animal Use (CEUA), Faculty of Veterinary Medicine and Animal Science, Botucatu – UNESP, obtaining a favourable opinion (Protocol 223/2011). The experimental protocol included epidemiological study based on information from owners on the animals' medical history, behavioural habits, reproductive history, contact with other dogs, population density in the living area, and previous treatments.

Cytological preparations of tumours were fixed, and then stained using the Giemsa technique (May Grünwald Giemsa 2%). A minimum of 100 cells per slide (2) were analysed using a light microscopy at ×400 magnification for tumour diagnosis and classification, according to the characteristics of

the predominant cell type, namely plasmacytoid, lymphocytoid or mixed, as described by Amaral *et al.*[1]

Once TVT diagnosis had been confirmed, the animals were anaesthetized for total cleansing of the tumour site, where sample collection was carried out by incisional biopsy, obtaining fragments of approximately 1 cm^3. All samples were taken from patients before undergoing chemotherapy. The samples were stored in saline and phosphate solution (PBS) pH 7.4 in RNA Later (Qiagen®, Venlo, Limburg, the Netherlands), until processing of the material.

TVT primary culture and compound test concentration

Insulations of TVT cultures were made according to the protocol described by Bassani-Silva *et al.* and Hsiao *et al.*[46,91] Thus, aseptic fragments of TVT, placed in saline PBS pH 7.4 (Invitrogen, Life Technology, Waltham, MA, USA), were transported to the Laboratory for *in vitro* Fertilization and Cellular Cultures in the Department of Animal Reproduction and Veterinary Radiology FMVZ – UNESP, Botucatu Campus.

There, samples were ground using a stainless steel scalpel. Subsequently the material was transferred to a trypsin solution (TrypLE Select; Invitrogen, Life Technology) at 37.5 °C, and kept for 40 min with a magnetic homogenizer, after which the solution was passed through a filter of 70 μm (70 μm Falcon® cell strainers, Corning, NY, USA). Cells resulting from this process were placed in a falcon tube over a Percoll gradient to 42% (Amersham Pharmacia Biotech, Piscataway, NJ, USA), and centrifuged (820 × *g*, 4 °C, 25 min). TVT cells located in the air–liquid interphase were collected, and then the pellet was resuspended and conditioned in 25 cm two flasks (Sarstedt, Germany) with 5 mL of DMEM high glucose culture (Dulbecco's modified essential medium – Gibco). This material was supplemented with 10% foetal calf serum (FCS) (Gibco, Life Technologies), and with the combination of 100 U mL^{-1} penicillin and 100 mg mL^{-1} streptomycin (Life Technologies, Gibco) and 3μg mL^{-1} amphotericin B (Life Technologies, Gibco). The initial isolation purity was confirmed using Hemacolor (Merck, Whitehouse Station, NJ, USA).

Following, samples were maintained in a CO_2 incubator at 5%, moisture 95% and a temperature of 37.5 °C. Cell viability and concentration were assessed by an exclusion test using trypan blue, and cells resuspended in DMEM high glucose culture (DMEM – Gibco, Life Technologies).

It is important to highlight that, in order to verify that cells coming from cultures as pertaining to TVT, samples were subjected to immunocytochemistry as well as analyses of chromosome numbers in the Animal Genetics Laboratory of the Institute of Biosciences UNESP – Botucatu (data not yet published).

Vincristine (Sigma-Aldrich, St. Louis, MO, USA), at different concentrations (0.25, 0.5 and 1.0 μM L^{-1}), was utilized for crop treatment. Taking into account the molecular weight of the drug (923.04 μmol L^{-1}), compound test concentrations were 0.023, 0.046 and 0.092 μmol L^{-1}.

Cytotoxicity and cell survival tests

For evaluation of cytotoxicity and cell survival, XTT (Roche Diagnostics, Mannheim, Germany) was used. First, cells were seeded in 96-well plates at respective cell concentrations of 5×10^3 to 1.5×10^3 for the cytotoxicity and survival tests. After 24 h, cells were treated with 0.25, 0.5 and 1.0 μM L^{-1} vincristine (Sigma-Aldrich) for 24 h. Untreated cells were used as control.

For the cytotoxicity test, immediately after treatment with test compound, cells were washed with 1× Hanks solution (0.4 g KCl, 0.06 g KH_2PO_4, 0.04 g Na_2HPO_4, 0.35 g $NaHCO_3$, 1 g glucose and 8 g NaCl in 1 L·H_2O). Next, 180 μL of DEM without phenol red (Invitrogen, Life Technologies), and 20 μL of XTT (XTT labelling solution 1 mL/20 μL of electron-coupling reagent) were added to the cells, being further incubated for 2 h at 370 °C in 5% CO_2. Subsequently, the medium was removed with XTT, and taken for reading.

For testing cell survival after treatment, specimens were washed with 1× Hanks solution, culture medium changed and again incubated at 37 °C for 5 days. After this period, the incubation was

performed using the XTT kit in the same manner already described for the cytotoxicity assay.

Dye absorption was read by enzyme-linked immunosorbent assay (ELISA) system, Spectra Count, at 450 and 690 nm wavelength, with the result being proportional to the number of viable cells in the test sample. All tests were performed in triplicate.

Cell cycle kinetics and apoptosis detection

For both tests, 2×10^5 cells were seeded in 6-well plates. After 24 h cells were treated with 0.25, 0.5 and 1.0 μM L^{-1} of vincristine (Sigma-Aldrich). After 48 h, they were washed with Hanks solution (0.4 g KCl, 0.06 g KH_2PO_4, 0.04 g Na_2HPO_4, 0.35 g $NaHCO_3$, 1 g glucose and 8 g NaCl in 1 L·H_2O), detached with trypsin (TrypLE Select – Invitrogen, Life Technologies) and then resuspended in fresh medium.

To assess the cell cycle, samples were centrifuged at 600-g for 10 min. The pellet was resuspended in 200 of HSF (50 mcg propidium iodide, 0.1% sodium citrate, 0.1% Triton X-100), stored on ice, and protected from light for 30 min. The percentages of cells in G0/G1, S and G2/M were measured using the software GUAVA Cytosoft, version 4.2.1.

For apoptosis, cells were resuspended in 100 μL of Guava nexin reagent kit (Millipore Merck®, Darmstadt, Germany), protected from light for 20 min, and immediately analysed in easyCyte Guava flow cytometer (Millipore). Annexin V was utilized to identify the externalization of phosphatidylserine on the cell membrane, as well as 7-ADD as an indicator of cell membrane integrity. The analysis was performed using the software Guava System. Five thousands cells per sample were analysed.

Cell status was defined as follows: unstained cells – viable; cells stained only with Annexin – initial apoptosis; cells stained with 7-AAD and annexin – late apoptotic; and cells stained with only 7-AAD – necrosis. All analyses were performed in triplicate.

MDR-1, *TP53*, *BCL-2* and *BAX* expression by qPCR

Total RNA from cultures was extracted using the kit RNeasy Mini (Qiagen) according to the manufacturer's instructions. After being purified, RNA was treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA) for 30 min at 37 °C to avoid false positive results arising from the genomic DNA amplification. The quality of the extracted RNA was evaluated on 2% agarose gel stained with ethidium bromide, and analysed using the equipment NanoVue (GE Healthcare). The samples were stored in a freezer at −80 °C.

The complementary DNA (cDNA) synthesis was performed to 1 μg of RNA using the kit High Capacity (Applied Biosystems®). The reaction was performed with 6 μL of Random Primer (10×), 6 μL of RT buffer (10×), 2.5 μL dNTPs (25×), 3 μL of MultiScribe (50 μm mL^{-1}) and RNase-free H_2O, according to the manufacturer's protocol. The reaction was incubated at 25 °C for 10 min, then at 37 °C for 120 min, and finally stored at 4 °C. Samples were kept at a temperature of −20 °C.

The qPCR steps were performed in an automatic thermocycler (ABI Prism 7500 Sequence Detection System FAST, Applied Biosystems). A sequence amplification of primers is detailed in Table 4. The qPCR reaction consisted of 4 μL of sample cDNA, 200 nM of each primer, 10 μL GoTaq qPCR Master Mix (Promega), and nuclease-free water, giving a final volume of 20 μL.

The reaction conditions for all genes were: initial denaturation of 95 °C for 2 min, followed by 40 cycles of amplification (95 °C for 15 s for denaturation, 60 °C for 1 m for annealing and extension), and the dissociation curve (95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s). As a negative control, nuclease-free water was used in place of the sample. The relative standard curve for each gene was generated using serial dilutions of cDNA, which was considered a reference sample. The lowest dilution of the standard was considered in relation to a relative value of 100. Following serial dilutions of 1/10, the three points were 10, 1 and 0.1. The relative concentration of the studied genes were normalized according to Larionov *et al.*, through the most

Table 4. Sense and antisense genes used in RT-qPCR

Gene	Sense	Antisense
BAX[a]	GGTTGTTGCCCTCCT CTACT	GTAAGCACTCCAGCC ACAAA
BCL-2[b]	TGGATGACTGAGTAGC TGAA	GGCCTACTGACTTCAC TTAT
TP53[c]	CGCAAAAGAAGAAGCC ACTA	TCCACTCTGGGCATC CTT
MDR-1[c]	CAGTGGTTCAGGTGGC CCT	CGAACTGTAGACAAA CGATGAGCT
RPS5[d]	GAGGCGTCAGGCTGTC GAT	AGCCAAATGGCCTGA TTCAC
RPS19[d]	GGGTCCTCCAAGCCC TAGAG	CGGCCCCCATCTTGGT
ACTB[d]	GGCATCCTGACCCTCA AGTA	CTTCTCCATGTCGTCC CAGT

[a]Sano *et al.* 2005.[93]
[b]Klopfleisch *et al.* 2009.[94]
[c]Culmsee *et al.* 2004.[95]
[d]Brinkhof *et al.* 2006.[96]

stable endogenous control from the three endogenous genes tested (RPS5, RPS19 and ACTB).[92] All reactions were performed in duplicate. A QR value <0.5 was defined as decreased expression, whereas QR > 2.00 was considered increased expression.

Statistical analysis

Descriptive statistics were performed to analyse the results. Data from *in vitro* assays were expressed as an average percentage ± of the standard deviation, and compared by Kruskal–Wallis test, followed by multiple comparisons with Dunn's test. ANOVA test was conducted for the gene expression analysis, followed by the Tukey multiple comparisons test,[97] using the programme GraphPad Prism 5.0. Values of $P < 0.05$ were considered significant.

Acknowledgements

The authors would like to thank the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), São Paulo, SP, Brazil for its financial support to develop this project. This sponsor did not have any influence on the study design, on the collection, analysis and interpretation of data, or on the writing of the manuscript and decision to submit for publication. Proc 2012/19285-2. M. M. F. designed the study, analysed and interpreted the data, made a critical review of the manuscript and wrote the manuscript; H. B. F. critically revised and formatted the manuscript; G. N. S. made a critical review of and wrote the manuscript; J. A. A. substantially contributed to conceiving the study; R. Y. helped to draft the study; J. P. A. substantially contributed to conceiving the study, acquired the data and helped produce the draft; N. S. R. supervised and coordinated the study, provided clinical and pathology advice, and critically revised the manuscript. All authors read and approved the final manuscript.

Conflict of interests

The authors declare that they have no competing interests.

References

1. Amaral AS, Bassanil-Silva S, Ferreira I, Fonseca LS, Andrade FH, Gaspar LFJ, *et al.* Cytomorphological characterization of transmissible canine venereal tumor. *RPCV* 2007; **103**: 563–564.
2. Drumond KO, Quessada AM, Silva SMMS, Costa FAL, Silva LS, de Pinho FA, *et al.* Transmissible venereal tumor treated with autohemotherapy. *Acta Sci Vet* 2013; **41**: 1107.
3. Ganguly B, Das U and Das AK. Canine transmissible venereal tumour: a review. *Vet Comp Oncol* 2013; **11**: 1–12.
4. Erünal-Maral N, Findik M and Asalan S. Use of exfoliative cytology for diagnosis of transmissible venereal tumour and controlling the recovery period in the bitch. *Dtsch Tierarztl Wochenschr* 2000; **107**: 175–180.
5. Sousa J, Saito V, Nardi AB, Rodaski S, Guérios S and Bacila M. Características e incidência do tumor venéreo transmissível (TVT) em cães e eficiência da quimioterapia e outros tratamentos. *Arch Vet Sci* 2000; **5**: 41–48.
6. Athanasius EC, Kene RO and Anyanwu HC. Comparative efficacy of surgery, vincristine sulphate and combined therapy of levamisole and Bacille Calmette Guerin vaccine in the treatment of transmissible venereal tumour-infected dogs. *Comp Clin Pathol* 2014; **23**: 1263–1267.
7. Rogers KS. Transmissible venereal tumour. *Compend Contin Educ Pract Vet* 1997; **19**: 1036–1045.
8. Kunakornsawat S, Yippaditr W, Jamjan N, Bootcah R, Netramai S, Viriyarumpa J, et al. Surgical correction of transmissible venereal tumor with vincristine resistance using episiotomy and vulvo

vagino plasty in female and subtotal penile amputation and scrotal ablation in male dogs. In: *Proceeding of 48th Kasetsart University Annual Conference: Veterinary Medicine*, Bangkok, Kasetsart, 2010: 191–200.
9. Sudjaidee P, Theewasutrakul P, Techarungchaikul S, Ponglowhapan S and Chatdarong K. Treatment of canine transmissible venereal tumor using Vincristine Sulfate combined with L-Asparaginase in clinical vincristine-resistant cases: a case report. *Thai J Vet Med* 2012; **42**: 117–122.
10. Andrade SMF. *Manual de terapêutica veterinária*. 3rd edn., São Paulo, ROCA, 2008.
11. Gaspar LFJ, Amaral AS, Bassani-Silva S and Rocha NS. Imunorreatividade à glicoproteína-p nos diferentes tipos citomorfológicos de tumor venéreo transmissível canino. *Vet em Foco* 2009; **6**: 1–6.
12. Gaspar LFJ, Ferreira I, Colodel MM, Brandão CVS and Rocha NS. Spontaneus canine transmissible venereal tumor: cell morphology and influence on P-glycoprotein expression. *Turk J Vet Anim Sci* 2010; **34**: 447–454.
13. Gerardi DG, Tinucci-Costa M, Silveira ACT and Moro JV. Expression of P-glycoprotein, multidrug resistance associated protein, glutathione-S-transferase pi and p53 in canine transmissible venereal tumor. *Pesq Vet Bras* 2014; **34**: 71–78.
14. Mendelsohn J, Howley I and Thompson G. *The Molecular Basis of Cancer*. 3rd edn., Saunders, Elsevier, 2008.
15. Hodges LM, Markova SM, Chinn LW, Gow JM, Kroetz DL, Klein TE, *et al.* Very important pharmacogene summary: ABCB1 (MDR1, P-glycoprotein). *Pharmacogenet Genomics* 2011; **21**: 152–161.
16. Alexandrova R. Multidrug resistance and p-glycoprotein. *Exp Path Parasitol* 1998; **1**: 62–66.
17. Thomas H and Coley H. Overcoming multidrug resistance in cancer: an update on the clinical strategy of inhibiting p-glycoprotein. *Cancer Control* 2003; **10**: 159–165.
18. Maia RC and Rumjanek VM. Mecanismos moleculares de resistência a múltiplas drogas. In: *Oncologia Molecular*. CG Ferreira and JC Rocha Eds., São Paulo, Atheneu, 2004: 113–122.
19. Szakács G, Paterson JK, Ludwig JA, Booth-Genthe C and Gottesman MM. Targeting multidrug resistance in cancer. *Nat Rev Drug Discov* 2006; **5**: 219–234.
20. Tsujimura S and Tanaka Y. Treatment strategy based on targeting P-glycoprotein on peripheral lymphocytes in patients with systemic autoimmune disease. *Clin Exp Nephrol* 2012; **16**: 102–108.
21. Binkhathlan Z and Lavasanifar A. P-glycoprotein inhibition as a therapeutic approach for overcoming multidrug resistance in cancer: current status and future perspectives. *Curr Cancer Drug Targets* 2013; **13**: 326–346.
22. Wang LH, Song YB, Zheng WL, Jiang L and Ma WL. The association between polymorphisms in the MDR1 gene and risk of cancer: a systematic review and pooled analysis of 52 case–control studies. *Cancer Cell Int* 2013; **13**: 46.
23. Soussi T and Béroud C. Assessing TP53 status in human tumours to evaluate clinical outcome. *Nat Rev Cancer* 2001; **1**: 233–240.
24. Ozaki T and Nakagawara A. p53: the attractive tumor suppressor in the cancer research field. *J Biomed Biotechnol* 2011; **2011**: 13.
25. Moro JV, Tinucci-Costa M, Silveira AC, Gerardi DG and Alessi AC. Reactivity of p53 protein in canine transmissible venereal tumor. *Arq Bras Med Vet Zootec* 2010; **62**: 318–323.
26. Harris CC and Hollstein M. Clinical implications of the p53 tumor-suppressor gene. *N Engl J Med* 1993; **329**: 1318–1327.
27. Suzuki K and Matsubara H. Recent advances in p53 research and cancer treatment. *J Biomed Biotechnol* 2011; **2011**: 7.
28. Kanayan N, Okuda M, Toyama N, Oikawa T, Inokuma H, Morimoto M, *et al.* Detection of the anti-P53 antibodies in dogs with tumors. *J Vet Med Sci* 2002; **64**: 973–979.
29. Choi YK and Kim CJ. Sequence analysis of canine LINE-1 elements and p53 in canine transmissible venereal tumor. *J Vet Sci* 2002; **3**: 285–292.
30. Sánchez-Servín A, Martínez S, Córdova-Alarcon E and Fajardo R. TP53 polymorphisms allow for genetic sub-grouping of the canine transmissible venereal tumor. *J Vet Sci* 2009; **10**: 353–355.
31. Vázquez-Mota N, Simón-Martínez J, Córdova-Alarcon E, Lagunes L and Fajardo R. The T963C mutation of TP53 gene does not participate in the clonal origin of canine TVT. *Vet Res Commun* 2008; **32**: 187–191.
32. Stockmann D, Ferrari HF, Andrade AL, Cardoso TC and Luvizotto MC. Detection of the tumour suppressor gene TP53 and expression of p53, Bcl-2 and p63 proteins in canine transmissible venereal tumor. *Vet Comp Oncol* 2011a; **9**: 251–259.
33. Chao DT and Korsmeyer SJ. BCL-2 family: regulators of cell death – review. *Annu Rev Immunol* 1998; **16**: 395–419.
34. Czabotar PE, Lessene G, Strasser A and Adams MJ. Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. *Nat Rev Mol Cell Biol* 2014; **15**: 49–63.

35. Delfino AB, Barreto EC, Jr ETS, Mendonça RG and Ornellas MH. O envolvimento de genes e proteínas na regulação da apoptose – carcinogênese. *Rev Bras Cancerol* 1997; **43**: 173–186.
36. Fett-Conte AC and Salles ABCF. A importância do gene p53 na carcinogênes humana. *Rev Bras Hematol Hemoter* 2002; **24**: 85–89.
37. Zhou H, Chen J, Meagher JL, Yang CY, Aguilar A, Liu L, *et al.* Design of Bcl-2 and Bcl-xL inhibitors with subnanomolar binding affinities based upon a new scaffold. *J Med Chem* 2012; **55**: 4664–4682.
38. Stockmann D, Ferrari HF, Andrade AL, Lopes RA, Cardoso TC and Luvizotto MC. Canine transmissible venereal tumors: aspects related to programmed cell death. *Braz J Vet Pathol* 2011b; **4**: 67–75.
39. Frenzel A, Grespi F, Chmelewskijwand W and Villunger A. Bcl2 family proteins in carcinogenesis and the treatment of cancer. *Apoptosis* 2009; **14**: 584–596.
40. Amaral AS, Ferreira I, Colodel MM, Fávero DM and Rocha NS. DNA damage in canine transmissible venereal tumor. *Rev Lus Ciên Med Vet* 2011; **4**: 1–5.
41. Athanasou NA. Cell and organ culture in the understanding of bone and its neoplams. In: *Pathology of Bone and Joint Neoplams*. TR Helliwell Ed., Philadelphia, W.B. Saunders, 1999: 34–58.
42. Adams EW, Carter LP and Sapp WJ. Growth and maintenance of the canine venereal tumor in continuous culture. *Cancer Res* 1968; **28**: 753–757.
43. Mohanty GC and Rajya BS. Growth and morphological characteristics of canine venereal tumor cell in vitro. *Vet Pathol* 1977; **14**: 420–425.
44. Okamoto Y, Fujinaga T, Tajima M, Hoshi N, Otomo K and Koike T. Isolation and cultivation of canine transmisible sarcoma cells. *Nihon Juigaku Zasshi* 1988; **50**: 9–13.
45. Beschorner WE, Hess AD, Nerenberg ST and Epstein RB. Isolation and characterization of canine venereal tumor-associated inhibitory and blocking factors. *Cancer Res* 1979; **39**: 3920–3927.
46. Bassani-Silva S, Sforcin JM, Amaral AS and Sousa NR. Propolis effect in vitro on canine transmissible venereal tumour cells. *RPCV* 2007; **102**: 261–265.
47. Montoya FL, Bersano OP, Ferreira LJ, Pedraza FJ and Rocha NS. Caracterización inmunofenotípica de cultivos primarios de tumor venéreo transmisible canino. *Rev Col Cienc Pec* 2013; **3 Suppl**: 380.
48. Bergman PJ, Ogilvie GK and Powers BE. Monoclonal antibody C219 immunochemistry against P-glycoprotein: sequential analysis and predictive ability in dogs with lymphoma. *J Vet Inter Med* 1996; **10**: 354–359.
49. Lee JJ, Hughes CS, Fine RL and Page RL. P-glycoprotein expression in canine lymphoma: a relevant, intermediate model of multidrug resistance. *Cancer* 1996; **77**: 1892–1898.
50. Steingold SF, Sharp NJ, McGahan MC, Hughes CS, Dunn SE and Page RL. Characterization of canine MDR1 mRNA: its abundance in drug resistant cell lines and in vivo. *Anticancer Res* 1998; **18**: 393–400.
51. Honscha KU, Schirmer A, Reischauer A, Schoon HA, Einspanier A and Gäbel G. Expression of ABC-transport proteins in canine mammary cancer: consequences for chemotherapy. *Reprod Domest Anim* 2009; **44**(Suppl. 2): 218–223.
52. Zandvliet M, Teske E and Schrickx JA. Multi-drug resistance in a canine lymphoid cell line due to increased P-glycoprotein expression, a potential model for drug-resistant canine lymphoma. *Toxicol In Vitro* 2014; **28**: 1498–1506.
53. Breier A, Štefanková S, Barancík M and Tribulová N. Time dependence of [3h]-vincristine accumulation by L1210 mouse leukemic cells. Effect of P-glycoprotein overexpression. *Gen Physiol Biophys* 1994; **13**: 287–298.
54. Burkhart CA, Kavallaris M and Band Horwitz S. The role of beta-tubulin isotypes in resistance to antimitotic drugs. *Biochim Biophys Acta* 2001; **1471**: O1–O9.
55. Ambudkar SV, Kimchi-Sarfaty C, Sauna ZE and Gottesman MM. P-glycoprotein: from genomics to mechanism. *Oncogene* 2003; **22**: 7468–7485.
56. Sulová Z, Mislovicová D, Gibalová L, Vajcnerová Z, Poláková E, Uhrík B, *et al.* Vincristine-induced overexpression of P-glycoprotein in L1210 cells is associated with remodeling of cell surface saccharides. *J Proteome Res* 2009; **8**: 513–520.
57. Veldhoen N, Stewart J, Brown R and Milner J. Mutations of the p53 gene in canine lymphoma and evidence for germ line p53 mutations in the dog. *Oncogene* 1998; **16**: 249–255.
58. Lee CH, Kim WH, Lim JH, Kang MS, Kim DY and Kweon OK. Mutation and overexpression of p53 as a prognostic factor in canine mammary tumors. *J Vet Sci* 2004; **5**: 63–69.
59. Sui X, Jin L, Huang X, Geng S, He C and Hu X. p53 signaling and autophagy in cancer: a revolutionary strategy could be developed for cancer treatment. *Autophagy* 2011; **7**: 565–571.
60. Lacroix M, Toillon RA and Leclercq G. p53 and breast cancer, an update. *Endocr Relat Cancer* 2006; **13**: 293–325.
61. Haupt S, Berger M, Goldberg Z and Haupt Y. Apoptosis – the p53 network. *J Cell Sci* 2003; **116**: 4077–4085.

62. Liu FS, Jan YJ, Lai CR, Twu NF, Lu CH, Hung MJ, *et al.* Expression analysis of apoptosis-related markers TP53, BCL-2, BAX and c-MYC in female genital tract sarcomas. *J Chin Med Assoc* 2008; **71**: 628–634.
63. Fernald K and Kurokawa M. Evading apoptosis in cancer. *Trends Cell Biol* 2013; **23**: 620–633.
64. Llambi F and Green DR. Apoptosis and oncogenesis: give and take in the BCL-2 family. *Curr Opin Genet Dev* 2011; **21**: 12–20.
65. Topham CH and Taylor SS. Mitosis and apoptosis: how is the balance set? *Curr Opin Cell Biol* 2013; **25**: 780–785.
66. Groth-Pedersen L, Ostenfeld MS, Høyer-Hansen M, Nylandsted J and Jäättelä M. Vincristine induces dramatic lysosomal changes and sensitizes cancer cells to lysosome-destabilizing siramesine. *Cancer Res* 2007; **67**: 2217–2225.
67. Mosmann T. Rapid colormetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983; **65**: 55–63.
68. Jordan MA, Thrower D and Wilson L. Mechanism of inhibition of cell proliferation by Vinca alkaloids. *Cancer Res* 1991; **51**: 2212–2222.
69. Rixe O and Fojo T. Is cell death a critical end point for anticancer therapies or is cytostasis sufficient? *Clin Cancer Res* 2007; **13**: 7280–7287.
70. Galán-Malo P, Vela L, Gonzalo O, Calvo-Sanjuán R, Gracia-Fleta L, Naval J, *et al.* Cell fate after mitotic arrest in different tumor cells is determined by the balance between slippage and apoptotic threshold. *Toxicol Appl Pharmacol* 2012; **258**: 384–393.
71. Silverman JA and Deitcher SR. MARQIBO® (vincristine sulfate liposome injection) improves the pharmacokinetics and pharmacodynamics of vincristine. *Cancer Chemother Pharmacol* 2013; **71**: 555–564.
72. Dixon GJ, Dulmadge EA, Mulligan LT and Mellett LB. Cell culture bioassay for vincristine sulfate in sera from mice, rats, dogs, and monkeys. *Cancer Res* 1969; **29**: 1810–1813.
73. Mujagic H, Chen SS and Geist R. Effects of vincristine on cell survival, cell cycle progression and mitotic accumulation in Asynchronously growing sarcoma 180 cells. *Cancer Res* 1983; **43**: 3591–3597.
74. Jordan MA, Wendell K, Gardiner S, Derry WB, Copp H and Wilson L. Mitotic block induced in HeLa cells by low concentrations of paclitaxel (Taxol) results in abnormal mitotic exit and apoptotic cell death. *Cancer Res* 1996; **56**: 816–825.
75. Tanaka NM, Maeda MSCF, Pereira LI, Araújo RC, Kemper B, Gonçalves RC, *et al.* Atividade do sulfato de vincristina em cultivos primários de tumores mamários caninos. *Rev Acad Ciên Anim* 2013; **11**: 87–96.
76. Jackson DV and Bender RA. Cytotoxic thresholds of vincristine in a murine and a human leukemia cell line in vitro. *Cancer Res* 1979; **39**: 4346–4349.
77. Ehrhardt H, Pannert L, Pfeiffer S, Wachter F, Amtmann E and Jeremias I. Enhanced anti-tumour effects of Vinca alkaloids given separately from cytostatic therapies. *Br J Pharmacol* 2013; **168**: 1558–1569.
78. He D, Wu H, Ding L and Li Y. Combination of BCL11A siRNA with vincristine increases the apoptosis of SUDHL6 cells. *Eur J Med Res* 2014; **19**: 34.
79. Shi J, Orth JD and Mitchison T. Cell type variation in responses to antimitotic drugs that target microtubules and kinesin-5. *Cancer Res* 2008; **68**: 3269–3276.
80. Hsiao YW, Liao KW, Hung SW and Chu RM. Effect tumor infiltrating lymphocytes on the expression of MHC molecules in canine transmissible venereal tumor cells. *Vet Immunol Immunopathol* 2002; **87**: 19–27.
81. Stettner N, Brenner O, Eilam R and Harmelin A. Pegylated liposomal doxorubicin as a chemotherapeutic agent for treatment of canine transmissible venereal tumor in murine models. *J Vet Med Sci* 2005; **6**: 1133–1139.
82. Gascoigne KE and Taylor SS. How do anti-mitotic drugs kill cancer cells? *J Cell Sci* 2009; **122**: 2579–2585.
83. Panvichian R, Orth K, Day ML, Day KC, Pilat MJ and Pienta KJ. Paclitaxel-associated multimininucleation is permitted by the inhibition of caspase activation: a potential early step in drug resistance. *Cancer Res* 1998; **58**: 4667–4672.
84. Chen JG and Horwitz SB. Differential mitotic responses to microtubule -stabilizing and -destabilizing drugs. *Cancer Res* 2002; **62**: 1935–1938.
85. Orth JD, Loewer A, Lahav G and Mitchison TJ. Prolonged mitotic arrest triggers partial activation of apoptosis, resulting in DNA damage and p53 induction. *Mol Biol Cell* 2012; **23**: 567–576.
86. Huang Y, Fang Y and Wu J. Regulation of Vinca alkaloid-induced apoptosis by NF-kappaB/IkappaB pathway in human tumor cells. *Mol Cancer Ther* 2004; **3**: 271–277.
87. Rogero SO, Lugão AB, Ikeda TI and Cruz AS. Teste in vitro de citotoxicidade: Estudo comparativo entre duas metodologias. *Mat Res* 2003; **6**: 317–320.
88. Moudi M, Go R, Yien CY and Nazre M. Vinca alkaloids. *Int J Prev Med* 2013; **4**: 1231–1235.

89. Gidding CE, Kellie SJ, Kamps WA and Graaf SS. Vincristine revisited. *Crit Rev Oncol Hematol* 1999; **29**: 267–287.
90. Morales-Ramírez P, Vallarino-Kelly T and Cruz-Vallejo V. Kinetics of micronucleus induction and cytotoxicity caused by distinct antineoplastics and alkylating agents in vivo. *Toxicol Lett* 2014; **224**: 319–325.
91. Hsiao YW, Liao KW, Chung TF, Liu CH, Hsu CD and Chu RM. Interactions of host IL-6 and IFN-gamma and cancer-derived TGF-beta1 on MHC molecule expression during tumor spontaneous regression. *Cancer Immunol Immunother* 2008; **57**: 1091–1104.
92. Larionov A, Krause A and Miller W. A standart curve based method for relative real time PCR data processing. *BMC Bioinformat* 2005; **6**: 62.
93. Sano J, Oguma K, Kano R, Yazawa M, Tsujimoto H and Hasegawa A. High expression of Bcl-xL in delayed apoptosis of canine neutrophils induced by lipopolysaccharide. *Res Vet Sci* 2005; **78**: 183–187.
94. Klopfleisch R and Gruber AD. Differential expression of cell cycle regulators p21, p27 and p53 in metastasizing canine mammary adenocarcinomas versus normal mammary glands. *Res Vet Sci* 2009; **87**: 91–96.
95. Culmsee K, Gruber A, Himmelstjerna G and Nolte I. Quantification of MDR-1 gene expression in canine tissues by real-time reverse transcription quantitative polymerase chain reaction. *Res Vet Sci* 2004; **77**: 223–229.
96. Brinkhof B, Spee B, Rothuizen J and Penning L. Development and evaluation of canine reference genes for accurate quantification of gene expression. *Anal Biochem* 2006; **356**: 36–43.
97. Pagano M and Gauvreau K. *Princípios de Biostatística*. São Paulo, Cengage Learning Edições LTDA, 2004.

