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SHORT COMMUNICATION

Evaluation of proliferative status of bitch mammary gland osteosarcoma using modified methods of AgNOR staining

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ABSTRACT

Canine mammary osteosarcoma is a rare but highly aggressive extra medullary tumour, predominantly affecting older female dogs and marked by rapid progression and a high metastatic potential. While histopathology is the standard diagnostic and prognostic tool, it does not always accurately represent the true proliferative activity of tumour cells, necessitating more sensitive adjunct techniques. Nucleolar organizer regions (NORs), which contain argyrophilic proteins involved in ribosomal RNA synthesis, can be visualized using silver staining (AgNOR). AgNOR quantification has emerged as a valuable adjunct to routine histopathology, as increased AgNOR counts correlate with higher metabolic activity, faster cell cycles, and greater tumour proliferation. Recent advances involving zinc and silver co-localization have further improved the sensitivity and consistency of AgNOR-based assessments. The present study investigated naturally occurring mammary gland osteosarcoma in bitches. Tumour tissues were collected, formalin-fixed, and subjected to routine histopathology, followed by AgNOR staining and zinc–silver co-localization analysis. Proliferative activity was evaluated through quantitative assessment of cell-cycle phases and mitotic figures. Results revealed a significant positive association between mitotic counts and proliferative phases of the cell cycle, along with a negative correlation with the G1 phase across all staining methods. These findings highlight the reliability of AgNOR techniques and zinc-silver co-localization as sensitive markers for assessing tumour proliferation and biological aggressiveness in canine mammary osteosarcoma.

Key words: AgNOR, Bitch, Co-localization, grading, mammary osteosarcoma, mitotic index, proliferative status

INTRODUCTION

Cancer remains a leading cause of death in animals due to limited therapeutic options and poor prognosis. Mammary tumours are the second most common tumour in dogs, accounting for 52% alone of all tumour cases[1]. Although histopathological grading remains the gold standard, it often fails to reflect true proliferative activity, necessitating newer techniques. Histochemistry is a potent supplement to conventional histopathological procedures because it improves diagnostic accuracy, offers functional insights and directs targeted therapy[2]. Histochemical techniques localize and demonstrate specific chemical constituents such as biomolecules and nucleic acids within cells and tissues. Nucleolar organizing regions having a group of argyrophilic proteins that are preferentially stained with silver techniques. Nucleolar organizing regions (NOR) contains a set of argyrophilic proteins, which can be selectively stained by silver methods. AgNOR ((Argyrophilic nucleolar organizer region) staining technique can be used as an adjunct to histopathology in differentiating benign and malignant tumours, particularly borderline cases. AgNOR staining is one of them that is becoming more and more popular since it is simple to use, correlates with the proportion of cell cycling, and is more intense when the cell cycle speed increases. rRNA is responsible for the production of many such proteins in the cell. Protein synthesis is an important step in cell development. Therefore, a relationship between NOR and cell proliferation has been suggested[3]. After silver staining, AgNOR appears as discrete brown to black dots in formalin-fixed tissues. The AgNOR index is a count of the number of such black dots, in a cell nucleus. The AgNOR index is a marker used to assess tumour proliferation rates and differentiate between benign and malignant tumours. They have the advantage that their numbers are increased only in actively and dividing cells. Their increased number indicates high metabolic activity which probably correlates with increased speed[4]. These silver-stained sites correspond to zinc-binding nucleolar proteins involved in replication and transcription[5]. Co-localization studies of zinc and silver binding sites may improve understanding of AgNOR formation and provide a more reliable, sensitive marker for tumour proliferation assessment[6].

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MATERIALS AND METHODS

Samples from 11 different adult bitches of different breeds, showing lesions of swelling and growth in the mammary gland, were collected from cases presented in the Teaching Veterinary Clinical Complex, Bihar Veterinary College, Patna or the nearby pet clinics for treatment. The bitches mostly aged between 8–10 years, were affected six to eight months back for which they were treated but showed no response. The growths of variable sizes were seen in different mammary glands in different cases, however, the growth were unanimously hard and firm with few ulcerated points (Fig. 1). These cases were therefore, suspected for mammary tumour. Clinically these animals were found anaemic and dull.

The representative tissue samples were collected and preserved in 10% formalin for further laboratory examination for diagnosis of neoplasia and its proliferative behaviour. These samples were subjected for evaluation of their biological aggressiveness in light of co-localization of zinc and argyrophilic sites in nuclei and counting of number of cells in different phases of cell cycle developed[7].

Samples collected from different regions of the tumour mass were subjected to routine tissue processing, paraffin embedding, and microtome sectioning. Four serial sections were prepared from each tissue block. One section was stained with hematoxylin and eosin (H&E) for histopathological classification, tumour grading, and mitotic figure counting[8]. Tumour grading was performed using the Nottingham modification of the Scarff–Bloom–Richardson system, based on tubule formation, nuclear pleomorphism, and mitotic activity[9]. Mitotic figures were counted in 50 high-power fields, and the mitotic index was calculated accordingly[10]. The remaining serial sections were subjected to AgNOR staining. To enhance AgNOR visibility, co-localization of silver with zinc was performed using Timm's method[11]. Accordingly, three serial sections were stained using Zn-AgNOR-Dithizone, Zn-AgNOR, and AgNOR techniques. These stains were used to quantify cells in different phases of the cell cycle and assess tumour proliferative behaviour[12]. For Zn-AgNOR-Dithizone staining, deparaffinized and rehydrated sections were treated with a solution of $ZnSO_4$, Na_2SO_3, and acetic acid, followed by AgNOR staining using silver nitrate and formic acid–gelatin. Sections were then treated with sodium thiosulphate, exposed to dithizone, cleared in chloroform, and mounted in glycerol. Zn-AgNOR staining followed a similar protocol without dithizone treatment, while AgNOR staining alone involved silver impregnation and mounting in DPX.

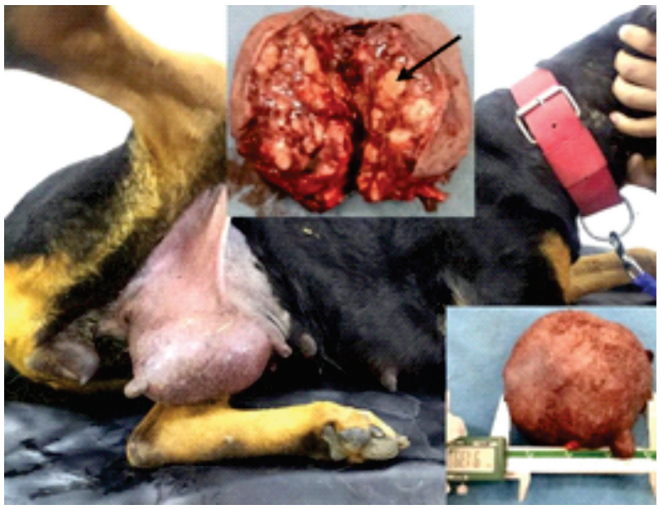

Fig.1. Mammary tumour (91.39 x 85.08 mm) on the right inguinal mammary gland in a Rottweiler (8yr/28.5 kg). Cut section showing hard, bony deposition in tumour mass (black arrow).

RESULTS

Gross examination, in general, revealed fluctuant mass with variable consistency, exhibiting both firm and soft areas. On cut section, the tissue appeared yellowish and contained multiple cavernous spaces filled with greyish fluid.

Histologically, the tumour was characterized by islands of osteoid formation (Fig. 2) and the presence of neoplastic osteoblasts. These cells were short spindle- to triangular-shaped, with plump oval nuclei, arranged in a haphazard manner rather than in organized bundles (Fig. 3). The hallmark feature was the production of osteoid matrix, portions of which were calcified, while other areas remained uncalcified (Fig. 4). In several regions, osteoid was irregularly deposited as fibrillar stroma between pleomorphic cells, with anaplastic cells embedded within the matrix, forming a lace-like pattern. Newly formed osteoid appeared dense, eosinophilia and fibrillar frequently entrapping osteocytes. Occasional multinucleated giant cells and poorly organized cartilaginous islands were also observed. Based on the gross and histopathological findings, the tumours were confirmed as mammary gland osteosarcoma.

The proliferative fraction and growth rate were evaluated by assessing cell-cycle phase distribution using Zn–AgNOR–Dithizone, Zn–AgNOR, and AgNOR staining techniques[13]. Representative histological images showing NOR dots with these three staining methods are presented in Fig. 5, 6 and 7 respectively. The observations are summarized as follows:

Zn-AgNOR-Dithizone Staining

The comparison of mean values of cells in different phases of cell cycle has been presented in Table 1 and Fig. 8. The mean count of cells in S/G2, M as well as S/G2+M phases taken as proliferative fraction of tumour

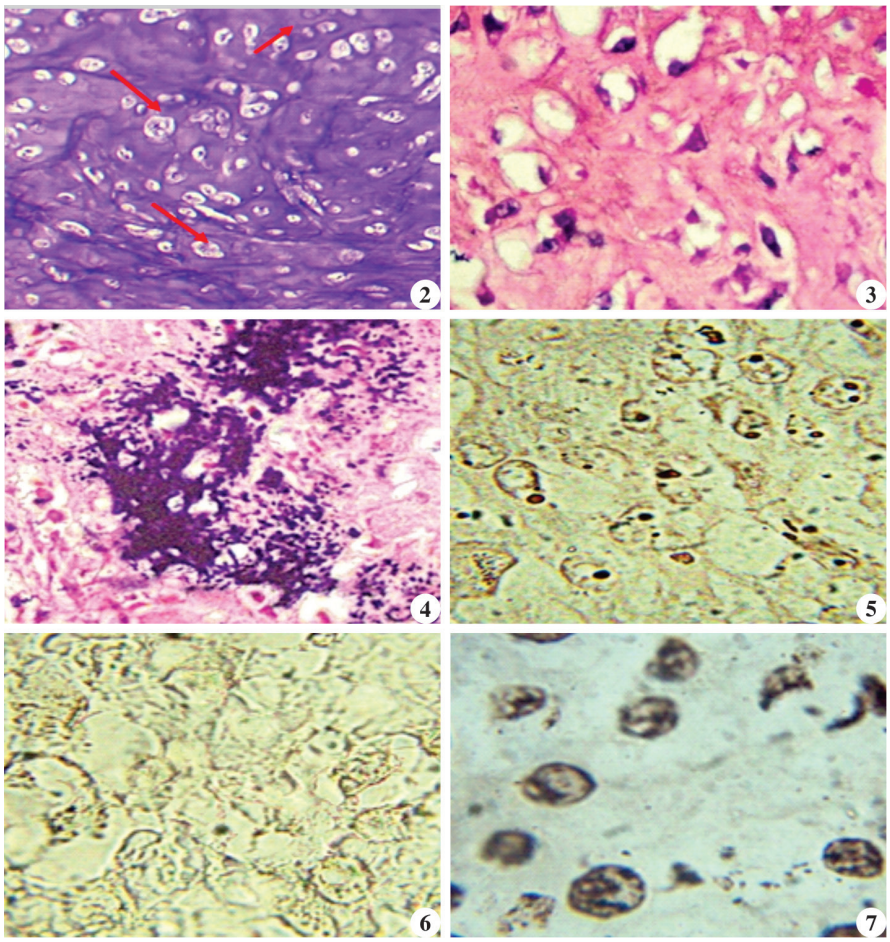

Fig.2. Osteosarcoma of mammary gland characterised by island of basophilic osteoid formation. Mitoses can be seen (H&E x400); **Fig.3.** Osteosarcoma with anastomosing mineralized osteoid (H&E x100); **Fig. 4.** Showing osteoblasts having pleomorphic spindle or triangular shaped nuclei incorporated into bony matrix (H&E x400); **Fig. 5.** Showing cells in different stages of cell cycle in Mammary Osteosarcoma (Zinc-AgNOR-Dithizone x1000); **Fig.6.** Showing cells in different stages of cell cycle in Mammary Osteosarcoma (Zn-AgNOR x1000); **Fig.7.** Showing cells in different stages of cell cycle in Mammary Osteosarcoma (AgNOR x1000).

cells along with mitotic figures were significantly higher in Grade III tumour as compared to Grade II while the count of cells in G_1 phases were significantly lower in Grade III than in Grade II. This clearly indicated more proliferative population of cells in tumours of Grade III than Grade II. At the same time the finding of proliferative population in accordance with the histological behaviour of osteosarcoma support the ability of the system to identify the cells in different phases of the cycle.

Zn-AgNOR Staining

The comparison of mean values is presented in Table 2 and Fig. 9. The observed proliferative fractions, in agreement with the histological behaviour of osteosarcoma, support the capability of this system to identify tumour cells in different phases of the cell cycle. The mean counts of cells in the S/G2 and M phases, as well as the combined S/G2+M proliferative fraction, along with mitotic figures, were significantly higher in Grade III tumours compared to Grade II tumours. Conversely, the mean count of cells in the G1 phase was significantly lower in Grade III than in Grade II tumours.

AgNOR Staining

The comparison of mean values

Table 1. Showing Comparison of counting of percentage of cells in different phases of cell cycle with Zn-AgNOR-Dithizone staining in different Grades of Mammary Osteosarcoma.

	SCORE	G1	S/G2	M	S/G2+M	Abnormal	Mitotic figure
GRADE II	6.21±0.14	67.57±2.75	21.03±1.89	9.59±0.58	29.67±2.38	2.59±0.45	21.71±1.05
GRADE III	9.02±0.00	38.38±1.89	43.49±1.68	13.01±0.51	55.015±2.02	5.41±0.35	28.54±1.18
t- value	-	8.17**	8.17**	3.78**	8.00**	3.93**	4.23**

NS Non Significant, *P<0.05, **P<0.01

Table 2. Showing Comparison of counting of percentage of cells in different phases of cell cycle with Zn-AgNOR stain in different Grades of Mammary Osteosarcoma.

	Score	G1	S/G2	M	S/G2+M	Abnormal	Mitotic figure
Grade II	6.33±0.11	66.35±3.25	20.50±2.28	10.48±0.79	31.00±2.85	2.67±0.55	21.70±1.07
Grade III	9.01±0.00	37.86±3.34	41.51±3.27	13.59±0.54	55.10±3.39	7.08±0.49	28.44±1.19
t- Value	-	6.00**	5.28**	3.29**	5.36**	4.91**	4.25**

NS Non Significant, *P<0.05, **P<0.01

Table 3. Showing Comparison of counting of percentage of cells in different phases of cell cycle with AgNOR stain in different Grades of Mammary Osteosarcoma.

	Score	G1	S/G2	M	S/G2+M	Abnormal	Mitotic figure
Grade II	6.29±0.12	75.81±1.90	10.95±1.37	11.00±0.67	21.00±1.73	3.17±0.40	21.70±1.06
Grade III	8.99±0.00	57.12±1.50	21.76±1.15	14.95±0.96	36.69±1.54	6.21±0.41	28.45±1.14
t- value	-	7.39**	4.80**	4.09**	6.44**	5.50**	4.25**

[NS] Non Significant, *P<0.05, **P<0.01

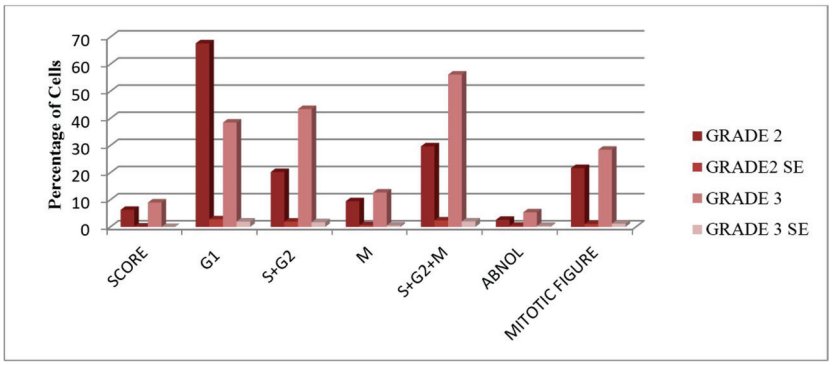

Fig. 8. Showing Comparison of mean values of percentage of cell counts and standard error in different phases of cell cycle with Zn-AgNOR- Dithizone staining in different grades of mammary gland osteosarcoma.

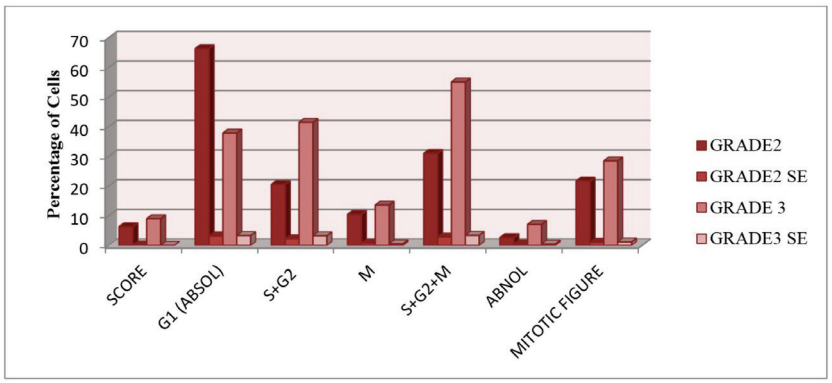

Fig. 9: Showing Comparison of mean values of percentage of cell counts and standard error in different phases of cell cycle with Zn-AgNOR stain in different grades of mammary gland osteosarcoma.

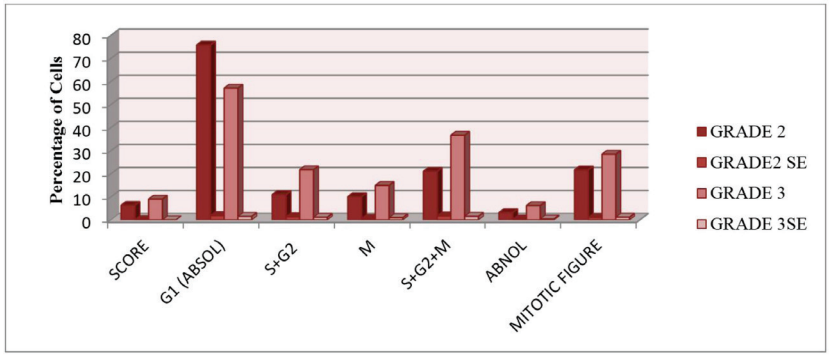

Fig. 10: Showing Comparison of mean values of percentage of cell counts and standard error in different phases of cell cycle with AgNOR staining in different grades of mammary gland osteosarcoma.

is presented in Table 3 and Fig. 10. The observed proliferative rate and growth kinetics, in accordance with the histological behaviour of osteosarcoma, support the effectiveness of this system in identifying tumour cells in different phases of the cell cycle. The mean counts of cells in the S/G2 and M phases, as well as the combined S/G2+M phase considered as the proliferative fraction, along with mitotic figures, were significantly higher in Grade III tumours compared to Grade II tumours. In contrast, the mean count of cells in the G1 phase was significantly lower in Grade III than in Grade II tumours. These findings clearly indicate a higher proliferative cell population in Grade III osteosarcomas.

DISCUSSION

Comparative evaluation of the different staining methods revealed that the percentage of cells in the S/G2 and M phases was consistently highest with Zn–AgNOR–Dithizone staining, followed by Zn–AgNOR, and lowest with AgNOR alone. This finding indicates that Zn–AgNOR–Dithizone staining is more sensitive in identifying S/G2- and M-phase cells, which constitute the most proliferative fraction of the tumour[14]. All three staining techniques demonstrated a significantly higher proportion of cells in the S/G2, M, and S/G2+M (proliferative fraction) phases, along with a lower proportion of cells in the G1 phase, in Grade III tumours compared with Grade II tumours, reflecting increased proliferative activity in higher-grade lesions[15].

Statistical correlation analysis demonstrated a positive and significant association between mitotic figure counts and proliferative phases (S/G2, M, and S/G2+M), and a negative correlation with the G1 phase across all staining

methods, thereby confirming the accuracy of cell-cycle phase identification[16]. Comparative assessment further established that Zn–AgNOR–Dithizone staining consistently detected higher numbers of S/G2- and M-phase cells than Zn–AgNOR or AgNOR alone, underscoring its superior sensitivity and precision in identifying highly proliferative tumour fractions.

Increased cellular proliferation shortens the cell-cycle duration and enhances metabolic activity. Elevated metabolic states are often associated with the presence of multiple extranuclear dots, as reported previously[17]. Mesenchymal cells are intrinsically active in protein synthesis, and tumours of mesenchymal origin exhibit similar behaviour. The intensified protein synthesis accompanying rapid cell cycling is supported by increased transcriptional and translational activity.

It is well established that rRNA ribosomal protein complexes are processed within the nucleolus with the involvement of zinc or silver-binding proteins. Under conditions of accelerated translational activity, these proteins may translocate toward sites of active protein synthesis. Additionally, several translation factors are zinc- or silver-binding proteins, and their increased abundance may contribute to the formation of extranuclear AgNOR granules, reflecting heightened and rapid protein synthesis[18].

Overall, the findings suggest that mammary gland osteosarcoma contains a relatively small but highly proliferative cell population. The tumour is believed to originate from rapidly dividing myoepithelial cells that undergo differentiation and transformation into osteoid tissue under the influence of osteogenic gene expression. During prolonged tumour progression, multiple subclonal populations may emerge, including rapidly proliferating subsets. A notable additional observation in higher-grade osteosarcomas was the presence of intracytoplasmic Zn–AgNOR dots following Zn–AgNOR–Dithizone, Zn–AgNOR, and AgNOR staining, further supporting enhanced proliferative and metabolic activity in advanced lesions.

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Conflicts of Interest: None

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