



Gross, histopathological and electronmicroscopic studies of canine mammary gland tumour treated with *NSI* gene of canine parvo virus

A K VERMA¹, N S JADON², PRIYANKA PANDEY³ and A K TIWARI⁴

Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, Uttarakhand 263 145 India

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ABSTRACT

Female dogs (10) affected with mammary gland tumour (MGT) included in this study were treated with *NSI* gene of canine parvo virus @ 200 µg intratumoural at weekly interval up to 4 week interval. Physical and clinical symptoms were noticed before and after treatment i.e at 0, first, second, third and fourth week of the treatment. The efficacy of the treatment was assessed on the basis of regression of tumour mass of mammary gland tumours, histopathological and electronmicroscopic (SEM and TEM) studies of biopsy samples of mammary tumour tissues. The parameters observed in this study revealed that the *NSI* gene have apoptotic activity in regression of mammary gland tumours.

Key words: Female dog, MGT, *NSI* gene

Mammary tumours are the second most common group of neoplasms in dogs, following skin tumours (Moulten 1999). It comprised 52% of all neoplasms of the mammary gland tumors diagnosed in female dogs, and of that 41 to 53% were diagnosed as malignant (Brody *et al.* 1985). Mammary carcinomas may exhibit rapid growth, doubling in size within a few weeks. However, the size and appearance of these neoplasms may vary greatly. Inflammatory carcinomas usually have diffuse involvement of multiple mammary glands. Dogs with inflammatory carcinomas are more likely to have generalized weakness with anorexia and weight loss (Alenza 2001). Effective treatment method with prompt diagnostic procedure is the prime importance for this life threatening neoplasm. Therefore, accurate and additional aids are required to identify patients at high risks. Recent studies have discovered another weapon in the form of canine parvovirus for stopping the tumorous growth in human and animals. The frequent isolation of parvovirus from tumour cells, as contaminants of tumour viruses, from tumour-bearing animals initially suggested a role of these viruses as etiological agents of cancer (Siegl 1984). In view of the above facts, this study was undertaken with a primary objective to study the effect of recombinant *NSI* gene derived from canine parvovirus on gross, histopathological

and electronmicroscopic (SEM and TEM) structure of mammary gland tumours tissue in female dogs.

MATERIALS AND METHODS

Investigation was carried on 10 clinical cases of female dogs, suffering with mammary gland tumours at College of Veterinary Science, Pantnagar, during July –December 2012. All the animals were included in this study on the holistic request of their owners. Out of 10 cases of canine mammary gland tumour subjected for this experiment, 8 female dogs were between 10 and 15 years of age and 2 female above 15 years of age. Three were German Shepherd, 4 Pomeranian and rest 3 were non-descript mongrel dogs.

Each animal was administered *NSI* gene of canine parvo virus @ 200µg, intratumoural at weekly interval for 4 consecutive weeks. *NSI* gene used was isolated by using standard procedure, virus infected culture fluid was treated with RNase A at a concentration of 40µg/ml and incubated at 37°C for 30 min. Thereafter, proteinase K was added to a final concentration of 250µg/ml and incubated for 15 min at 37°C followed by addition of 0.5% SDS and the mixture was re-incubated at 37°C for 1 h. The viral DNA was extracted once with equal amount of phenol: chloroform and once with chloroform: iso-amyl alcohol. Aqueous phase containing DNA was recovered by centrifugation at 10,000×g for 5 min. Finally the DNA in aqueous phase was precipitated by adding 1/9th volume of sodium acetate (3M, pH 5.2) and 2.5 volumes of chilled ethanol at –20°C overnight. After incubation, mixture was centrifuged at 12,000×g for 15 min. The pellet obtained was washed with 70% ethanol and DNA pellet recovered by centrifugation for 10 min at 12,000×g was air dried and re-suspended in

Present address: ¹M.V.Sc. Student (amitkumarverma011@gmail.com), ²Professor and Head (drjadonnns12@rediffmail.com), ³SRF (shuklapriyanka5@gmail.com), College of Veterinary and Animal Science. ⁴Principal Scientist (aktiwari63@gmail.com), Division of Animal Biotechnology, IVRI, Izatnagar.

30 µl of nuclease free TE buffer.

Amplification of *NS-1* gene sequence was carried out in 25 L reaction volume. Briefly, 2.5 ml of 10X PCR buffer, 1.5 mM Mg⁺⁺, 10 pmol of each forward and reverse primers, 200 mM of dNTPs mix, 2.5 U of Taq DNA polymerase and 3 l of DNA was taken (Senthilkumar *et al.* 2006). The reaction was carried out in a thermal cycler using following standard protocol.

Size of amplified product- 428 bp

Primer used was

Forward-5'GAAACAAAGCGCGGGAGAGA3'

Reverse- 5'ATGGCTTGAGCAATGATAGAT 3'

PCR product was analyzed by electrophoresis on 1% agarose gel after staining with ethidium bromide (0.5 µg/ml) under UV transilluminator. The PCR amplicon was gel purified. For this, 1% preparative agarose gel was prepared and PCR product was electrophoresed to separate the DNA bands. DNA band of specific PCR product was excised and DNA from gel was eluted using a kit. The gel slice containing required DNA band was mixed with 3 volumes of buffer QG and heated for 10 min at 50°C, and 1 gel volume of isopropanol was added and mixed by inverting the tube several times. The mixture was loaded to elute column and centrifuged for 1 min. The column was washed once with 500 ml buffer QG and then 750 ml of buffer PE. The DNA from column was eluted with 20 ml of elution buffer. Concentration and purity of eluted DNA was checked again on 1% agarose gel. PCR products were cloned in T/A cloning vector using pTZ57R/T cloning kit. Approx 25 ng purified PCR product was ligated to 25 ng pTZ57R/T vector using T-4 DNA ligase at 16°C for 2 h followed by 16 h at 4°C. The ligated mixture was used to transform competent DH5a strain of *E. coli*.

For preparing competent cells, frozen culture of *Escherichia coli* strain DH5α was revived at 37°C overnight in LB broth and next day 200 µl of freshly grown DH5α cells was to be inoculated in 20 ml LB medium. The cells were again incubated at 37°C under constant shaking until OD₆₀₀ was between 0.25 and 0.35. The cells were centrifuged at 2,000 ×g for 10 min at 4°C and the pellet was resuspended 1/10 (2ml) volume of TSS buffer and kept on ice for 1 h. After that cell was aliquoted in 1.5 ml microcentrifuge tubes (200 µl each) and stored at -20°C until used.

The ligated product in a 10 µl volume was diluted with TCM buffer to make final volume to 200 µl and kept on ice for 30 min. It was then mixed with 200 µl of competent cells and kept again on ice for 1h. The cells were subjected to heat shock at 45°C for 2 min, followed by immediate chilling on ice for 5 min. Thereafter, 600 µl of SOC medium was added to each tube and cells were incubated at 37°C for 1h under constant shaking. The transformed cells were plated on LB agar plates containing ampicillin (80 µg/ml), IPTG (25 µg/ml) and X-gal (25 µg/ml) and incubated overnight at 37°C. The plates were observed for presence of colonies. The recombinant clones were grown in LB broth and plasmids was isolated.

The white colonies were picked up and inoculated in 3 ml LB broth containing ampicillin (80 µg/ml) and incubated overnight at 37°C under constant shaking. The plasmid DNA was extracted following an improvised alkali lysis method (Birnboim and Dolly 1979). Overnight grown culture (1.5 ml) was pelleted in a microcentrifuge tube at 12,000×g for 30 sec and resuspended in 300 µl of buffer B1 by vortexing. To this bacterial suspension, 300 µl of buffer B2 was added, mixed gently and incubated at room temperature for 5 min. Then 300 µl of chilled buffer B3 was added and lysate was mixed gently and kept in ice for 5 min. After incubation, mixture was centrifuged at 12,000×g for 10 min; the supernatant was transferred gently to another microfuge tube. The supernatant was extracted once with phenol: chloroform by mixing vigorously and centrifuged at 12,000×g for 15 min. The upper aqueous phase containing the plasmid DNA was precipitated with 0.8 volume of isopropanol. The pellet obtained by centrifuging at 12,000×g for 10 min was washed with 70% ethanol, air dried and reconstituted in 40 µl of TE (10mM Tris HCl pH 7.5 and 1mM EDTA). The 428bp fragment released by KpnI and Apal restriction enzyme digestion of recombinant pTZ57R/T was electrophoresed in 1% agarose gel. The products were visualized in long-range UV light and the band representing the NS-1 gene was excised and purified using kit. The pcDNA3.1 (+) vector was also digested with same and KpnI and Apal2020bp restriction enzymes and gel purified as described above. The insert was cloned into pcDNA 3.1(+) eukaryotic expression vector at KpnI and Apal2020bp restriction enzyme site. The recombinant pcDNA cpv NS-1 was selected.

Transfection grade recombinant plasmid containing complete coding sequence of NS-1 gene in right orientation (pcDNA.cpv.NS-1) was prepared by using kit as per the manufacturer's protocol. About 250 ml of overnight grown bacterial culture was pelleted by centrifugation at 6,000 ×g and pellet was resuspended in 10 ml of buffer B1 containing RNase A (25 µg/ml). To this, 10ml of buffer B2 was added and mixed gently by inverting 4–6 times and kept at room temperature for 5 min. After adding 10 ml of chilled buffer B₃, mixed immediately and incubated on ice for 20 min. The tubes were centrifuged at 10,000 ×g for 30 min at 4°C. The supernatant collected was recentrifuged to collect clear supernatant. QIAGEN-tip 500 was equilibrated by applying 10 ml buffer QBT. Supernatant from step 4 was applied to the equilibrated QIAGEN – tip and allowed to enter the resin bed by gravity flow. The column was washed twice with 30ml buffer QC for each wash. The DNA was eluted with 15 ml buffer BF and precipitated by adding 0.7 volumes of isopropanol at room temperature. The sample was centrifuged immediately at 12,000×g for 30 min at 4°C to pellet plasmid DNA. The DNA pellet was withheld with 70% ethanol, air-dried and resuspended in 400 µl of TE buffer.

Physical and clinical symptoms were noticed before and after treatment i.e at 0, first, second, third and fourth week of the treatment. The subjected animals were kept under

observation for 3 months after the end of experiments for untoward symptoms as well as recurrence of tumour. The extent of apoptotic effect of gene therapy was determined by observing and assessing the clinical size of tumour mass and comparison of the regression of tumour mass with their initial base size before treatment. The tumor mass at zero week interval was considered as base value. For histopathological studies the tissues were preserved in 10% natural buffer formalin. Routine paraffin embedding processing and staining by H & E (Luna 1992) was adopted for this study.

Ultrastructural (SEM and TEM) study of tumour cells at different time intervals was undertaken. Degree of ultrastructural differentiation, cytological changes and cellular characteristics were recorded in all animals. A piece of tumour tissue was collected for the scanning electron microscopic study. The samples were immediately fixed in 2.5% glutaraldehyde solution in 0.1M phosphate buffer (pH-7.4) for 24 h. Specimens were washed 3 times in 0.1M phosphate buffer for 5 min each and kept for 2 days at 4° C. Dehydration of specimens was done gradually through increased concentrations of acetone, as 30, 50, 70% up to dry (absolute) alcohol. After dehydration specimens were dried by critical point drying technique. After completion of drying process specimens were mounted on the aluminum stubs with adhesive tape. Gold coating on the specimen was done after mounting. Scanning electron microscopic studies were carried out using JSM-6610LV scanning electron microscope operated at appropriate acceleration voltage and magnification range. A portion of previously fixed tissue sample was processed and semi thin section was stained with toluidine blue and optimal areas thin section was selected. Thin section was stained in uranyl acetate and lead citrate and transmission electron microscopic studies were made with appropriate acceleration voltage and magnification range.

RESULTS AND DISCUSSION

Out of 10 mammary gland tumours single nodules were found in 7 and multiple nodules in 3 cases. Seven mammary tumour masses were irregularly hard and 3 were soft in consistency. Most of mammary gland tumours were found in glands 4 (5), 5 (2), 3 (1) and in 1(2). The present findings related with the location of mammary gland tumours were also supported by Goldschmidt *et al.* (2011) as the majority of mammary gland tumours occur in glands 4 and 5 (60–70%), possibly due to the fact that the 2 most caudal pairs of glands contain the most mammary tissue. *NSI* gene had positive impact on regression of all 10 mammary gland tumours, in which 6 mammary gland tumours were completely regressing, 3 cases were moderately regressed and 1 case was partially regressed. In completely regressed cases, 3 cases were regressed after 3 weeks of treatment, and remaining 3 cases regressed after fourth week of gene administration (Figs A to F). Reduction in mammary gland tumour was started rapidly after first injection of *NSI* gene but after 4 weeks of treatment the remarkable regression of

tumour growth was noticed. On physical examination, 3 animals showed 70% reduction in tumours growth after 2 weeks and after third week, they showed complete regression of tumor mass and other 3 cases took 4 weeks for complete regression. A comparatively slow rate in the regression process of tumour growth was also recorded in 1 case. Follow up of the entire regressed tumours for 3 months. No untoward effect of gene therapy was observed in any animal. Very limited work has been reported in regard to regression effect of *NSI* gene in canine mammary gland tumors. Priyanka *et al.* (2012) have reported that VP3 gene of CAV induces apoptosis in malignant canine mammary tumour (CMT) cells only. *NSI* gene of canine parvovirus plays important role in prevention of tumor establishment, thus prolonging the life of tumour bearing animals (Cornelis *et al.* 2006).

Histopathological examination of biopsy sample of tumour at 0 week interval revealed more or less similar structure. The cells appeared as confluent arranged oval or round shape neoplastic cells clustered uniformly in a sheet or ribbon like pattern. Multifocal areas of haemorrhages were also noticed in few histopathological sections. The cells were closely packed together with the strands of fibrous connective tissue. The nuclei of tumour cells were centrally placed, large, round to oval in shape, granular and vesicular with the prominent nucleolus. The nuclear cytoplasmic ratio of tumour was more. The cytoplasm of tumour cells was faintly basophilic, vacuolated and moderate in amount. In high power magnification, the prominent nuclei with frequent mitotic figure were observed. The few mononuclear cells were also infiltrated throughout the tumour parenchyma. Besides above findings variable sized mammary gland alveoli with compact luminal spaces with tumorous cells and scanty secretions also seen (Fig. 1). Intervalveolar spaces were seen with hyperchromatic nuclear fibrous tissues. There was prominent infiltration of lymphocytes, plasma cells and few macrophages. Mitotic figures were also seen. The presence of round individual cells in an arborizing fibrovascular network could help in diagnosing the tumour histologically. These observations are in accordance with the findings of Krithiga *et al.* (2005). The nucleus of the tumour cells was round to oval in shape and centrally placed. Anisonucleoliosis was prominent in the nucleus of the tumour cells. The nucleoli were basophilic and the number varied from 1 to 3. The nuclear chromatin pattern was coarse to reticulate (Thangathurai *et al.* 2008). Histopathological examination at fourth week interval showed a large area of necrosis of neoplastic tissue. The regressing tumours alveoli are seen with space occupying new connective tissues with collagen and fibroblast. Neoplastic cells were highly vacuolated with oval or round, large or small nucleus with coarse and granular chromatin and the interlaced intercellular collagen fibres. The nucleus was absent in most of the degenerative places of necrosed area. In high power magnification, the cytoplasm was abundant with vacuolation and had low mitotic figures. At multiple places, there was necrosis of individual or group

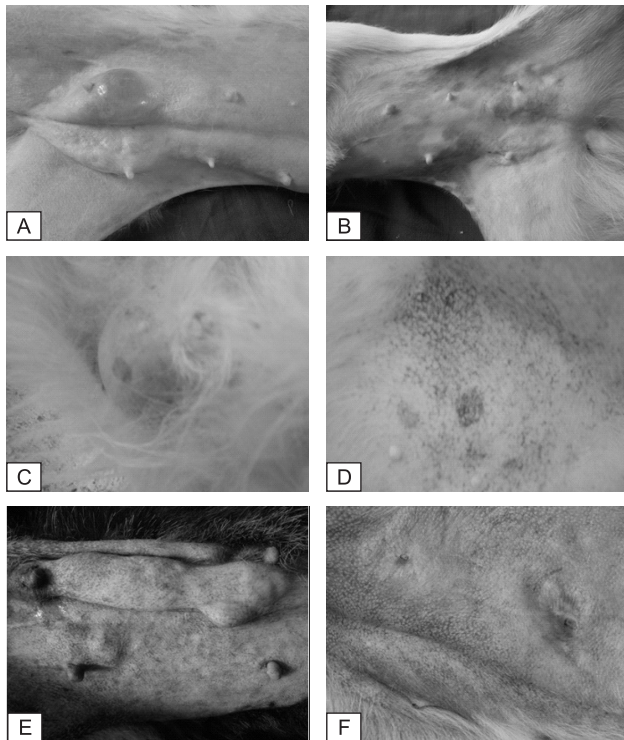


Fig A–F. A. Before treatment. B. After treatment. C. Before treatment. D. After treatment. E. Before treatment. F. After treatment.

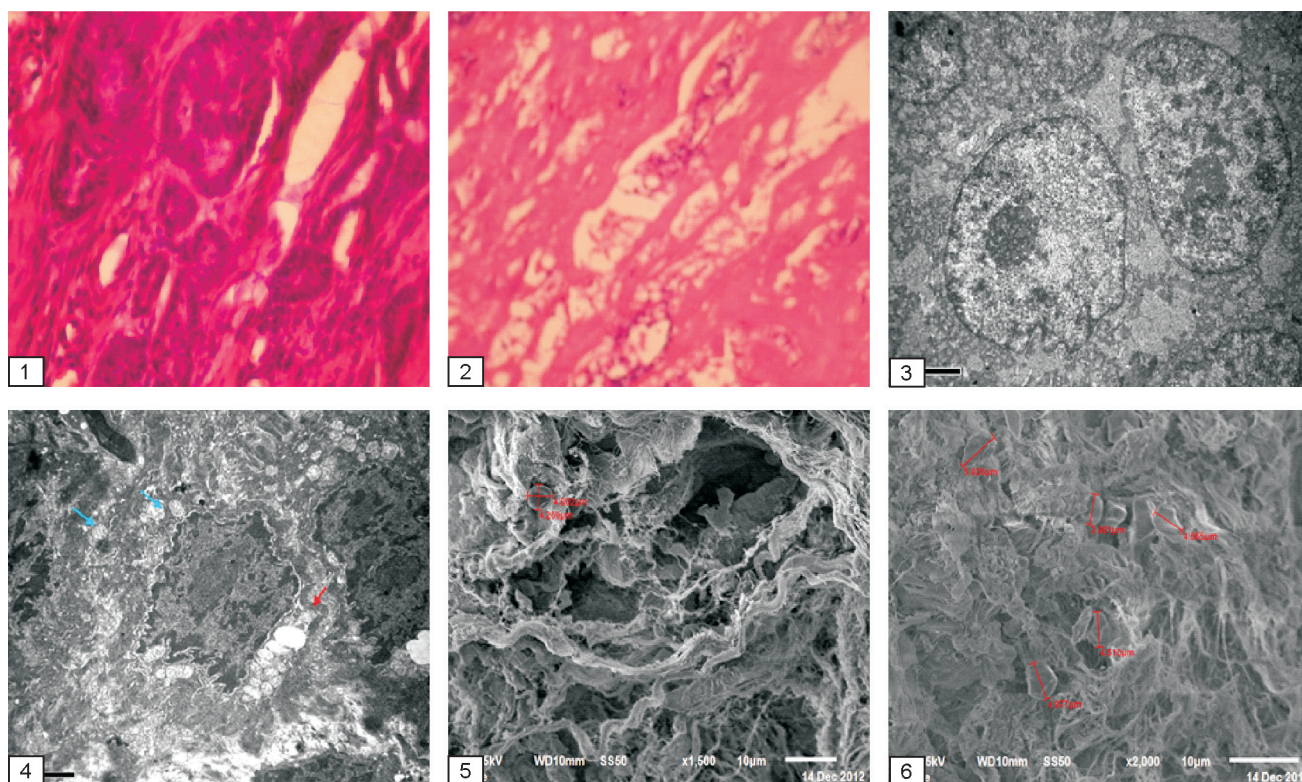
of neoplastic cells, which appeared as irregular empty spaces seen throughout the section of tumour. The number of apoptotic cells was more in comparison to biopsy sample of 0 week intervals (Fig. 2). Presence of shrunk isolated apoptotic cells, with a condensed chromatin and fragmented nucleus and apoptotic bodies were also observed. Abundant inter-cellular stromal collagen accumulation was revealed in the progressive regression of tumour mass. At some places newly forming blood vessels with mature connective tissue were seen in histopathological section of the animals.

Ultrastructural examination of the tissue revealed a monotonous and confluent population of round and less commonly elongated cells admixed with stromal elements. The nucleus was seen with irregular margin and the nuclear integrity was lost with karyorrhexis (destructive fragmentation of the nucleus of a dying cells whereby its chromatin is distributed irregularly throughout the cytoplasm), it is usually preceded by pyknosis and is followed by karyolysis. The nucleolus has a nucleolemal pattern of pars amorpha (the part of the nucleolus that occupies irregular spaces in the nucleolonema and contain finely filamentous matter) with few endoplasmic reticulum. The rough endoplasmic reticulum desintegrated and ruptured. The rough endoplasmic reticulum was seen with cloudy inner substances. The RER mass is compact and seen in groups (normally spaces present between them). The scanty groups of ribosomes were seen attached with corners of rough endoplasmic reticulum.

The cytoplasm was seen granular and vacuolar indicating degeneration of the cells. Cytoplasm of regressing tumour

cells was seen with numerous vacuoli with granular homogenous streak of cytoplasm containing disintegrating mitochondria with few cloudy cytoplasmic substances. The cell density increased when the round cell become tightly clustered. The cytoplasm contained mitochondria with a few irregular cristae, abundant rough endoplasmic reticulum, polyribosomes, Golgi apparatus, occasional lipid droplets and numerous tubular and vesicular profiles. Occasionally, microvilli were observed, more easily in the areas where cells were less cohesive. Cells, considered to degenerate, had a darker cytoplasm, swollen mitochondria and endoplasmic reticulum, a widened perinuclear space and coarsely clumped chromatin. The early degenerative neoplastic cell was determined by cytoplasmic changes. In the degenerating cells, there were few ribosomes and a diminished amount of endoplasmic reticulum. However in advanced stages, the granular ER formed an extensive network throughout the cell. The cisternae of the granular ER was continuous in many areas with a prominent perinuclear space. Degenerating neoplastic cells had an increased clumping of nuclear chromatin along the nuclear envelope and a widened dilated perinuclear space. Disintegrating nucleous with nuclear blebs (irregular buldge in the plasma membrane of nucleous) and irregularly distributed nucleoplasm and nucleolus with vacuoles. Variable size mitochondria were seen with constricted endoplasmic reticulum and disintegrating golgi bodies with free ribosomes in the cytoplasm. The Ultrastructural studies revealed the presence of both healthy and degenerating tumour in all the neoplasm with variable figure. The healthy round neoplastic cells were more at 0 week in comparison to second and third week of studies.

In degenerative stages, the tumour was observed to be composed of tumour cells, macrophages, lymphocytes plasma cells, stroma of reticular cells and blood vessels. Generally, the neoplastic cells have large nuclei with irregular and most of them have invaginated nuclear membrane. The nucleoplasm was homogeneous except where the chromatin was condensed against the border of the nuclear membrane. A large characteristic nucleolus was also present. In the cytoplasm, ribosomes were observed either in free or in the form of polysomes and were irregularly encountered. A few mitochondria observed in various numbers, were round to oval and swollen with few thin cristae. Large electron dense granules were occasionally observed. The Golgi body was small, paranuclear, and associated with centrioles. Vacuolation as well as electron dense granules were observed in the cytoplasm. The surface of the cells had a characteristic arrangement of microvilli which gave the impression of multilayered membranous system between the cells. A few spindle shaped cells were observed with 2 highly electron dense nucleolus and prominent swollen endoplasmic reticulum. The cytoplasm was characterized by moderate to severe vacuolation of tubules of endoplasmic reticulum and Golgi apparatus. Clustered ribosomes were occasionally observed. The cisternae of the granular ER were continuous in many areas



Figs 1–6. 1. Variable sized mammary gland alveoli (black arrow) with compact luminal spaces with tumorous cells at 0 week. 2. Regression of tumorous tissue around mammary gland alveoli with space occupying new connective tissues with collagen and fibroblast at fourth week. 3. Microphotograph showing the degenerating cells, having few ribosomes and a diminished amount of endoplasmic reticulum at 0 week. 4. Microphotograph showing vacuolation (blue arrow) as well as electron dense granules (red arrow) were observed in the cytoplasm at fourth week. 5. Microphotograph showing irregularly sized mammary tumour cells wrapped in a mesh of fibrous connective tissues at 0 week. 6. Microphotograph showing increased vascular CT in form of RBC (4.1–4.65 μm) seen from engorged blood vessels in the fibrous CT with few alveolar packets and some tumorous cells at fourth week.

with a prominent perinuclear space. Mitochondria appeared as double membrane bound vacuoles without cristae. Mitochondria were observed to be more swollen. Electron dense membrane bound granules were frequently observed. In degenerative tumour cell the mitochondria was observed as round to oval with few cristae which showed variable degrees of swelling generally characteristic of tumour cells.

TEM is considered the gold standard to confirm the apoptosis. This is because of categorization of an apoptotic cell, which is irrefutable if the cell contain ultrastructural morphological characteristics. These characteristics include electron dense nucleus (marginalization in the early phase), nuclear fragmentation, and intact cell membrane even in the cell disintegration phase, disorganized cytoplasmic organelles, large clear vacuole and bleb in the cell surface. As apoptosis progress, these cells lose the cell to cell adhesion and separate from neighboring cells. During later phase of apoptosis, the cell gets fragment into apoptotic bodies with intact cell membrane and contains cytoplasmic organelles with or without nuclear fragments. Phagocytosis of apoptotic bodies was also be appreciated with TEM. Purohit (2009) has also reported that tumours in progressive stage have round cells with microvilli while regressing tumours had transitional fusiform cells.

SEM image revealed that highly entangled messy pattern

of fibrous connective tissues was seen in a mature tumour mass. The irregularly sized mammary tumour cells wrapped in a mesh of fibrous connective tissues. Tumours cell are seen with finger like blebs which indicate apoptotic changes. Densely entangled numerous variety of fibrous connective tissues is seen with few apoptotic tumour cells in between. Variable sized luminal accini are seen with dense intervoven fibrous connective tissues in between. The increased vascular connective tissue is seen from engorged blood vessels in the fibrous connective tissues after tumour regression with few alveolar packets. The spaces surrounded by fibrous connective tissues are indicative of wearing off of regressed tumour masses. Apoptosis was revealed by variety of morphological changes including blebbing and changes in cell membrane. The apoptotic cells have lost its cell membrane asymmetry and attachment. The shrinkage of healthy impact cell was also observed. The spindle shape structure of tumour cells in the present study was due to its advancement of age. During regression of tumour cells, the evidence of cellular degradation into fibroblast like cell was also observed by Saha *et al.* (2013).

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