HISTOPATHOLOGICAL AND CYTOLOGICAL ANALYSIS OF TRANSMISSIBLE VENEREAL TUMOR IN DOGS AFTER TWO TREATMENT PROTOCOLS

Fabiana Aguena Sales Lapa [1], Silvia Franco Andrade [2], Eduardo Roberto Gervazoni [3], Vanessa Massumi Kaneko [1], Osimar Carvalho Sanches [4], Luis Roberto Almeida Gabriel Filho [5]

[1] Discente do Mestrado em Ciência Animal da UNOESTE, Presidente Prudente – SP. [2] Docente do Mestrado em - Ciência Animal da UNOESTE. [3] Aluno do Curso de Medicina Veterinária da UNOESTE. [4] Docente do Curso de Medicina Veterinária da UNOESTE. [5] Docente da UNESP, Tupã - SP.

ABSTRACT
The transmissible venereal tumor (TVT) is a contagious neoplasm of round cells that frequently affect dogs. The treatment consists of chemotherapy being more effective the vincristine alone, however the resistance emergence to this agent due multidrug resistance of the P-glycoprotein (P-gp), a transporter protein encoded by the MDR1 gene, has been taking the association with other drugs. Recent studies demonstrated the antitumoral effect of the avermectins when associated to the vincristine in the treatment of some neoplasms. Therefore, the objective of the present study was to compare the effectiveness of standard treatment of TVT with vincristine only when compared to combined treatment with vincristine and ivermectin, evaluated through number of applications of the two protocols, histopathological and cytological analysis from 50 dogs diagnosed with TVT during the period of 2007 to 2010. The combined protocol significant reduced the number of applications and cytological and histopathological findings collaborate with the hypothesis that the combination of vincristine and ivermectin promotes faster healing than the use of vincristine alone. Combination treatment with vincristine and ivermectin could be in the future an excellent therapeutic alternative for the treatment of TVT for probably reducing the resistance to vincristine, simultaneously reducing the cost of TVT treatment and promoting a faster recovery of the dog.
Keywords: transmissible venereal tumor; ivermectin; vincristine; cytology; histopathology.

ANÁLISE HISTOPATOLÓGICA E CITOLÓGICA DO TUMOR VENÉREO TRANSMISSÍVEL EM CÃES APÓS DOIS PROTOCOLOS DE TRATAMENTO

RESUMO
O tumor venéreo transmissível (TVT) é uma neoplasia contagiosa de células redondas que afeta frequentemente cães. O tratamento consiste no uso de antineoplásicos, sendo mais efetiva a vincristina somente, porém o aparecimento de resistência a este agente devido à resistência multidrogas da P-glicoproteína (P-gp), uma transportadora de proteína codificada pelo gene MDR1, tem levado a associação com outras drogas. Estudos recentes demonstraram o efeito antitumoral das avermectinas quando associadas à vincristina no tratamento de alguns tipos de neoplasias. Portanto, o objetivo do presente estudo foi comparar a eficácia do tratamento padrão do tumor venéreo transmissível (TVT) somente com vincristina com o tratamento combinado com vincristina e ivermectina, avaliado através do número de aplicações dos dois protocolos e das análises citológica e histopatológica de 50 cães diagnosticados com TVT durante o período de 2007 a 2010. O protocolo combinado reduziu significativamente o número de aplicações e as análises citológicas e histopatológicas colaboram com a hipótese de que a combinação de vincristina e ivermectina promove uma cura mais rápida do que o uso somente da vincristina. O tratamento combinado com vincristina e ivermectina pode ser no futuro uma excelente alternativa terapêutica para o tratamento do TVT por provavelmente reduzir a resistência à vincristina, simultaneamente reduzindo o custo do tratamento e promovendo uma recuperação mais rápida do cão.
Palavras–chave: tumor venéreo transmissível; ivermectina; vincristina; citologia; histopatologia.

INTRODUCTION

The transmissible venereal tumor (TVT) is a naturally occurring round cell neoplasm that affects dogs in tropical and subtropical countries (MACEWEN, 1996). TVT is located mainly in the mucosa of the external genitalia of both sexes, but there are reports of cases in oral and nasal cavities, eyes, skin, tonsils, liver, pharynx, spleen, kidneys, brain, ovary, and foreskin, as well as the anal and perianal region (ROGERS et al., 1998; DAS; DAS, 2000).

The methods used to treat TVT are cryosurgery, radiotherapy, surgical resection and antineoplastic chemotherapy that is the protocol of choice in routine clinical treatment (NAK et al., 2005). Antineoplastic treatment may combine two or more chemotherapeutic agents (for example, vincristine and cyclophosphamide combined with methotrexate), or it can involve a single agent as vincristine (in which case is most effective or doxorubicin) (MACEWEN, 1996; NAK et al., 2005). However, the extensive use of vincristine in recent years resulted in the emergence of TVT resistance to the drug in Brazil with more applications than described in the literature (on average of four to six applications) (SILVA et al., 2007; GASPAR et al., 2009). Furthermore, some TVT may not respond to multiple administrations of chemotherapy (HARMELIN et al., 1995).

It is known that the main factor responsible for the emergence of multidrug resistance is the P-glycoprotein (P-gp), a transporter protein encoded by the MDR1 gene that exists in normal tissues (CNS, intestinal cells, renal tubular cells and bile canaliculi) and tumor tissues. The P-gp is a substrate for various molecules, including for the antineoplastic drug vincristine (KORYSTOV et al., 2004) and other drugs such as vinblastine, doxorubicin, avermectins and loperamide (MEALEY et al., 2003). The mechanism of action of P-gp is not yet fully elucidated, but it is known that it is involved in the efflux of substances from within the cell, as an important drug efflux pump that is expressed on the membranes of blood-brain barrier (BBB) for example, thus allowing these to be excreted rather than absorbed by the body (DOWLING, 2006). Neurotoxicity is observed in Collies when ivermectin is administered in therapeutic dosages, due to a mutation in the MDR1 gene (DOWLING, 2006; MEALEY et al., 2002; HOPPER et al., 2002).

Recently, a study showed the antitumor effect of ivermectin in the treatment of some cancers in rats (DRINYAEV et al., 2004) and another study demonstrated the synergistic effect of a combination of avermectin and vincristine that may increase the antitumor effect of the antineoplastic agent and reduce resistance to vincristine, which has become increasingly common in recent years (KORYSTOV et al., 2004). Ivermectin, besides abamectin and selamectin, inhibits the P-gp dependent drug resistance profiles tumors cells and also is a potent substrate and inhibitor for P-gp in Caco-2 cells (POULIOT et al., 1997; DIDIER; LOOR, 1996; GRIFFIN et al., 2005).

Another study (ANDRADE et al., 2009) found that a combination of vincristine and ivermectin was more effective than treatment with vincristine alone, for the treatment of TVT. They reported that the former protocol required a decreased number of applications, in spite of not being statically significant, with no changes to physical or haematological analyses.

Therefore, this study aimed to verify the efficacy of the combination of vincristine and ivermectin and compare it with the isolated use of vincristine in the treatment of TVT, assessing the progress of healing through cytological and histopathological analyses of the tumor.

MATERIAL AND METHODS

Experimental design

Fifty dogs from the routine care of a Veterinary Teaching Hospital, during august of 2007 to march of 2010, received clinical, cytological and histopathological diagnosis of genital TVT. The animals were evaluated by physical examination, white and red blood count, cytology and biopsy of the tumor before administration(s) of the chemotherapeutic(s), weekly directly prior to treatment. Information on whether the clinician and pathologist evaluating was blinded to the treatment group. The samples were sent to the Laboratory of Pathological Anatomy and read by one and always the same pathologist. The number of administrations was determined by macroscopic evidence of tumor regression, mimicking what is routinely performed for treatment of TVT (MACEWEN, 1996; DAS; DAS, 2000). The treatment was suspended temporarily when the white blood cell count was below 3.000/mm^3 or platelets were below 50.000/mm^3 or also if the dog exhibited clinical signs of vomiting or severe diarrhoea (WHITE, 1991).

The dogs were divided randomly into two groups of 25 animals as follows: vincristine group (VG): 25 dogs (11 males and 14 females); age: 4.0 ± 2.4 years; weight: 13.7 ± 6.2 kg; with intravenous administration (IV) of vincristine sulphate (1 mg/mL) at a dose of 0.5 mg/m^2 once a week; vincristine with ivermectin group (VIG): 25 dogs (7 males and 18 females); age: 4.9 ± 2.2 years; weight: 11.8 ± 7.3 kg; with administration of the same dose of vincristine and the same intervals between doses as the VG group, plus administration of ivermectin (1.0 g/100 mL) therefore after administration of the antineoplastic agent, at a dose of 400 µL/kg subcutaneously, also once a week.

Cytological analysis

Cytology was performed by using a gynaecological brush, rubbed against the tumor; its contents were stained by Panotic (WRIGHT, 1989). The microscopic analysis of samples was based on general criteria for malignancy (pleomorphism; anisocitosis; anisokaryosis and number of neoplastic cells); nuclear criteria of malignancy (multiple and obvious nucleoli, coarse chromatin), and cytoplasmic criteria of malignancy (basophilia, vacuolation) in 10 microscopic fields, considering the following scores: (0) no change, (+) slight change, (+ +) moderate change, (+ + +) markedly change, evaluated under optical microscope in an increase of 400 X (RASKIN; MEYER , 2001).

Histopathological analysis

Histopathological examination was performed by removing a fragment of the mass, by cutting with scissors under infiltration of local anaesthesia (2% lidocaine chloridrate without vasoconstrictor). The fragments were fixed in formalin solution and 10% buffer (pH 7.0) for 24 to 48 hours and then washed in running water for 1 hour. After that, the tumor fragments were processed according to routine technique for optical microscopy and embedded in paraffin. We cut 5 μm sections of all the samples, which were stained with haematoxylin and eosin (TOLOSA et al., 2003). The microscopic analysis of biopsy samples of both group (GV) and the group (GVI) was based on the verification of malignancy, vascular changes and inflammatory infiltrate associated with the neoplasm, which was made by the average number of blood vessels and mitoses in 10 microscopic fields in increase of 400X. Were considered as criteria for malignancy (neoplastic infiltration in epidermis and dermis, regeneration of skin, number of mitosis and neoplastic cells), vascular changes (neovascularization and neoplastic infiltration) and

inflammatory infiltration (focal, multifocal, diffuse, lymphocytic, plasmocitic, neutrophilic and macrophages), considering the following scores: (0) no change, (+) slight change, (+ +) moderate change, (+ + +) markedly change; while the number of mitosis were classified by: (+) 1-3 mitoses; (+ +) 3 to 5 mitoses (+++) and 5 to 10 mitoses per field in an increase of 400 X (JONES et al., 1997).

Statistical analysis

The mean values of the number of administrations of the protocol treatment in VG and VIG, from dogs diagnosed with TVT were compared and analyzed by Student *t*- tests. A significance level of $P < 0.05$ was adopted. For assessment of microscopic variables, we adopted the "neoplastic infiltration in dermis" criterion in biopsies samples for independent observations in both groups. We used the binomial test for two proportions and BioEstat 5.0 statistical software, with a significance level of 5%. The sample size was sufficient to approximate binomial distribution to the normal curve.

RESULTS AND DISCUSSION

The number of administrations with VIG protocol was significantly reduced ($P < 0.05$) than VG protocol. In the VG protocol the numbers of administrations were 5.5±1.8 (range 4-9) while in VIG protocol was 3.2±1.8 (range 2-8). The criteria neoplastic infiltration in dermis was significant difference in both protocols. The VIG group showed earlier a significantly reduced ($P < 0.05$) than VG protocol in histopathological samples. It was statically and clinically significant the reduction of the number of administrations and the earlier reduction of the neoplastic infiltration in dermis in the VIG protocol. It probably reduces the resistance induction to vincristine, the costs and provides a faster recovery of the dog. It isn't in agreement of another similar study (ANDRADE et al., 2009) that analyzed the clinical and hematological alterations induced by the use of the same protocol and the reduction of the number of applications was not statically significant, probably by the limited number of the dogs sample (n=20) of this study.

The number of cytological samples collect was the same of the number of the administration protocol (Table 1). The cytological study showed varying degrees of cellular pleomorphism (Figure1). The malignant characteristics evaluated showed that cytoplasmic vacuolization and the presence of loose chromatin occurred frequently in the majority of samples from the dogs in both groups (Table 1). Cytological data also revealed that in samples related to the latest applications, in some cases, neoplastic cells were not present, even when the tumor was observed macroscopically and in biopsy. Cytological evaluation frequently demonstrated abnormal mitotic profiles and large amounts of inflammatory cells, as reported in the literature (MOZOS et al., 1996; RASKIN; MEYER, 2001). However, these alterations were seen only in the early collections, when the chemotherapy was beginning to exert its effect (Figure 1).

The number of histopathological samples collect was not the same of the number of the administration protocol due to the collection impossibility for there not being more size of appropriate mass for this procedure (Table 2). Histopathological study of animals that received only vincristine showed cellular pleomorphism ranging from moderate to market change. There was a predominance of neutrophil and leukocyte infiltration in the inflammatory profiles of both groups (Table 2). Some biopsies of VIG group showed proteinaceous material (Figure 2) that was not found in biopsies of VG group. The proteinaceous material is found in tumours with better prognosis (JONES et al., 1997) and this is

not found in biopsies of animals treated only with vincristine.

Another finding in some samples from biopsies was the presence of neoplastic cells in dermis when there was no in epidermis. Evaluation of malignancy characteristics showed the presence of cytoplasmic vacuolation in all animals, and large quantities of cells with coarse chromatin. The vacuoles play an important role in malignancy diagnosis, differentiating this condition from others such as mast cell tumor, histiocytoma and lymphosarcoma (DUNCAN; PRASSE, 1979; MEINKOTH; COWELL, 2002). The presence of numerous cells with coarse chromatin indicates a high rate of cell proliferation; this variation was observed until the last application of chemotherapy.

The inflammatory process revealed by the histopathological findings was similar to that reported in previous studies (DUNCAN; PRASSE, 1979), including the prevalence of lymphocytes and neutrophils in the collected samples. Immunohistochemical studies showed that these cells are largely composed of T cells, which are important in spontaneous regression of the TVT (MOZOS et al., 1996).

The absence of neoplastic cells in cytology in the latest episodes of treatment in some dogs, even in the presence on biopsy, may be indicative that the cytology exfoliative is inefficient as control of the treatment; if tumor healing occurs first in the surface, deeper layers of the skin would still harbor neoplastic cells. The biopsy was more effective for control of the treatment, because it draws deeper cells of the tumor or the skin of the animal. It is thus still possible to find tumor cells that may cause new tumor growth even after complete remission (MEIKOTH; COWELL, 2002). A possible alternative would be the collection of material by fine-needle aspiration cytology, which involves the collection of cells located in deeper tissue. Further research should be performed in order to compare the two techniques.

CONCLUSION

The combined protocol reduces the number of applications and cytological and histopathological findings collaborate with the hypothesis that the combination of vincristine and ivermectin promotes faster healing than the use of vincristine alone. The combined protocol represents a potential alternative therapy for reducing the resistance to vincristine, simultaneously reducing the cost of TVT treatment and a faster recovery of the dog.

The presence of neoplastic cells in dermis when they are absent from epidermis demonstrates the importance of histological monitoring during TVT treatment. When treatment is performed using only the macroscopic aspect or exfoliative cytology as a parameter for the interruption of treatment, tumor relapses is possible.

ETHICAL COMMITTEE

The experiment was approved by the Ethical Committee of Unoeste (protocol n.017/07).

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Table 1. Median and percentiles of the cytological analysis of samples taken from 50 dogs diagnosed with TVT and treated with vincristine (VG) and vincristine plus ivermectin (VIG).

Criteria of Malignancy	Samples Collect*/Number of Animals															
	VG Group									VIG Group						
General Criteria	1^0/25	2^0/25	3^0/25	4^0/25	5^0/15	6^0/7	7^0/4	8^0/4	9^0/4	1^0/25	2^0/25	3^0/10	4^0/7	5^0/3	6^0/2	7^0/2	8^0/1
Pleomorphism	2± 2;3	2± 2;3	1± 1;2	0± 0;2	1± 0;1	1± 1;1	1± 1;1	0± 0;0	0± 0;0	2± 1;3	1± 0;3	1± 0;3	0± 0;3	0± 0;2	2± 0;2	1± 0;1	0± 0;0
Anisocytosis	2± 2;3	2± 2;3	1± 1;2	0± 0;2	1± 0;1	1± 0;1	1± 1;1	0± 0;0	0± 0;0	2± 1;3	1± 0;3	1± 0;3	0± 0;3	0± 0;2	2± 0;2	1± 0;1	0± 0;0
Anisokaryosis	2± 2;3	2± 2;3	1± 1;2	0± 0;2	1± 0;1	1± 0;1	1± 1;1	0± 0;0	0± 0;0	2± 1;3	1± 0;3	1± 0;3	0± 0;3	0± 0;2	2± 0;2	1± 0;1	0± 0;0
Number of neoplastic cells[1]	3± 3;3	3± 2;3	2± 0;3	2± 0;3	2± 0;2	2± 0;2	1± 1;1	0± 0;0	0± 0;0	3± 2;3	2± 0;3	1± 0;3	1± 0;3	1± 0;2	1± 0;1	0± 0;0	0± 0;0
Nuclear Criteria	1^0/25	2^0/25	3^0/25	4^0/25	5^0/15	6^0/7	7^0/4	8^0/4	9^0/4	1^0/25	2^0/25	3^0/10	4^0/7	5^0/3	6^0/2	7^0/2	8^0/1
Multiple and obvious nucleoli	3± 2;3	2± 2;3	2± 2;3	0± 0;3	3± 0;3	3± 0;3	2± 2;2	0± 0;0	0± 0;0	3± 1;3	2± 2;3	1± 0;3	0± 0;3	0± 0;2	1± 0;1	1± 0;1	0± 0;0
Coarse chromatin	3± 3;3	2± 2;3	2± 2;3	0± 0;3	2± 0;2	2± 0;2	1± 1;1	0± 0;0	0± 0;0	3± 1;3	2± 0;3	1± 0;3	0± 0;3	0± 0;3	0± 0;0	0± 0;0	0± 0;0
Cytoplasmatic Criteria	1^0/25	2^0/25	3^0/25	4^0/25	5^0/15	6^0/7	7^0/4	8^0/4	9^0/4	1^0/25	2^0/25	3^0/10	4^0/7	5^0/3	6^0/2	7^0/2	8^0/1
Basophilia	3± 3;3	3± 2;3	1± 1;3	0± 0;3	2± 0;2	2± 0;2	2± 2;2	0± 0;0	0± 0;0	3± 1;3	1± 0;3	1± 0;3	1± 0;3	0± 0;3	0± 0;0	0± 0;0	0± 0;0

*Cytology sample collect of the tumor before administration(s) of the chemotherapeutic(s), weekly directly prior to treatment. (0) Absence of changes; (1) Discrete change; (2) Moderate change; (3) Marked change.

[1]Number of neoplastic cells: (0) absent; (1) 10-20 cells per field; (2) 20-40 cells per field; (3) >50 cells per field.

$P < 0.05$.

Table 2. Median and percentiles of the histopathological analysis of biopsies taken from 50 dogs diagnosed with TVT treated with vincristine (VG) and vincristine plus ivermectin (VIG).

Parameters	Samples Collect*/Number of Animals														
	VG Group								VIG Group						
Criteria of Malignancy	1^0/ 25	2^0/ 25	3^0/ 25	4^0/ 25	5^0/ 15	6^0/ 7	7^0/ 4	8^0/ 3	1^0/ 25	2^0/ 25	3^0/ 10	4^0/ 7	5^0/ 3	6^0/ 2	7^0/ 2
Neoplastic infiltration in epidermis[1]	1± 1;3	1± 0;1	1± 0;1	1± 0;2	1± 0;1	1± 0;1	1± 0;1	0± 0;0	2± 0;3	1± 0;2	0± 0;1	0± 0;1	0± 0;0	0± 0;0	0± 0;0
Neoplastic infiltration in dermis[1]	3± 2;3	3± 2;3	2± 1;3	1± 1;3	3± 2;3	3± 2;3	3± 1;3	2± 1;2	3± 2;3	2± 1;3	2± 1;3	2± 1;3	2± 2;2	2± 1;2	1± 1;1
Regeneration of epidermis[2]	0± 0;1	1± 0;2	2± 1;3	3± 0;3	0± 0;3	3± 0;3	3± 0;3	3± 1;3	0± 0;3	2± 0;3	2± 0;3	3± 2;3	3± 2;3	3± 0;0	3± 3;3
Number of mitoses at magnification of 400X[3]	2± 1;2	1± 0;2	0± 0;1	0± 0;2	0± 0;0	3± 0;3	2± 0;2	0± 0;0	2± 1;3	2± 0;2	1± 0;2	1± 0;2	1± 1;1	1± 1;1	1± 0;1
Vascular Changes	1^0/ 25	2^0/ 25	3^0/ 25	4^0/ 25	5^0/ 15	6^0/ 7	7^0/ 4	8^0/ 3	1^0/ 25	2^0/ 25	3^0/ 10	4^0/ 7	5^0/ 3	6^0/ 2	7^0/ 2
Neovascularization[4]	1± 1;2	1± 1;2	2± 1;2	2± 1;2	2± 1;2	1± 1;1	1± 1;1	1± 1;1	2± 1;3	2± 2;3	1± 1;2	2± 1;2	2± 1;2	1± 1;1	1± 1;1
Neoplastic infiltration[1]	0± 0;0	0± 0;0	0± 0;0	0± 0;0	0± 0;0	0± 0;0	0± 0;0	0± 0;0	0± 0;0	0± 0;0	0± 0;0	0± 0;0	0± 0;0	0± 0;0	0± 0;0
Inflammatory infiltration[5]	1^0/ 25	2^0/ 25	3^0/ 25	4^0/ 25	5^0/ 15	6^0/ 7	7^0/ 4	8^0/ 3	1^0/ 25	2^0/ 25	3^0/ 10	4^0/ 7	5^0/ 3	6^0/ 2	7^0/ 2
Focal	0± 0;0	0± 0;1	0± 0;2	0± 0;2	1± 1;1	1± 0;1	1± 0;1	1± 0;1	0± 0;1	0± 0;1	0± 0;2	0± 0;0	0± 0;0	0± 0;0	0± 0;0
Multifocal	1± 0;2	1± 0;1	1± 0;2	1± 0;2	0± 0;1	1± 0;1	1± 0;1	1± 0;1	0± 0;2	0± 0;2	1± 0;1	1± 0;1	0± 0;0	0± 0;0	0± 0;0
Diffuse	0± 0;2	0± 0;2	0± 0;0	0± 0;0	0± 0;0	0± 0;0	0± 0;0	0± 0;0	1± 0;3	1± 0;3	2± 0;2	1± 0;1	1± 1;1	1± 1;1	1± 0;1
Lymphocytic	0± 0;2	1± 0;2	2± 1;2	1± 0;3	1± 1;1	0± 0;0	0± 0;0	0± 0;0	1± 0;1	1± 0;2	1± 0;2	1± 1;2	2± 2;2	2± 1;2	2± 0;2
Plasmocitic	0± 0;1	0± 0;0	0± 0;1	0± 0;1	0± 0;0	1± 0;1	0± 0;0	0± 0;0	0± 0;2	0± 0;2	0± 0;1	0± 0;1	0± 0;1	0± 0;0	0± 0;0
Neutrophilic	2± 0;3	1± 0;2	1± 0;1	0± 0;1	0± 0;0	3± 0;3	0± 0;0	0± 0;0	2± 0;3	1± 0;2	1± 0;3	0± 0;1	0± 0;0	0± 0;0	0± 0;0
Macrophages	0± 0;1	0± 0;2	1± 0;2	1± 0;2	2± 0;2	0± 0;0	1± 0;1	1± 0;1	0± 0;3	0± 0;1	0± 0;1	0± 0;1	0± 0;0	0± 0;0	0± 0;0

*Biopsy sample collect of the tumor before administration(s) of the chemotherapeutic(s), weekly directly prior to treatment. [1]Number of neoplastic cells: (0) absent; (1) 10-20 cells per field; (2) 20-40 cells per field; (3) >50 cells per field. [2]Regeneration of epidermis: (0) without regeneration; (1) only basal layer; (2) basal and prickle cell layer; (3) complete regeneration. [3]Number of mitoses: (1) 1-3 mitoses per field; (2) 3-5 mitoses per field (3) and 5-10 mitoses per field (40 X magnification). [4]Neovascularization: (1) 1-3 capillaries per field; (2) 3-5 capillaries per field; (3) 5-7 capillaries per field. [5]Inflammatory infiltration: (0) absent; (1) ≤20 cells per field; (2) 20-40 cells per field; (3) >50 cells per field (40 X magnification). $P < 0.05$.

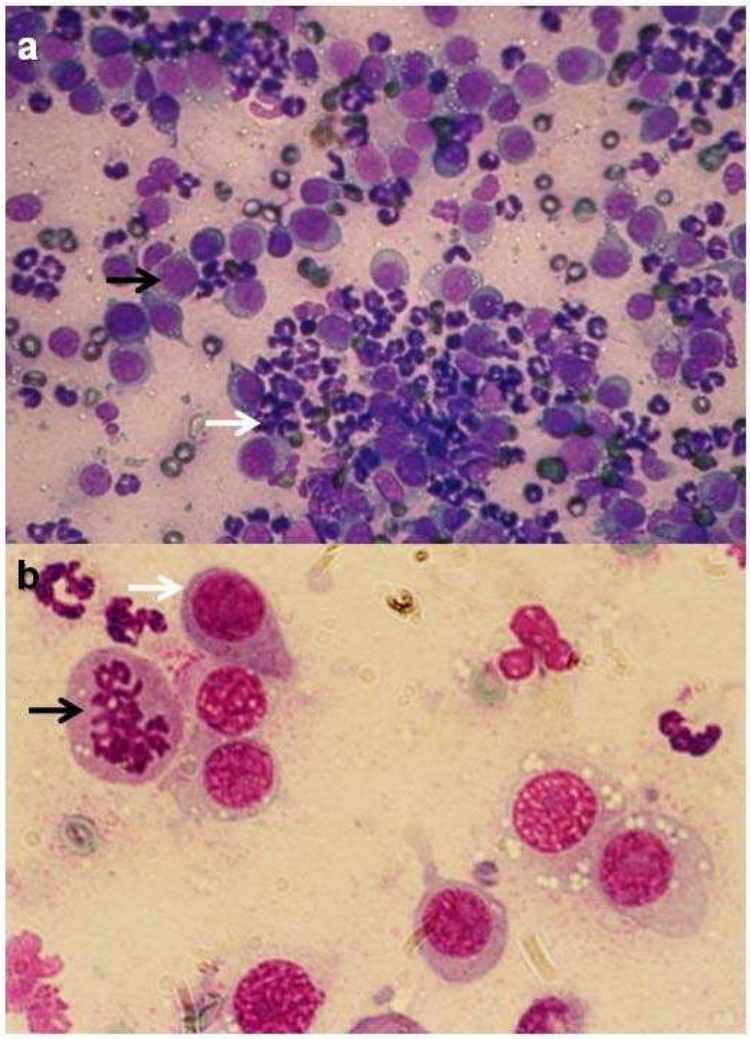


Figure 1. Cytological analysis of TVT (first collection). A) Large amount of TVT cells (black arrows) and inflammatory cells (white arrow) from a VG animal. 40 X magnification. B) Tadpole cell (white arrow) and abnormal mitosis (black arrow) in TVT smear from a VIG animal. 100X magnification.

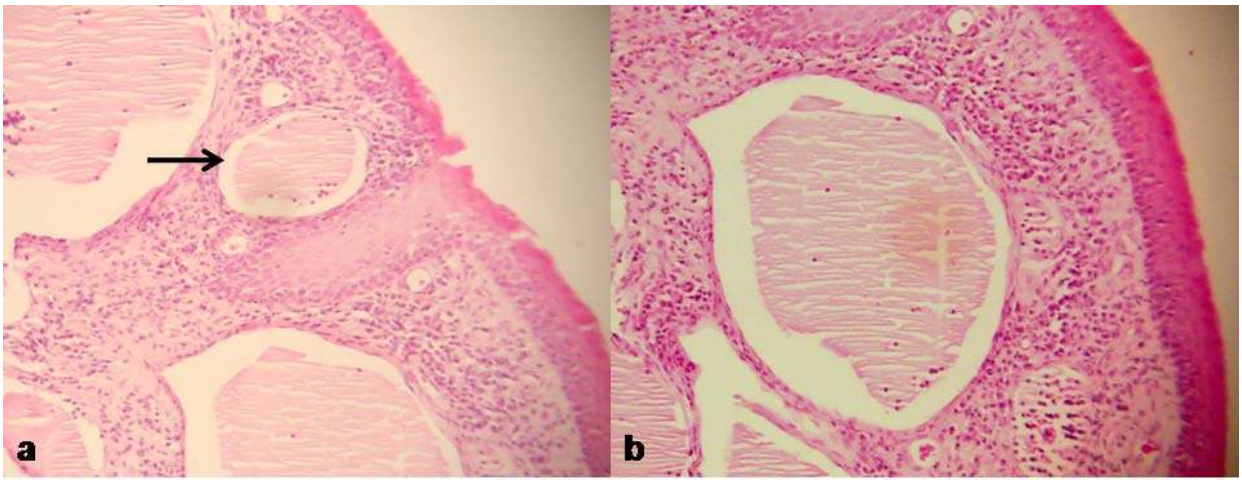


Figure 2. (A) A cystic formation (arrow) containing the protein material is observed in animal n. 5 Group GVI, in the microscopic visualization of 4x, (B) Microscopic view of 10x.