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Research Paper

Anti Tumorous Effect of Synthesized Vincristine Hydrogel Scaffolds on Transmissible Venereal Granuloma in Canine

Arun Kumar[1], Narendra Singh Jadon[2], M. G. H. Zaidi[3], Manjul Kandpal[4] and Rashmi Saini[5*]

[1,2,3,4,5]Department of Veterinary Surgery and Radiology, College of Veterinary and Animal Science, G. B. Pant University of Agriculture and Technology, Pant agar, Uttarakhand, India

Corresponding Author: * Rashmi Saini

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Abstract

Vincristine is considered a first-line chemotherapy drug for the treatment of venereal tumour (CTVT). There are many types of encapsulated antitumor drugs used to treat tumors, with increased drug solubility, better bioavailability, high stability, drug release, extended half-life, selective internal organs or tissues, and overall reduced dosage. Therefore, the aim of this study was to evaluate the antitumor safety of vincristine and its scaffold, which can be quickly and safely affected by many clinical physiological and blood biochemical parameters before and after treatment of infectious veneral tumors in dogs.The oncolytic effect of vincristine and its scaffolds was demonstrated using clinical methods and ability effects and histopathological changes in the tumor body and oncolytic effect at 0, 1, 2, 3 and 4 weeks. Dogs with CTVT used in this study (n = 12) were divided into two groups regardless of gender. Control group (A-vincristine; n=6) and experimental group (B-vincristine scaffold; n=6). Animals in groups A and B were injected with 0.025 mg/kg body weight of vincristine sulfate once a week for four consecutive weeks, and 0.025 mg/kg of vincristine sulfate was scaffolds injected once a week for four consecutive weeks. In conclusion, the use of vincristine hydrogel scaffolds is useful and effective in the treatment of CTVT.

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KEYWORDS: Vincristine sulphate, hydrogel scaffolds, CTVT and apoptosis

1. INTRODUCTION

Cancer is defined as disruption of the normal pattern of cell division and the growth of abnormal, uncontrolled cells. It is also known by many names, including canine transmissible venereal tumor (CTVT), canine venereal granuloma, canine genital warts, adhesive tumors, and infectious sarcoma. It is a cancer of dogs and other canines that usually affects the external genitalia and is spread through sex between animals, but it can also be spread by dog bites, kisses, or licking the tumor. Because it is frequently sexually transmitted and occurs mostly in young and sexually active animals (Kumar *et al.*, 2014, 2015) [16][17]. It is transferred during sex, across major histocompatibility complex (MHC) barriers within her members of the canine family. TVT accounts for 23-43% of all cancers in India. Abandonment and uncontrolled sexual behavior of many dogs is one of the major reasons why TVT rates are so high in Asian countries. Veneral tumors in dogs can be large and bleed, often causing pain and infection. Cytologically, TVT cells are highly variable, round to oval in shape, often containing mitotic figures, aggregated chromatin, and one or two prominent nucleoli. Histological evaluation of CTVT often shows cells growing in tight clusters

or confluent sheets. Treatment options include surgery, radiation therapy, immunotherapy, biological therapy, and chemotherapy. Surgery is commonly used for small local TVTs, but the recurrence rate can be as high as 50-68%. Methods to prevent recurrence after surgery include resection and cauterization, electrosurgery and cryosurgical resection, or surgical resection after chemotherapy. Drug Therapy has become a popular method using many drugs such as vincristine, doxorubicin and cisplatin. Vincristine sulfate is an excellent chemotherapeutic agent for the treatment of CTVT in dogs but has serious side effects (Verma *et al.,* 2014) [22]. Significant difficulties are encountered in treatment due to drug resistance and serious side effects. Therefore, good drugs need to be introduced into the body to reduce their toxicity and side effects. Therefore, it is predicted that treatment based on the use of vincristine and scaffolds will be effective in dogs with CTVT. Given the lack of clinical studies on this topic, the main aim of this study was to compare treatment outcomes with vincristine sulfate and hydrogel scaffolds alone.

2. MATERIALS AND METHODS

2.1 Animals, experimental design and ethics

Dogs of different breeds, ages and genders with genital warts were examined at the Veterinary Clinical Teaching Centre, G. B. Pant University of Agriculture and Technology, Pantnagar, Uttarakhand, India. The diagnosis of CTVT was confirmed by oncological examination. The treatment was evaluated in a clinical trial in which dogs were randomly assigned to two groups to receive treatment. In Group A, dogs treated with vincristine (A-Vincristine; n = 6) were administered vincristine sulfate intravenously at 0.025 mg/kg body weight per week for four consecutive weeks. Dogs in group B (B-vincristine scaffolds; n = 6) were treated with intravenous vincristine sulfate scaffolds at a dose of 0.025 mg/kg once a week for four consecutive weeks. Physical and clinical symptoms were observed before and after treatment, that is, at weeks 0, 1, 2, 3 and 4 of treatment. After the experiment, the experimental animals were monitored for two months for any adverse symptoms and tumor recurrence. All procedures for animals used in the study were approved by the Ethics Committee and Animal Care Ethics Committee, the clinical center of CTVT, with a letter of recommendation: IAEC/CVASC/VSR/362/dt.21/12/2018 Research to obtain a therapeutic dose is currently being carried out at the Veterinary Clinical Teaching Centre, G. B. Pant University of Agriculture and Technology, Pantnagar, Uttarakhand, India. Growth regression of sexual tumors has been studied in previous experiments on a dog with different dosage regimens of each drug.

2.2 Clinical examination and hemograms

Physical and clinical symptoms were observed before and after treatment, that is, at weeks 0, 1, 2, 3 and 4 of treatment. After the experiment, the experimental animals were monitored for two months for any adverse symptoms and tumor recurrence. The extent of the apoptotic effect of different drugs was determined by observing and measuring the clinical size of the tumor and comparing this with the return of the tumor to its previous size. Peripheral blood was collected and hemogram analysis including red blood count and white blood count of neutrophils, lymphocytes, monocytes, eosinophil and basophils (n x 103/µl). The results from the blood tests were compared to the reference values for the canine species as previously described by Thrall *et al.,* (2012) [27]. Check the difference in blood chemistry, including blood total protein, blood albumin, blood glucose, blood urea nitrogen, blood creatinine, blood aspartate aminotransferase (AST), blood alanine aminotransferase (ALT), and serum gamma Glutamyl transpeptidase (GGT).

2.3 Histopathology Examination

Paraffin embedding technique was used for histopathological investigation to evaluate the cytological changes of veneral tumor cells at different times and to determine the nature of the tumor. Tissue samples from different groups were collected in 10% formalin buffer at 0, 1, 2, 3 and 4 weeks of the experiment. Observe the normal and cytological changes of veneral tumor cells in different treatments. Serial sections of 6–8-micron thickness was cut from each sample using an automatic microtome and stained with hematoxylin and eosin. Examine the slides under a microscope and record the bacteria.

2.4 Statistical analysis

Statistical Analysis of the data was done with one way analysis of variance. Analysis of variance and C.D. test were done as per the methods described by the Snedecor and Cochran (1994).

3. RESULTS AND DISCUSSION

3.1 Gross appearance of canine venereal tumour

The data presented in this study have the same characteristics as TVT: external pollen-like nodules of various sizes (0.5-5 cm in diameter). In men, neoplastic masses usually appear as single, multiple, small to large, sessile or stalked soft nodular masses at the base of the penis. In women, the tumor often appears in the form of a single cauliflower and masks the external genital organs. The consistency of the tumor is soft and hard. All tumors have irregular, soft and fragile tissue. In rare cases, the tumor breaks down in nature and the animal becomes cachectic. In male dogs, genital tumors are usually found in the tail of the genital area, from the feet to the pupils or throat, and some occur on the foreskin. These findings are consistent with Rezae *et al.,* 2016.[21] Tumors in both male and female dogs are red to reddish in color due to their blood volume, and because they are fragile, they bleed easily. Patients affected by CTVT are often characterized by a foul-smelling, persistent or intermittent serous or bloody discharge, and animals often show a persistent soft spotting habit. Urinary incontinence (anal temperature, urination and defecation) is normal in all animals except in one case where excessive urination occurs due to enlargement of the urine. Current findings regarding the appearance and location of granuloma venereum are also supported by Das and Das (2000); (2008); Kisani and Adamu (2009) [13].

Regression of tumour

In this group, complete regression in tumor size was observed at the end of the third week (fig a, b and c, d). In group B (plate 1, Fig. e and f), complete regression of tumor size was observed on vincristine scaffolds. Veneral lesions in male dogs in group A disappeared after two weeks of treatment and turned from pink to purple. After injection of vincristine alone and vincristine stent in animals in groups A and B, respectively, veneral tumors began to develop rapidly. After one week of treatment in groups A and B, a significant increase and decrease in blood and foul odor were observed. During physical examination, a 50% decrease in tumor growth was observed in animals in groups A and B after the first week. The publication is completed after the second week. No tumor recurrence or adverse effects of vincristine stent were observed in patients affected by CTVT during the study period and within three months of treatment or follow-up. Vincristine sulfate and its scaffold induce apoptosis in association with caspase-3 and 9. The current finding of apoptosis or regression in CTVT is consistent with the observations of Decker *et al.,* (2015) [7] reported that the tumor regression observed so far with vincristine treatment may be due to its binding to tubulin, a type of tubulin in the brain that acts as an antibiotic. The spindle then fails to function in mitosis and arrests the cell cycle in metaphase (Garden, 2010; Ganguly *et al.,* 2016 [9][8] and Antonov, 2017) [1]. Sharma *et al.,* (2011) [23] and Antonov (2017) [1] reported that the most effective, safe and easy-to-use chemotherapy that can prolong life in CTVT patients with extragenital metastases is 0.025 mg/kg body weight vincristine sulfate 3-4 times a week.

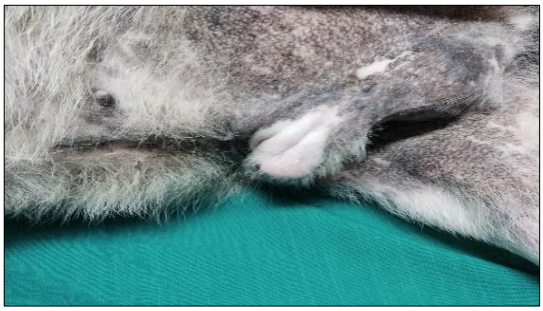


Fig (a)

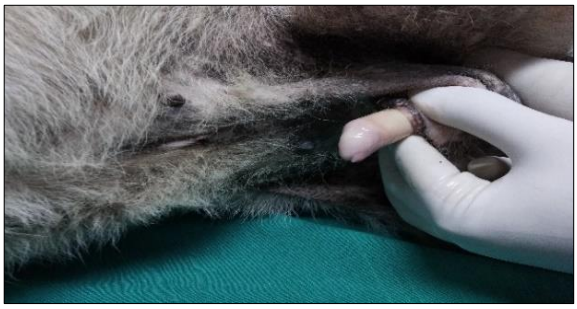


Fig (b)

Group A: Before and after treatment with vincristine sulphate

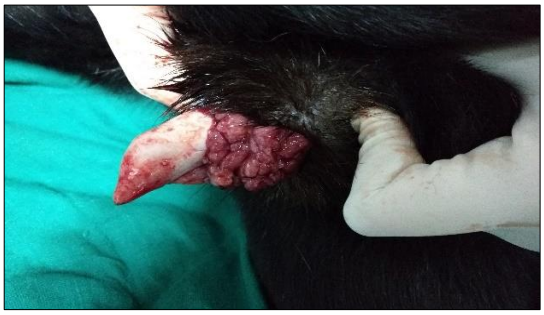


Fig (c)

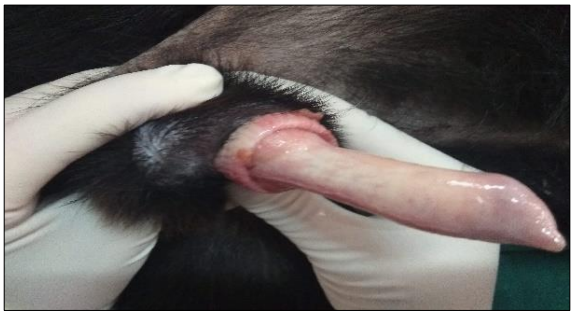


Fig (d)

Group A: Before and after treatment with vincistine

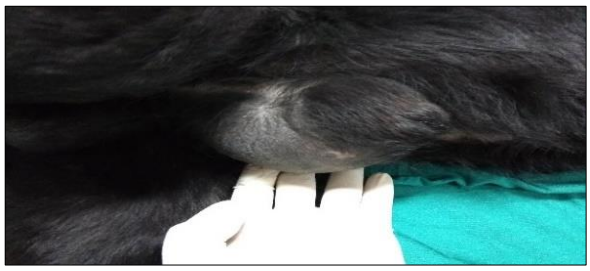


Fig (e)

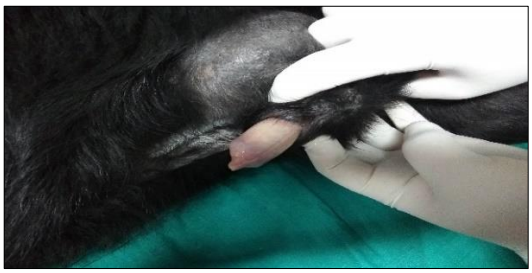
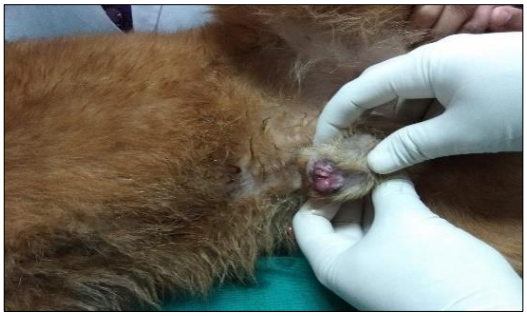


Fig (f)

Group B: Before and after treatment with vincristine scaffold



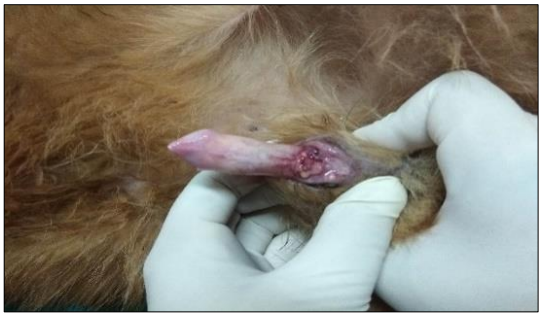
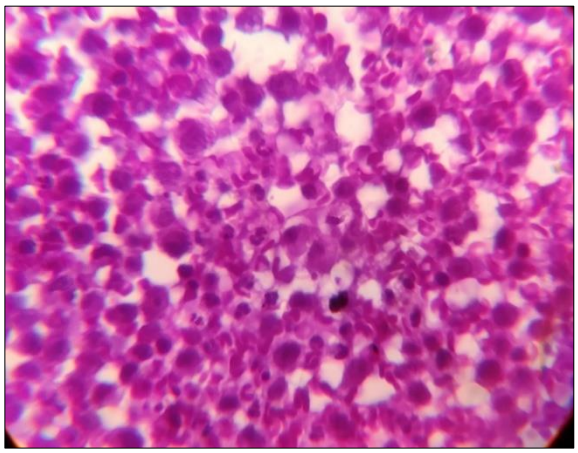


Fig (g) Fig (h)

Group B: Before and after treatment with vincristine scaffold

Plate No. 1: Photograph showing gross appearance of the venereal tumours before and after the treatments

Histopathological Studies: The histopathological staining at different time interval in various group of animals before and after administration of drugs are given photograph depicted in plate 5.



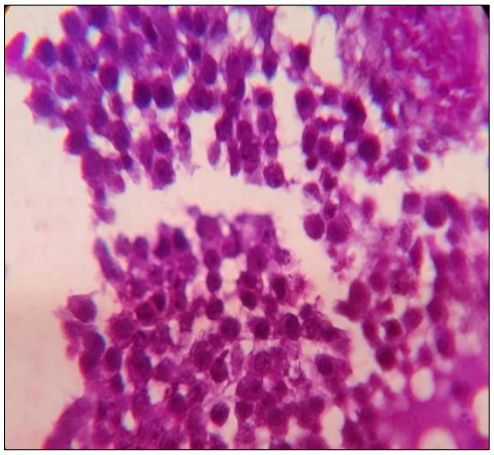
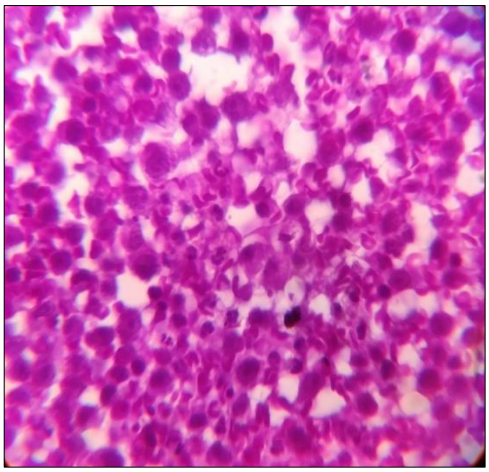


Fig. a: Group A (0 week) **Fig. b:** Group A (4 week)



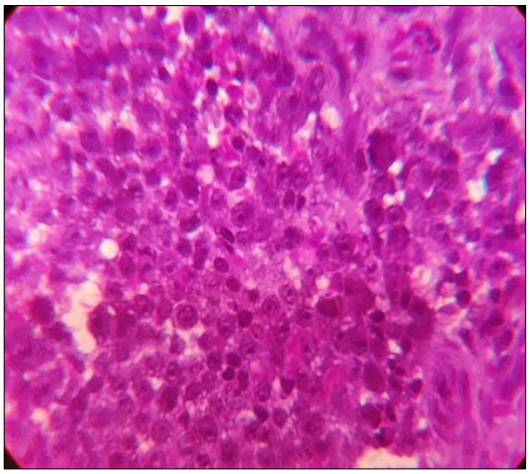


Fig. c: Group B (0 week) **Fig. d:** Group B (4 week)

Plate No 2: Photomicrograph showing histopathological section at different time intervals.

In Group A, the histopathological examination performed at the 3rd week showed widespread necrosis in the tumor. Tumor cells are highly vacuolated; oval or round nuclei, large or small, coarse-grained chromatin and staggered collagen fibers between cells. Most of the degeneration in the necrotic area has no nucleus. At high magnification, cytoplasm is abundant, vacuoles are present, and the number of mitoses is low. In many areas, there is necrosis of individual or groups of tumor cells that appear as irregular spaces throughout the tumor (**Figure 1**). A). The number of apoptotic cells was higher compared to the week 0 blood sample. Besides apoptotic bodies, the presence of shrunken isolated apoptotic cells with condensed chromatin and fragmented nuclei was also observed. Group A animals showed greater intercellular matrix collagen deposition in the progression of tumors in the fourth week (panel b) and group B animals in the third week of the study period (panel e). In the histopathological examination of group A animals, a mature tissue was observed in the fourth week. In histopathological staining, changes were observed in group A and B animals in the second and third weeks. Histopathological examination of the STD tumors in Group B at the second week revealed extensive necrosis of the tumor. Tumor cells are highly vacuolated; oval or round nuclei, large or small, coarse-grained chromatin and staggered collagen fibers between cells. Histopathological studies have revealed areas of round lesions with borders that are not easily distinguishable. Trompieri (2009) [28] reported that venereal tumors in dogs have a heterogeneous population of tightly packed, large, round or oval cells with dense but well-circumscribed cytoplasm. The nuclear chromatin is thin and dispersed, the nucleus is large and oval, and the nucleolus is prominent and in place.

Infiltration of lymphocytes, plasma cells and small amounts of macrophages is evident. Mitosis is also seen. The presence of a rounded base in the dendritic fibrovascular network aids in the histological diagnosis of the tumor. These observations are consistent with the findings of Krithiga *et al*., 2005 [14]. Significant changes in cellular (heterogeneous cell size) and nuclear morphology (heterogeneous cell size) were also observed. The nuclei of tumor cells are round to oval and in situ. Nuclear pleocytosis is evident in tumor cell nuclei. Nucleoli are basophilic and range from one to three. Nuclear chromatin structure varies from coarse to reticular (Thangathurai *et al*., 2008) [10]. The composition and amount of stroma present in transplanted tumors that degenerate is more distinct than in transplanted TVTs that grow similarly to genital TVTs. Section (2017) [11] and Murad *et al.*, [20] The regressed tumor showed cytoplasmic vacuolation. In some cases, neutrophil infiltration and extensive collagen tissue. After the tumor had completely regressed, a biopsy of the penile or genital epithelium of the tumor site revealed mature tissue with no evidence of tumor cells. The submucosa is filled with newly formed blood vessels and connective tissue.

Hematobiochemical parameters

There was no decrease in the Hb values of the animals in Group A after vincristine sulfate intake (P<0.05). It was also found that Hb in the third week (10.20 ± 0.46) was not significantly (P<0.05) lower than the results recorded in the short period of the second and fourth weeks. A non-significant (P>0.05) increase in Hb values was also observed in group B animals that received vincristine stent during the study period. Hb value reached its highest value at the basal value (10.24±0.45) in the short term of four weeks (11.18Â±0.43) However the value of 0^{th} week was not lower than the 1st, 2nd and 4th weeks (P>0.05). There was no significant difference between the levels of second and fourth weeks in animals of section of group B the mean haemoglobin percentage level increased insignificantly (P<0.05), probably due to improvement in tumor size at the time of loss of the ulcerated surface, as opposed to hemoglobin. The level decreased with Changchun alone. The low value of group A of neobase may be due to the blood loss and bone loss of this drug (Kumar *et al*., 2018 and Murad *et al*., 2019) [15] [20]. A significant (P<0.05) reduction in cell volume (PCV) was observed in Group A animals receiving vincristine sulfate administration throughout the study period. The PCV decreased from the base line value (43.78±3.12) to the third week (39.84±2.84, then increased until the fourth week (40.85±1.67). No significant change in PCV level was observed in animals of group B that received vincristine scaffolds throughout the study period (P>0.05), although there was a slight increase (42.75 ± 1.58) every 3 weeks. The decrease in PCV in group A animals receiving vincristine may be due to myelosuppressive anemia. The same results were observed by Srivastava *et al*. (2009) [25] in animals treated with vincristine. The lack of significance in group B, where vincristine stent was applied, may be due to reduced toxicity due to the slow release of the drug from the vincristine stent (Cunha *et al.*, 2017) [3].

There was a non-significant (P>0.05) decrease in the level of total leucocytes count (TLC) in the animals of group A up to three-week time interval and then tends to increase towards mean base value at fourth week. In the animals of group B subjected to treatment with vincristine scaffolds, the levels of TLC showed a significant (p<0.05) decrease throughout the period of study. The TLC values at second week (8.95±0.28), third week (8.85±0.31) and fourth week (8.62±0.25) were significantly (P<0.05) lower as compared to its base value (9.20±0.35).

Significant (P>0.05) changes in TLC level in animals of groups A and B in the present study may be due to depression of reticuloendothelial system (Birhan and Chanie, 2015) [2] along with mild myelosuppression with the use of vincristine sulphate (Dan *et al*., 2017) [4].

In the animals of group A and group B, a non-significant (P>0.05) changes in mean neutrophil values were observed throughout the period of study. The fluctuating increase or decrease in mean neutrophil values at various time intervals was very near to their base value at 0 week. In the animals of group A and B, a non-significant (P>0.05) change in mean neutrophil throughout the period of experiment might be due to myelosuppression. However, no significant changes in neutrophil level were observed by Das and Das (2000) [6] and Sharma, (2011) [23] in animals treated with anticancerous therapy.

A significant (P<0.05) change in mean lymphocyte value was observed throughout the period of experiment in the animals of group A and B subjected to administration of vincristine and its scaffolds respectively. The significant increase in mean lymphocyte value was very near to base value at different time intervals throughout the period of study. In the animals of group A, the lowest (27.50±2.74) and highest (37.45±1.55) mean lymphocyte level was observed at 0- and fourth-week times interval, respectively. In the animals of group B, the lowest (32.50±1.58) and highest (38.70±1.22) mean lymphocyte level was observed at 0 and fourth week times interval, respectively.The present finding of lymphocytosis may be due to myelosuppression and was also observed previously by Mason *et al.* (2014) [18]; Srivastava *et al.* (2014) [24] and Cunha *et al.* (2017) [3].

A gradual and non-significant (P>0.05) decrease in the serum protein was observed up to fourth weeks in the animals of group A, and B subjected to administration of vincristine only and its scaffolds. In both the groups, lowest level was at fourth week time interval. The decrease in the serum protein was within normal physiological limit.

The decreased concentration of total protein (TP) indicates the possibility of mild interference in absorption and assimilation of nutrient owing to intestinal disorders in dogs as a sequel to vincristine therapy. This is mainly because of primary action of antineoplastic drugs on fast dividing cells. The same observation has been reported by Dan *et al*., 2018 [5]. However, in group B the effect of vincristine and might be neutralize by action of scaffolds. Varughese *et al*. (2012) [29] and Ganguly *et al*. (2016) [8] have also observed low concentration of total protein in canine venereal granuloma like other benign and malignant tumour as compare to normal dogs.

A non-significant (P>0.05) change in the level of serum albumin was observed throughout the period of experiment in the animals of groups A and B subjected to administration of vincristine sulphate and its scaffolds respectively. The levels of serum albumin remained in the normal physiological limit in all the animals of both the groups at different time intervals throughout the period of the study. Non-significant change in albumin level at various time intervals in both the groups of animals does not indicates the harmful effect of vincristine on the hepatocytes of the liver (Antonov, 2017 and Murad *et al.*, 2019) [1][20].

In the animals of group A, a non-significant (P>0.05) decrease in mean glucose level was observed up to second week (61.50±3.58) followed by increase in its values at fourth week time intervals. In group A the mean glucose level at second week (61.50±3.58) was significantly different from their mean base value (74.40±2.85) as well as the value at fourth week (75.25±3.18). The mean glucose level at first, second-, and third-week intervals did not vary significantly (P<0.05). In the animals of group B, subjected to administration of vincristine scaffolds, a slight increase in mean base glucose values was observed at first week (89.50±8.25) followed by decrease in its values upto third week time interval.

Non-significant change in blood glucose level in animals of groups A, and B subjected to administration of vincristine sulphate and its scaffolds indicates that no considerable effect of these drug in the body. However, a significant decrease in the mean blood glucose level was a characteristic feature of venereal granuloma (Tella, 2004) [26]. The hypoglycemia in such cases is probably due to excessive glucose utilization by neoplastic cells, secretion of insulin like growth peptide or some other humoral induced alterations in the normal compensatory mechanism which prevents fasting hypoglycemia (Sudjaide *et al.*, 2012).

In the animals of group A and B, a non-significant (P>0.05) change in mean serum urea nitrogen level was observed. In the animals of group A, a slight and non-significant (P<0.05) increase in serum urea nitrogen was observed throughout the period of study. In the animals of group B, the fluctuating trend in serum nitrogen level was observed, however its level was in the normal physiological limit.

Non-significant change in serum urea nitrogen at various time intervals in all the animals of group A and B indicates the no harmful effect of drugs on renal, hepatic system and the other related system, Tella *et al.* (2004) [26] and Sharma *et al.* (2011)[23] have also observed similar changes in BUN level in CTVT dog.

In the animals of group A, a slight and non-significant (P<0.05) increase in serum creatinine was observed throughout the period of study. In the animals of group B, the fluctuating trend in serum creatinine level was observed, however its level was in the normal physiological limit and it was near to their base value. Non- significant change in creatinine level at various time intervals in group A and B subjected to administration of vincristine sulphate and scaffolds indicates the no harmful effect of drugs on renal and other related organs. Sharma *et al.* (2011)[23] have also observed similar change in creatinine level in canine CTVT dog.

There was non-significant (P>0.05) change in the level of alanine amino transferase (ALT) and serum aspartate amino transferase (AST) throughout the period of experiment in animlas of group A and B subjected to the administration of vincristine and its scaffolds. These values were in the normal physiological limit in all the animals of both the groups at different time intervals throughout the period of this study.

AST is found in high concentrations in cardiac and skeletal muscles as well as liver cells. Animals in group A treated with vincristine sulfate had no significant changes in AST over consecutive weeks of treatment; this is consistent with the analysis of Mello *et al.* (2013) [19]; Dan *et al.* (2018) [5] reported that vincristine did not harm the liver and muscles. In contrast to the current study, Sharma *et al*. (2011) [23] found a significant (P<0.05) increase in AST values after vincristine treatment in dogs with CTVT until the second week of treatment. ALT is a liver-specific enzyme abundantly found in the cytoplasm of hepatocytes. The liver is one of the body's main metabolic centers and its cells contain many important enzymes. When the liver is damaged, cell membranes will become more permeable or cell walls will rupture, allowing enzymes to enter the blood vessels and increasing levels in the bloodstream. Therefore, measuring the activities of these enzymes may reflect the integrity of the liver walls and provide an important method for assessing liver damage. Cytoplasmic enzymes such as alanine

aminotransferase and aspartate aminotransferase are affected by cell membrane permeability. During the entire experiment, there were no significant changes in blood gamma glutamyl transpeptidase (IU/L) and blood aspartate levels (P>0.05) between the two groups of animals, and the blood results were within normal physical limitations. All animals from different groups at different times throughout the study. Serum GGT is found in many tissues, but the main source is the liver. Microsomal (ribosomal and mitochondrial) enzymes such as gamma glutamate dehydrogenase must be damaged in the liver before they can increase. In this study, high blood GGT levels were observed in animals with liver disease; this confirmed the findings of Kaneko *et al*. (2008) [12]. The decrease in serum GGT after the start of treatment in various groups indicates the positive effect of the treatment.

4. CONCLUSION

Based on the limitations of this study, it was determined that the level of apoptosis of CTVT cells at the first and second week in animals treated with vincristine sulfate scaffolds was physically, clinically and hematologically demonstrated compared to the extent of vincristine sulfate alone. Vincristine alone is a good drug in the treatment of CTVT even at the site of metastasis, but vincristine stent is more effective due to tumor progression compared to vincristine alone. This may be because healthy cells have fewer side effects. Vincristine stents can be safely used by field veterinarians to treat TVT in dogs.

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Table 1: Mean±SE of heamtobiochemical parameters after administration of vincristine sulphate in animals of group A and cisplatin in animals of group B at different time intervals.

Parameters (Mean±SE)	group	Time intervals				
		0week	1week	2week.	3week	4 week
Haemoglobin(gm/dl)	1	11.00±0.23	10.86±0.47	10.22±0.55	10.20±0.46	10.24±0.45
	2	10.24±0.45	10.45±0.23	10.95±0.32	11.12±0.43	11.18±0.43
PCV (%)	1	43.78±1.58	42.25±2.15	40.60±2.45*	39.85±2.98*	40.85±1.67
	2	41.25±2.56	41.30±2.62	41.50±1.26	42.75±1.58	42.65±1.28
TLC(10^3/µl)	1	8.68±0.49	7.90±0.59	7.85±0.56	7.52±0.54	7.58±0.58
	2	9.20±0.35	9.11±0.33	8.95±0.28*	8.85±0.31**	8.62±0.25
Lymphocyte (%)	1	27.50±2.74	31.75±0.44	35.15±2.73*	37.28±1.28*	37.45±1.55
	2	32.50±1.58	34.25±2.26	36.50±1.70*	38.50±1.39	38.70±1.22*
Neutrophils (%)	1	70.50±3.27	71.76±1.75	68.00±2.32	69.00±2.22	70.00±1.81
	2	69.50±1.79	61.35±4.87*	57.75±2.75**	56.25±1.83*	57.80±2.16
Total Protein(g/dl)	1	5.98±1.51	5.52±1.46	5.48±1.40	5.23±0.28	5.27±0.88
	2	6.85±0.11	6.75±0.34	6.55±1.71	6.51±1.32	6.45±1.22
Albumin (g/dl)	1	3.15±0.40	3.00±0.46	3.20±0.50	3..32±0.61	3.35±0.44
	2	2.54±0.57	2.43±0.62	2.55±0.71	2.35±0.44	2.23±0.76
Glucose(mg/dl)	1	74.40±2.85	69.75±2.462	61.50±3.58	70.65±2.28	75.25±3.18
	2	81.40±6.32	89.50±8.25	85.80±4.89	83.60±6.24	85.60±6.16
Blood urea nitrogen(mg/dl)	1	29.50±2.35	29.75±1.68	30.15±2.56	30.21±1.57	31.01±1.32
	2	30.00±2.83	30.75±2.96	30.45±2.67	31.25±3.15	30.40±2.27
Creatinine(mg/dl)	1	1.16±0.19	1.20±0.44	1.26±0.47	1.25±0.84	1.27±0.32
	2	1.20±0.06	1.35±0.19	1.34±0.24	1.29±0.37	1.26±0.72
AST (IU/L)	1	37.25±1.44	37.65±3.29	38.10±3.05	38.22±1.05	38.25±1.02
	2	40.25±4.82	41.00±4.43	41.90±4.52	41.75±4.56	41.25±4.13
ALT(IU/L)	1	29.50±3.13	28.50 ± 3.03	28.75± 3.46	30.00± 2.45	31.25± 3.12
	2	31.00± 2.45	30.25± 3.44	31.50± 3.76	31.95± 2.55	31.75± 2.45
GGT(IU/L)	1	4.25± 0.54	4.15± 0.75	3.45± 0.60	3.95± 0.50	4.50± 0.80
	2	3.20± 0.69	4.25± 1.47	3.50± 1.47	4.25± 0.88	4.10 ± 0.89

* Significant at $p < 0.05$ difference with 0-week time interval.
** Significant at $p < 0.01$difference with 0-week time interval.