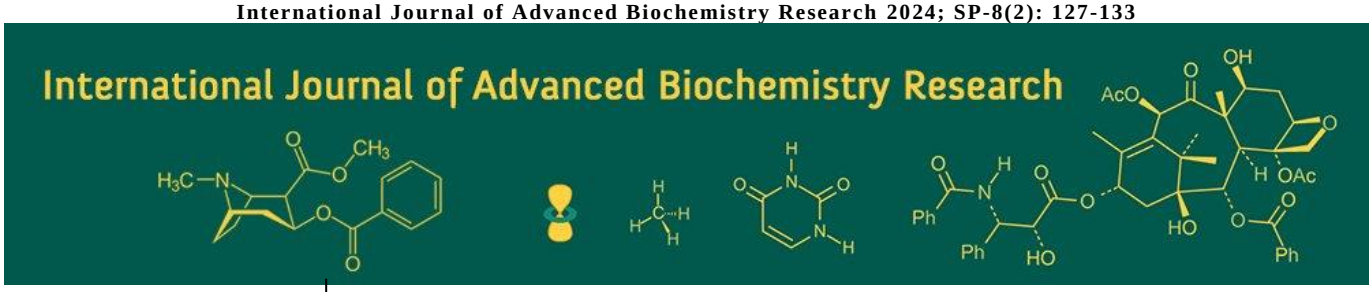

ISSN Print: 2617-4693
ISSN Online: 2617-4707
IJABR 2024; SP-8(2): 127-133
www.biochemjournal.com
Received: 02-11-2023
Accepted: 08-01-2024

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Epidemiological, cytological, and haemato-serological analysis of canine mammary gland tumours

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DOI: https://doi.org/10.33545/26174693.2024.v8.i2Sb.520

Abstract
When it comes to neoplastic situations, a clear and early diagnosis is crucial for both human and animal patients to determine the best course of therapy and improved prognosis. The study's objective was to analyse the epidemiological, cytological, and haemato-serological aspects of canine mammary gland tumours. This included examining the tumor's consistency, cytology, blood and serum parameter analysis, breed, sex, age, and reproductive status in addition to the number and location of affected mammary glands. Between January 2021 and April 2022, a total of 53 dogs were brought to the Veterinary Clinical Complex, DUVASU, Mathura, Uttar Pradesh, India, with the suspicion of having a mammary gland tumour. Of them, 42 dogs had neoplastic growth and 11 dogs had inflammatory growth based on cytological examination. A total of 102 cases of various dog cancers were documented throughout this time, indicating a 41.18% incidence of mammary tumours. Older female canines between the ages of 8 and 12 were the most affected, with the German Shepherd breed having the highest incidence. A haemato-serological examination revealed a drop in serum iron levels, haemoglobin concentration, and total erythrocyte count, but an increase in total leucocyte count, primarily neutrophils, and serum calcium level. When comparing the blood neutrophil count and serum calcium level in the neoplastic state to the control, a strong association was found.

Keywords: Canine, cytological, haemato-serological, mammary gland tumours, neoplastic

1. Introduction
One of the greatest risks to both human and animal life is cancer. When it comes to pets, this is the main reason for death. The most prevalent tumour in the modern world among human females is breast cancer, which is growing quickly because of alterations in environmental factors and lifestyle. One in ten new cases of cancer identified annually is female breast cancer, making it the most common malignancy in both industrialised and developing nations (Bray *et al.*, 2004) [4]. Like skin tumours, mammary tumours are the second most prevalent neoplasm in dogs. Dogs are twice as likely as people to develop cancer (Gupta *et al.*, 2012) [10].

Mammary tumours account for about 52% of all tumour cases in dogs (Varallo *et al.*, 2019) [29]. Most of these cases are documented in female dogs worldwide, with occasional occurrences of 1.7% to 8% occurring in male canines (Nithya *et al.*, 2018) [18]. Since dogs grow a wide range of tumours with clinicopathological characteristics and incidence rates comparable to human cancers, they are an excellent model for comparative oncology. Carcinogenesis risk factors that are the same in both species include obesity, environmental contaminants, and ageing. The canine mammary tumour serves as an excellent model for human breast cancer because of its similarities in morphology, behaviour, and immunohistochemistry with human breast cancer (Gray *et al.*, 2020) [9]. According to Kumar *et al.* (2018) [14], the mean leukogram of dogs with mammary carcinoma cases showed an insignificant increase in the total WBC count and neutrophils, a drop in lymphocytes, and an insignificant decrease in mean haemoglobin in dogs with mammary tumours.

2. Materials and Methods

From January 2021 to April 2022, a prospective study was conducted at the Veterinary Clinical Complex, DUVASU, Mathura, Uttar Pradesh, India. A total of 53 dogs with suspected canine mammary gland tumours were seen during this time; 42 of them had neoplastic development on their mammary glands, and 11 of them had inflammatory growth, according to the results of fine needle aspiration biopsy and ultrasonography tests. The Institutional Animal Ethics Committee accepted the study under application number IAEC/22/22. Following the case's presentation, a thorough clinical examination was performed, and the following observations were recorded: the owner's contact information; animal's age, sex; breed and body weight; location of the lesion(s); number of glands involved; size of the affected gland; colour, texture, and consistency of the neoplasm; length of the illness; history of prior inflammation or injury; history of parity and spaying; and an ultrasonographic examination to determine whether the tumour had spread distantly to lymph nodes and visceral organs. The modified WHO TNM staging approach was used as the basis for the clinical staging of tumours (Matos *et al.*, 2012) [16]. Out of 42 neoplastic cases 39 cytological samples, 33 blood samples and 21 serum samples were obtained for further examination.

2.1 Sample collection for blood and serum examination

The dog's saphenous and cephalic veins were used to get the samples. The drawn blood was moved right away to a plain vial (3 ml) for the analysis of serological parameters and gently-filled in EDTA vial (2 ml) for the investigation of total blood parameters. The plain vial was tilted for twenty to twenty-five minutes in order to extract the serum. After centrifugation at 2000 rpm for three minutes, the serum was extracted. In order to examine the serological parameters, the collected serum was appropriately labelled with the relevant information and stored at -20 °C. Five healthy animals served as the source of the control samples, with the consent of pet owners.

To obtain complete blood parameters, blood taken in an EDTA vial was gently mixed and placed under an automated blood counting machine (NIHON KOHDEN MEK-6550 CELLTAC α). Inductively coupled plasma-optical emission spectroscopy (5800 ICP-OES, Agilent, CA, USA) was used to analyse the minerals in serum, which included zinc (Zn), iron (Fe), copper (Cu), calcium (Ca), phosphorus (P), and magnesium (Mg). The wavelengths (nm) for zinc, iron, copper, calcium, phosphorus, and magnesium were 213.857 nm, 238.204 nm, 327.395 nm, and 422.673 nm, respectively. For the analysis of the minerals, the instrument's settings were as follows: 12 L/min plasma gas flow, 0.7 L/min nebulizer gas flow, and 1 L/min Aux flow. The viewing mode was axial at 8 mm height. Every sample was tested triplaicate. To estimate calcium and phosphorus, the following standards were created using ICP multi-element standard solution IV (Merck Chemicals, Darmstadt, Germany): 0, 0.5, 2, 5, and 10 ppm. Plotting the absorbance versus the concentration allowed for the creation of a calibration curve for these minerals using these standards. The system used ICP Expert software to automatically compute the concentration of minerals in the sample after plotting the calibration curve. The standard's operating parameters were akin to those of the samples. The student unpaired t-test was used to statistically analyse the blood and serum sample data.

2.2 Fine needle aspiration fluid collection for cytology

A 22 G fine needle fitted to 5ml syringe, was carefully inserted into the affected mammary gland region, approximately up to the centre of the growth in 2-3 different directions, after the affected area had been thoroughly sterilised with a sterile swab soaked in 70% alcohol. The fluid was then aspirated by keeping the needle inside the growth. After removing the syringe, the aspirated fluid was gently spread out over a clean, grease-free glass slide to create a smear. The smear obtained from fine needle aspirates was stained with Giemsa stain for cytological analysis in order to determine the kind of neoplastic cells and analyse their properties in order to categorise the tumour into several groups. Robinson's grading system was used for the cytological grading of canine mammary tumours (Robinson *et al.*, 1994) [22].

3. Results and Discussion

A total of 102 canine neoplastic cases were recorded at the Veterinary Clinical Complex, DUVASU, Mathura, Uttar Pradesh, India, during the study period. 42 cases of canine mammary gland tumours were found among the 102 neoplastic cases, accounting for 41.18% of the total. Based on cytological and ultrasonographic examination, the majority of canine mammary tumours were malignant. Of the 42 total neoplastic cases, 2 had distant metastases discovered in the lungs on ultrasonographic examination, and 3 had regional lymph node involvement based on cytological examination.

3.1 Epidemiological variables

A total of ten dog breeds with mammary gland tumours were included in the current investigation. Among these ten breeds, the German Shepherd (12/42), Labrador Retriever (10/42), and Pomeranian (6/42) were the three breeds in which the majority of incidents occurred. Nondescript (4/42), Rottweiler (3/42), Doberman (2/42) Great Dane (2/42), American cocker spaniel (1/42), St. Bernard (1/42) and cross-bred (1/42) were the other affected breeds. The results of the study showed that German Shepherds had the highest incidence of canine mammary tumours (28.57%), with pure breeds being most affected. Breed distribution patterns vary, therefore breed predisposition also depends on the research area's geographic location. Genetics may have a part in the development of canine mammary tumours, as evidenced by the increased occurrence in purebred dogs compared to non-descript and cross-bred dogs (Sarli *et al.*, 2002; Srivastava *et al.*, 2009; Nithya *et al.*, 2018) [25, 27, 18].

Older bitches that were more than six years of age were frequently affected animals. The age group of 7 to 12 years old had the highest incidence of cases (32/42 - 76.2%). While neoplastic instances were seen in older age groups, the majority of non-neoplastic cases were observed in younger age groups. According to cytological grading, the incidence of benign tumours peaked in the age range of 5-7 years, whereas the incidence of malignant tumours peaked in the age range of 8-12 years. This suggests that the risk of malignancy increases with age. This may be the result of an accumulation of tumorigenic factors that rises with age (Zatloukal *et al.*, 2005; Toniti *et al.*, 2009; Pastor *et al.*, 2018) [34, 28, 20].

41 out of 42 cases included female dogs, and one case involved a male dog (non-descript). The results were consistent with earlier research, which found that canine mammary tumours are more common in female dogs and extremely uncommon in male dogs. Hormonal differences between the sexes were the cause of this (Schneider *et al.*, 1969; Alenza *et al.*, 2000) [26, 2].

Out of the 42 neoplastic cases that were presented, 68 tumour masses were found. The majority of patients had many tumour masses in several mammary glands. In comparison to anterior pairs of mammary glands, posterior pairs of mammary glands had a higher frequency of afflicted glands. Inguinal (35.29%), caudal abdominal (29.41%), cranial abdominal (16.18%), caudal thoracic (16.18%), and cranial thoracic (2.94%) glands were involved, in decreasing order. The caudal pair of mammary glands was more impacted than the cranial pair in both the thoracic and abdominal pairs. The right side of the animals was more impacted than the left in the majority of cases. This could be the result of posterior glands changing more proliferatively in response to hormonal influences, particularly oestrogen. Since these are the largest glands, there is a chance that they will experience more stress and physiological changes, which increases the susceptibility to develop mammary cancer (Nithya *et al.*, 2018) [18].

Only 6 animals (14.28%) out of the 42 animals in the current study had undergone spaying, whereas 36 animals (85.72%) had not. These findings are related to reproductive history. Spaying was performed on animals who were at least 4 years old. Although parity information was not consistently gathered, the majority of cases showed that the cancer developed after the second or third parity. This may be because, according to earlier research (Moulton *et al.*, 1970; Salas *et al.*, 2015) [17, 24], there are hormonal status differences between spayed and intact bitches and the time of spaying.

After a thorough examination of 42 instances, it was discovered that the tumour's diameters ranged widely, from 2 to 20 cm. CMTs were shaped differently, showing nodules, irregularities, elongations, rounds, and ovoids. Most tumours were soft to solid, hard, and granular masses in consistency. Few tumours were cystic, while some had ulcerations. Some of the tumorous growths had a greyish look, while others ranged in hue from pink to whitish. 42 instances in total were classified into 5 TNM staging categories in the current investigation; of them, 4.76% were in stage 1 (2/42), 7.14% in stage 2 (3/42), 76.19% in stage 3 (32/42), 7.14% in stage 4 (3/42), and 4.76% in stage 5 (2/42). The TNM stage 3 category has the highest number of instances.

3.2 Haematological parameters

Of the 42 cases of neoplasm in the current investigation, 33 blood samples and 21 serum samples were obtained. The student unpaired t-test was used to evaluate the mean, standard error of mean, range, and percent difference of tumour condition from control as shown in table.1.

The average WBC value was 19.13 ± 1.93 × 103 / µl, indicating a 74.54% rise in total leucocyte count in the tumour state relative to the control. However, because of the large diversity in clinical values, these differences did not reach the statistically significant threshold. The mean neutrophil count in the tumour condition was 80.57 ± 1.26%, indicating a highly significant (P value < 0.0001) increase in neutrophil count compared to control. According to Childress (2012), neutrophilic leucocytosis may result from either acute or chronic inflammation or tissue necrosis linked to a cancer disease. According to Kumar *et al.* (2018) [14], IL1 and TNF-α can also cause neutrophilia by promoting the synthesis of growth factors. In order to fight against infection, leucocytes and phagocytic cells-primarily neutrophils-produce reactive oxygen and nitrogen species, which are normally produced in moderation. However, in chronic inflammatory conditions, there is a greater generation of these reactive species, which react to form peroxynitrite, a mutagenic agent. Therefore, repeated tissue damage and regeneration in the presence of highly reactive oxygen and nitrogen species released from inflammatory cells interact with the DNA of proliferating epithelium, leading to permanent genomic alterations such as point mutations, deletions, and rearrangements, among other effects. This ultimately causes the tissue to become more susceptible to tumour development, which in turn promotes the growth of neoplastic tissue in the mammary gland tissue (Coussens and Werb, 2001) [6].

The lymphocyte count in the tumour condition was found to be significantly lower (P value < 0.0001) than in the control group, with a mean value of 15.38 ± 1.1%. Since T lymphocytes are an essential component of the blood cell population and aid in the removal of cancer cells, their decline in circulation was the primary cause of lymphopenia. Disease development results from immune system changes brought on by ageing, especially to circulating T-cells. These changes reduce cellular immunity. Immunosuppressive substances such as cytokines (IL-6, IL-10), transforming growth factor (TGFβ), and T-cell degradation are produced by cancer cells. These compounds further suppress the immune system. As a result, these variables likely to cause a drop in the overall lymphocyte count in tumour conditions, which accelerates the progression of the cancer. This is also a promising explanation for why the majority of tumours develop in elderly people (Karayannopoulou *et al.*, 2017) [13].

When comparing the tumour condition to the control, there was a highly significant (P value = 0.0001) drop in the mean monocyte count (3.37% ± 0.32%). may be caused by myeloid cells, which are innate immune cells that make up the mononuclear phagocytic system and have dendritic cells, monocytes, and macrophages as their progenitors. Given the critical role that myeloid cells play in stimulating and modulating immune responses, the soluble factors released by tumour cells suppress the activation and functions of canine myeloid cells, which in turn causes dysregulation of canine myeloid cells and, ultimately, cancer-related immunosuppression, which manifests itself in a number of ways, including decreased monocyte counts and metastatic spread (Wasserman *et al.*, 2012) [31].

The eosinophil count mean value was 0.68 ± 0.09%, indicating a 97.05% increase in eosinophil count in the tumour condition compared to the control. However, because of the large diversity in clinical values, these differences did not reach the statistically significant threshold. In cases of tumours, eosinophilia may develop as a result of allergic responses or parasite infections. According to certain research, mammary cancer cases have also been linked to paraneoplastic eosinophilia, which is brought on by the IL-5 cytokine and eosinophilic chemotactic factor (Losco, 1986) [15].

The total erythrocyte count was 5.63 ± 0.26 × 106 / µl on average. The result shows that, when compared to the control, the total RBC count in the tumour condition decreased by about 8%. The mean haemoglobin concentration in the tumour condition was 11.72 ± 0.47 g/dl, indicating a small drop of 9.98% when compared to the control. Given that there were no structural changes to the RBCs, the anaemia might be understood as normocytic normochromic anaemia (Hristov and Binev, 2018) [12]. This could be the result of cytokines secreted by the tumour cells, such as IL-1, which reduces the half-life of erythrocytes by sequestering iron, TNF-α, and IL-6, which reduces the release of erythropoietin, which in turn lowers the concentration of haemoglobin and the number of red blood cells (Kumar *et al.,* 2018) [14]. One of the main causes of paraneoplastic anaemia is blood loss connected to tumour haemorrhage (Finora, 2003) [8]. The mean platelet count in the tumour condition was 247.7 ± 25.3 × 103 /µl, indicating a drop of 29.07% when compared to the control group. However, because of the large diversity in clinical values, these differences did not reach the statistically significant threshold. This may be the result of direct bone marrow injury leading to a decreased ability for producing thrombocytes (Hristov and Binev, 2018) [12]. The other cause of thrombocytopenia is immune-mediated thrombocytopenia, which occurs when there is no obvious reason for the body to produce antiplatelet antibodies. According to Rozanski *et al.* (2002) [23], thrombocytopenia promotes surface bleeding, which results in superficial blood loss and eventually corresponds with anaemia in tumour conditions.

Table 1: Representation of haematological profiles of healthy control and canine mammary gland tumour condition (n=33)

Blood parameter	Values				% Difference
	Control		Tumour		
	Mean	Range	Mean	Range	
WBC (×10^3/µl)	10.96±0.57	9.8-13.1	19.13±1.93	6.4-57.1	74.54% ↑
Neutrophils (%)	60.60±1.82	58.4-62.7	80.57±1.26	59-92.3	P-value: < 0.0001↑
Lymphocytes (%)	32.10±0.43	30.9-33.4	15.38±1.11	4.72-32.4	P-value: < 0.0001↓
Monocytes (%)	6.96±0.33	6.2-7.8	3.37±0.32	1-7.9	P-value: = 0.0001↓
Eosinophils (%)	0.34±0.07	0.2-0.6	0.68±0.09	0.1-2	97.05% ↑
RBC (×10^6/µl)	6.13±0.2	5.54-6.8	5.63±0.26	1.96-8.24	8% ↓
Hb (g/dl)	13.02±0.18	12.5-13.6	11.72±0.47	4.9-14.6	9.98% ↓
Platelet (×10^3/µl)	349.2±3.97	338-362	247.7±25.3	12.6-557	29.07% ↓

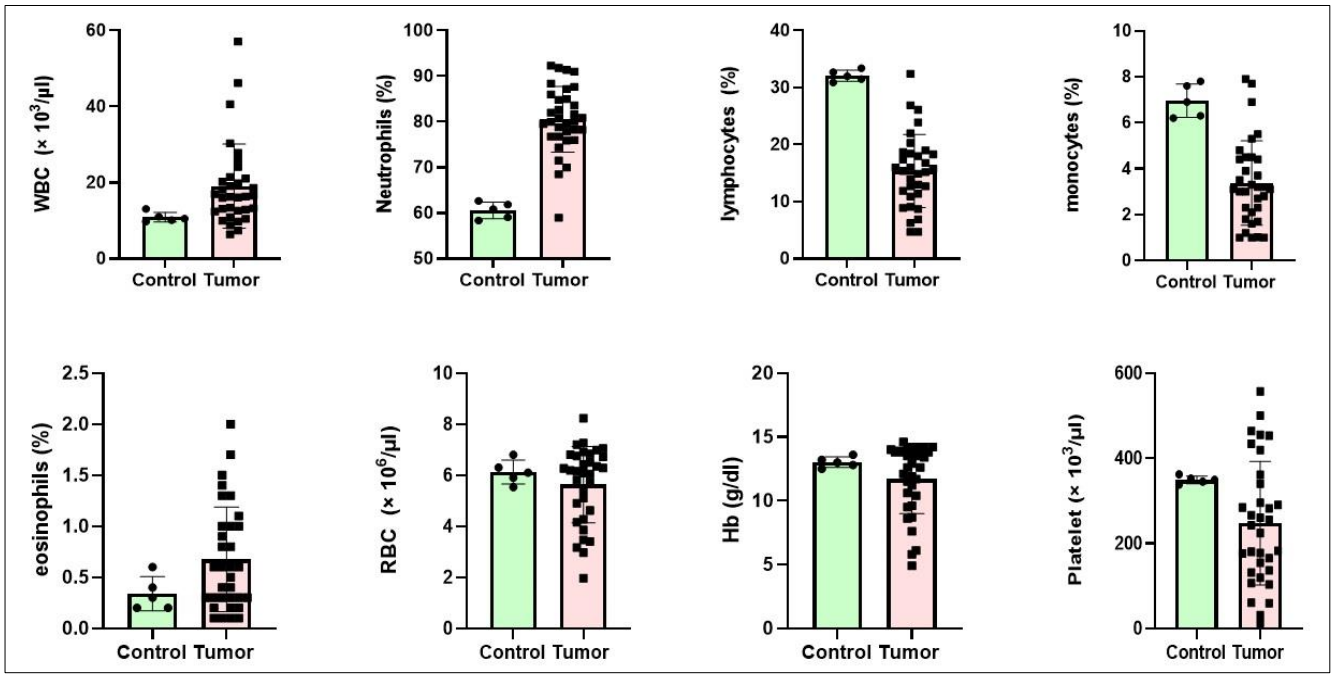

Fig 1: Graphical representation of different haematological parameters showing comparison between control and tumour cases.

3.3 Serological Parameters

The total serum calcium level in the tumour condition was significantly higher (P value < 0.05) than in the control group, with a mean value of 19.25 ± 1.55 mg/dl. The generation of parathyroid-related peptides may be the cause of humoral hypercalcemia, which is the mechanism behind the development of hypercalcemia. This peptide and parathyroid hormone are closely linked, and they both function similarly in stimulating osteoclastic bone resorption and increasing renal calcium resorption. Additionally, tumours induce localised osteolytic reactions that activate osteoclast cells and lead to lytic hypercalcemia (Bae *et al.,* 2007; Finora, 2003) [8].

Serum iron levels in tumour conditions were significantly lower (P value < 0.05) than in controls, with a mean value of 0.51 ± 0.04 mg/dl. This happens because cancer cells divide to support their development, DNA replication, and metastatic processes, which require a significant quantity of iron consumption (Aapro *et al.,* 2012) [1]. tumour cell secretions of cytokines, such as IL-1, lead to iron

sequestration, which lowers blood iron levels and, in turn, shortens red blood cell half-lives, resulting in anaemia under tumour conditions (Kumar *et al.*, 2018) [14].

The total serum phosphorus level was 23.21% higher in the tumour condition compared to the control group, with a mean value of 9.50 ± 0.42 mg/dl. Acute tumour lysis syndrome, which is brought on by chemotherapy or radiation-induced fast lysis of malignant cells, may be the cause of the elevated serum phosphorus level. The concentration of phosphorus in malignant cells is higher than in normal cells. When these cells are destroyed, their intracellular contents—such as phosphorus, potassium, and purines—enter the systemic circulation and eventually surpass the kidney's excretory capacity. This results in metabolic and electrolyte disturbances, such as hyperphosphatemia and hyperkalaemia (Vickery and Thamm, 2007; Yarpuzlu, 2003) [30, 32].

The serum zinc level in the tumour condition was 32.26% higher than in the control group, with a mean value of 0.41 ± 0.07 mg/dl. However, because of the large diversity in clinical values, these differences did not reach the statistically significant threshold. The mean blood copper and magnesium levels were 0.17 ± 0.00 mg/dl and 0.023 ± 0.00, respectively. This represents a very minor increase of 6.25% and 9.50% in serum copper and magnesium levels, respectively, and shows no discernible change in the tumour condition when compared to the control. Nevertheless, there is a paucity of information about the results in dogs. The serum level of copper was shown to have slightly increased in the current investigation (Yucel *et al.*, 1994; Zowczak *et al.*, 2001) [33, 35]. According to Gupte and Mumper (2009) [11], elevated copper levels in cancer patients may have a promising explanation as copper plays a major role in angiogenesis as it is the chief and key component of ceruloplasmin (> 75% copper), which acts as an endogenous angiogenic stimulator and is essential for the development of new blood vessels in growing tumours.

Table 2: Representation of serological profiles of minerals in healthy control and canine mammary tumour condition (n=21)

Serum parameter (mg/dl)	Values				% Difference
	Control		Tumour		
	Mean	Range	Mean	Range	
Calcium	10.81±0.51	9.49-12.31	19.25±1.55	11.03-44.83	P-value: < 0.05↑
Phosphorus	7.71±0.17	7.18-8.20	9.50±0.42	4.19-12.53	23.21% ↑
Iron	0.78±0.15	0.29-1.29	0.51±0.04	0.17-0.97	P-value: < 0.05↓
Zinc	0.31±0.04	0.24-0.44	0.41±0.07	0.15-1.8	32.26% ↑
Copper	0.16±0.00	0.15-0.16	0.17±0.00	0.10-1.10	6.25% ↑
Magnesium	0.021±0.00	0.020-0.021	0.023±0.00	0.01-0.04	9.5% ↑

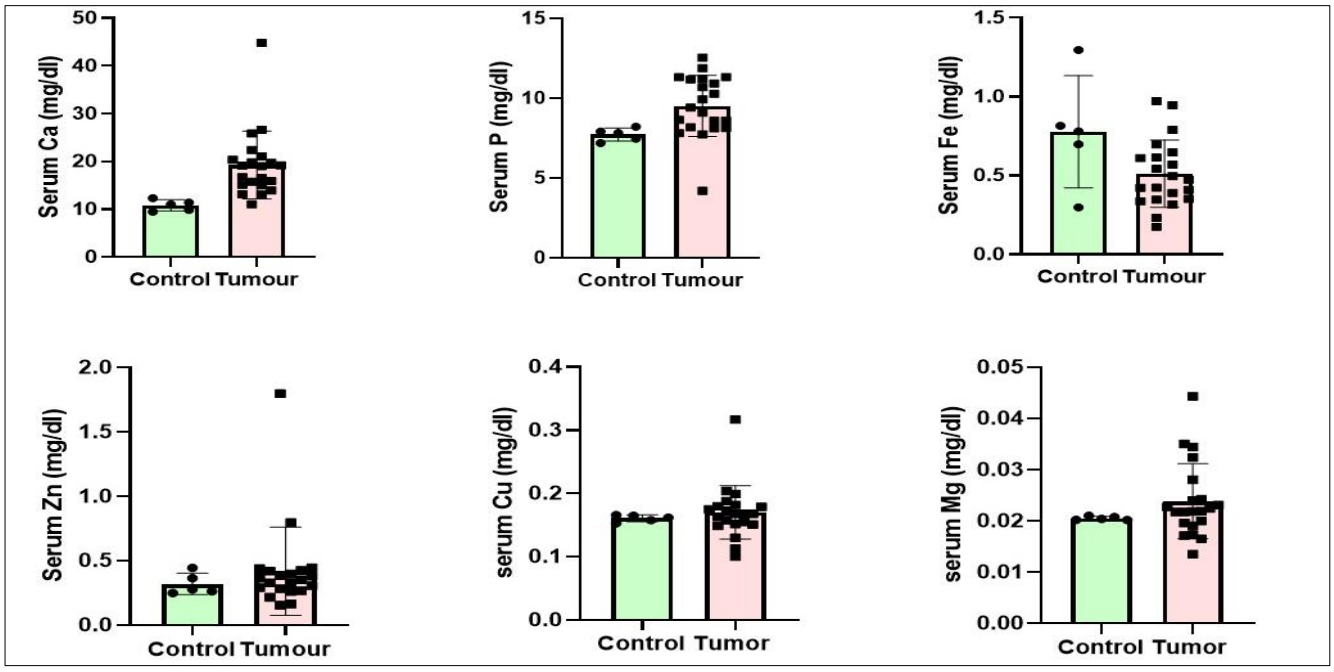

Fig 2: Graphical representation of different serological parameters showing comparison between control and tumour cases.

3.4 Cytological examination of FNAC smears

There were 42 neoplastic cases in all, of which 39 cytology samples were obtained using the fine needle aspiration method. The majority of samples (56.41%) fell into the grade II group of the cytological grading system. These results agreed with earlier research (Phukan *et al.*, 2015; Pal *et al.*, 2016) [21, 19]. Based on cytological testing, cancers classified as grade 2 or 3 were deemed malignant, whereas grade 1 tumours were deemed benign (Dolka *et al.*, 2018) [7]. (Figures 3–8).

Table 3: Percentage of different grades of tumour samples based on cytology (n=39)

Grade of tumour	No. of samples	Percentage (%)
Grade I	11	28.21%
Grade II	22	56.41%
Grade III	6	15.38%
Total	39	100%

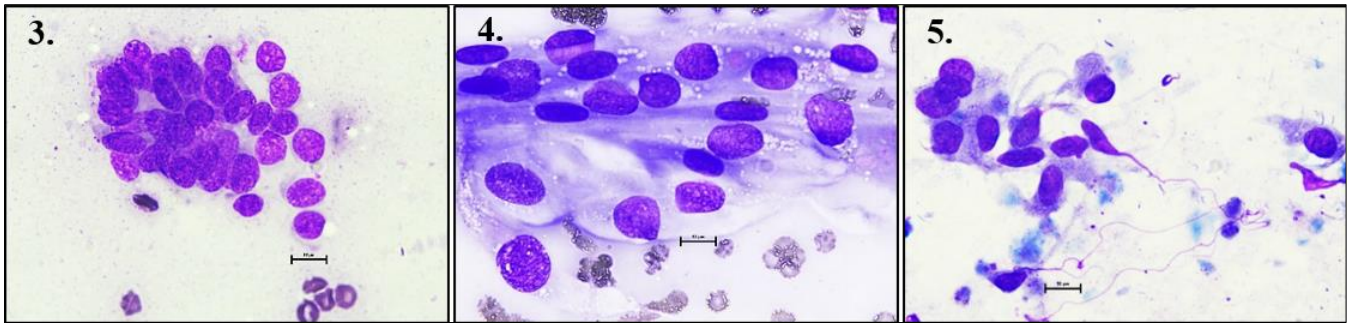

Fig 3: Uniform cell population grouped into densely packed clusters. There is relatively little cytoplasm and the entire cell is occupied by the nucleus. The nucleus has an inconspicuous nucleolus and smooth borders with granular chromatin. Grade I category. Giemsa stain, 1000X.

Fig 4: Highly pleomorphic cells in a mixed population that are grouped loosely and also organised singly. Large and hyperchromatic nucleus having uneven nuclear borders with buds and clefts having granular chromatin and an indistinct nucleolus. Cytoplasm is vacuolated. Grade II category. Giemsa stain, 1000X.

Fig 5: A population of mixed, moderately pleomorphic cells with some myoepithelial cells having elongated, spindle-shaped nuclei distributed singly and in loose clusters. Nucleus is large and hyperchromatic having granular chromatin and an indistinct nucleolus with slightly uneven nuclear borders. Grade II category. Giemsa stain, 1000X.

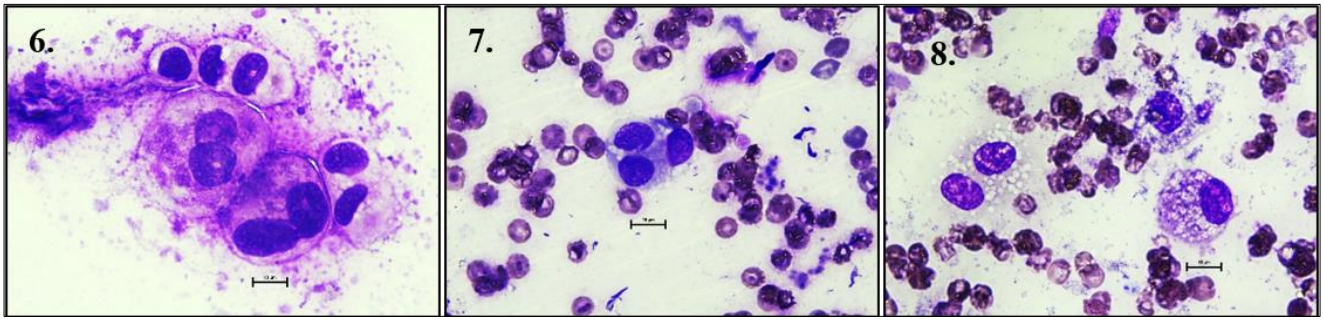

Fig 6: Highly pleomorphic and enlarged cells grouped singly. A massive cancerous cell exhibiting both cytokinesis and karyokinesis is seen in the quadra-nucleate stage. Cell sizes exceed five times those of RBCs. Clumps of chromatin and an ill-defined nucleolus characterise the hyperchromatic nucleus. Grade III category. Giemsa stain, 1000X.

Fig 7: Enlarged cell arranged singly showing tri-nucleate stage. The size of cells exceeds 5 times of RBC. The nucleus is hyperchromatic with irregular margins and having clumped chromatin and indistinct nucleolus. Grade III category. Giemsa stain, 1000X.

Fig 8: Singly arranged highly pleomorphic cells with vacuolated cytoplasm. The nuclei are hyperchromatic with slightly irregular nuclear margins having clumped chromatin and indistinct nucleoli. Grade III category. Giemsa stain, 1000X.

4. Conclusion

A strong correlation was found between the cytological and haemato-serological parameters, as significant variation was noted in the haemato-serological parameters in those cases which were malignant according to cytological examination. This suggests that the haemato-serological parameters alone may not be an accurate enough preliminary tool for identification of neoplastic condition, as research data regarding these tests is limited in canines. Therefore, in addition to it, cytological, histopathological, and molecular tests should be conducted to reach the appropriate diagnosis.

5. Acknowledgement

We express our gratitude to the Veterinary Pathology Laboratory, Veterinary Clinical Complex, and the administration of Veterinary University in Mathura-281001, Uttar Pradesh, India, for their invaluable support in successfully conducting this research study, as well as to all the animals and their owners who participated in the study.

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