

# HETEROLOGOUS TRANSPLANTATION OF BOVINE ETHMOID CARCINOMA CELLS USING CYCLOSPORINE-A\*

Ajith Jacob George<sup>1</sup>,  
K.M. Ramachandran<sup>2</sup>, A. Rajan<sup>3</sup>,  
K.T. Punnoose<sup>4</sup> and C.B. Manomohan<sup>5</sup>

Krishi Vigyan Kendra, Kerala Agricultural University  
Sadanandapuram, Kottarakkara 691550  
Kollam, Kerala

The prevalence of carcinoma of the ethmoturbinate was reported in cattle from many parts of India. Tumour transplantation studies have great importance in experimental cancer research, which could be used for studying information regarding cell kinetics of the neoplastic cells.

Borel and Merzados (1980) showed that cyclosporine-A (CS-A) acts on immunocompetent T cells in the very early stage of lymphocyte stimulation and this effect was reversible. Galfande *et al.* (1987) observed that CS-A depressed the IL-2 induced proliferation of IL-2 receptor expressing cells. Gunn *et al.* (1981) showed that CS-A has a preferential effect on T cell-mediated immunity. White *et al.* (1979) observed that CS-A inhibited proliferation of porcine T lymphocytes, lymphocytes which were not proliferating and leukocyte migration. The study was undertaken to assess the heterologous transplantability of bovine ethmoid carcinoma cells using CS-A.

## Materials and Methods

Seven cattle bearing tumour of the ethmoturbinate mucosa, twenty weaned white rats (Wistar) and white mice (Swiss albino) each and twelve weaned New Zealand white rabbits were utilized for the study.

CS-A was administered orally to ten mice and ten rats at the rate of 15 mg/kg body weight for 14 days and to six rabbits orally for seven days at the rate of 30 mg/kg body weight. Administration of CS-A commenced twenty four hours prior to transplantation. Six rabbits and ten each of mice and rats were maintained without immunosuppression as control group.

Hank's balanced salt solution (Hi-Media) (HBSS) and TC 199 media (Hi-Media prepared as per the manufacturers' direction by dissolving in deionized double distilled water and filtered through 0.2 micro membrane filter (Sartorius) under positive pressure were used as maintenance and culture media respectively. Culture media was supplemented with 10 per cent foetal calf serum (CSIR, New Delhi). The antibiotic mixture added contained penicillin G – 200 IU/ml, streptomycin – 150 mg/ml, gentamycin – 50 mg/ml and nystatin – 100 IU/ml were used.

Trypsin (0.25 per cent) (1:250 Difco) in phosphate buffered saline (Ca and Mg free) (Hi-Media) (PBS) was prepared and sterilized by filtering through 0.2 micro membrane filter.

\* Part of M.V.Sc. thesis submitted by the first author to KAU, Thrissur

<sup>1</sup>. Assistant Professor

<sup>2</sup>. Since deceased;

<sup>3</sup>. Dean (Retd.), CoVAS., Mannuthy

<sup>4</sup>. Professor & Head (Retd.), Dept. of Veterinary Microbiology, CoVAS., Mannuthy

<sup>5</sup>. Associate Director of Extension (CZ), Mannuthy, KAU

All the seven tumour bearing cows were euthanised by exsanguination after stunning with captive bolt pistol. Fresh soft healthy tumour tissue was collected under sterile condition from the deeper portion avoiding the necrotic area. The tumour tissue was collected in HBSS with antibiotics. A part of the tumour tissue was taken in 10% formalin for histopathology.

The tumour tissue was washed several times using PBS containing antibiotics, to remove the debris. It was transferred into a Petri dish containing PBS. The superficial fascia was removed from the tumour mass. The tissue was then cut into small cubes of one mm size and washed several times with PBS.

A few cubes of the tumour tissue were transferred into a beaker containing 100 ml of 0.25 per cent trypsin solution in PBS. The beaker was placed on a magnetic stirrer and stirred for 10 minutes using a magnetic stirring paddle. The supernatant was decanted and replaced with fresh 100 ml of 0.25 per cent trypsin and again stirred using magnetic stirrer for another 10 minutes. Foetal calf serum (three ml) was added to the suspension to neutralize the trypsin. The suspension was sieved through a double layered sterile muslin cloth into a sterile flask. The suspension was transferred to a centrifuge tube and was centrifuged at 1000 rpm for five minutes. The supernatant was poured off and the cell pellet was suspended in media with antibiotic. The viable cell concentration was adjusted to  $1 \times 10^6$  viable cells per 0.25 ml after estimating live cell concentration by trypan blue staining.

Single cell suspension obtained by trypsinization was injected subcutaneously into all experimental animals, at the rate of  $1 \times 10^6$  viable cells per inoculum. The thickness of the skin was measured immediately and weekly after inoculation. The site of inoculation was observed visually and by palpation daily from the third day onwards after inoculation. All the experimental animals were observed for one month. All animals were sacrificed after the observation period. The site of inoculation, spleen, lungs, heart, liver and kidney were taken for histopathology. At the time of sacrifice blood was collected in EDTA for total leukocyte count and lymphocyte percentage calculation.

## Results and Discussion

The tumour tissue for transplantation was obtained from seven affected cows. The ethmoid tumour specimens were diagnosed as adenocarcinoma. The gross and histopathological findings confirm that the tumour was a primary cancer arising from the ethmoid turbinate mucosa. Similar observations were made by Chakrabarthy *et al.* (1988); Muralimanohar (1988) and Gangadharan (1992).

Control animals revealed local oedema at the inoculation site twenty-four hours after transplantation and attained maximum size by the first week. It gradually decreased in size and completely disappeared after the second week as shown in Table (1). Histopathologically, in the inoculated site of all the control animals there was congestion of subcutaneous blood vessels and infiltration with a few lymphocytes and macrophages. Neoplastic cells were not

Table 1. Skin thickness after transplantation

Weeks	Mice		Rat		Rabbit	
	Immuno-suppressed (mm)	Control (mm)	Immuno-suppressed (mm)	Control (mm)	Immuno-suppressed (mm)	Control (mm)
0	0.040 $\pm$ 0.006	0.058 $\pm$ 0.019	0.140 $\pm$ 0.006	0.139 $\pm$ 0.008	0.166 $\pm$ 0.004	0.157 $\pm$ 0.005
1	0.043 $\pm$ 0.006	0.059 $\pm$ 0.005	0.136 $\pm$ 0.007	0.142 $\pm$ 0.008	0.151 $\pm$ 0.003	0.159 $\pm$ 0.002
2	0.052 $\pm$ 0.004	0.062 $\pm$ 0.053	0.133 $\pm$ 0.005	0.137 $\pm$ 0.006	0.153 $\pm$ 0.003	0.145 $\pm$ 0.004
3	0.066 $\pm$ 0.005	0.065 $\pm$ 0.005	0.132 $\pm$ 0.005	0.133 $\pm$ 0.013	0.153 $\pm$ 0.004	0.146 $\pm$ 0.004
4	0.074 $\pm$ 0.004	0.067 $\pm$ 0.084	0.132 $\pm$ 0.150	0.131 $\pm$ 0.020	0.154 $\pm$ 0.003	0.148 $\pm$ 0.005

detected at the site of inoculation. The lack of tumour growth in control animals is due to the destruction of the neoplastic cells by the host immune cells as observed microscopically by infiltration of inflammatory cells at the site.

The total leucocyte count and lymphocyte percentage are shown in Table (2 and 3).

**Table 2.** Total leucocyte count ( $\times 10^3/\text{mm}^3$ )

Species	Immuno suppressed	Control
Rat	$2.17 \pm 0.39$	$13.28 \pm 0.58$
Mice	$2.65 \pm 0.32$	$9.72 \pm 0.28$
Rabbit	$2.97 \pm 0.62$	$10.65 \pm 0.58$

**Table 3.** Lymphocyte percentage

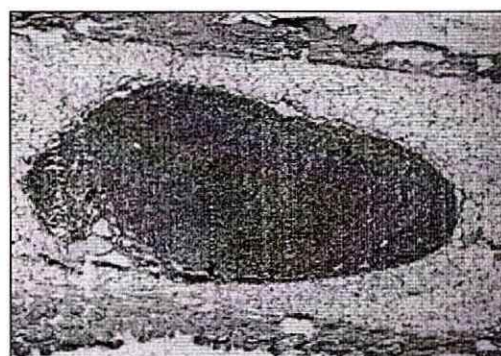
Species	Immuno suppressed	Control
Rat	$31.70 \pm 0.47$	$72.60 \pm 1.39$
Mice	$33.60 \pm 0.52$	$64.90 \pm 4.75$
Rabbit	$33.83 \pm 0.79$	$53.10 \pm 0.54$

There was significant difference between the control and immunosuppressed animals in these parameters. The total leucocyte count in all the immunosuppressed animals was very low when compared to the control. The lymphocyte percentage of the immunosuppressed animals was very low when compared to the control. Among the different animals treated with CS-A, rats had the lowest lymphocyte percentage than mice and rabbits. The eosinophil percentage did not reveal any variation from the normal. Basophil count in all the cases was zero.

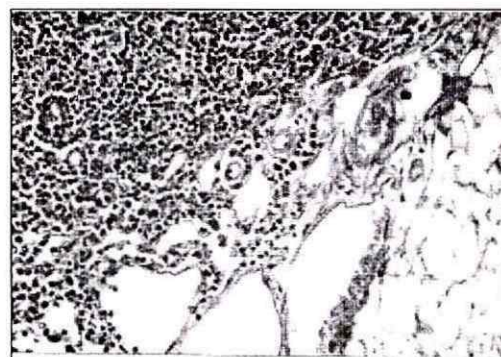
In all immunosuppressed heterologous transplantations, the initial swelling completely disappeared within twenty-four hours. There was no evidence of tumour growth except in mice immunosuppressed with CS-A. The growth was a small fluctuating firm nodule by the end of second week. The nodule increased in size progressively and by the end of the fourth week it was easily palpable.

In eight mice immunosuppressed with CS-A and inoculated with trypsinised single cell suspension of adenocarcinoma, neoplastic foci were identified microscopically. A round or oval zone of proliferating tumour cells was seen embedded in the

subcutaneous fat of mice (Fig. 1). In all the cases the proliferating tumour mass were encapsulated by a very thin layer of fibrous tissue and were always in close proximity to the blood vessels. All the tumours were well vascularised from the murine epidermis, with few blood vessels within the proliferating tumour mass (Fig. 2).



**Fig. 1.** Transplanted tumour growth in mice



**Fig. 2.** Tumour vascularization

The tumour growth was well differentiated with diffuse acini formation. The acini were lined by a single layer of columnar cells with a basally placed hyperchromatic vesicular nucleus. The cells in the area without acini were polygonal in shape with hyperchromatic vesicular nucleus. Pleomorphism was also observed. The cells were in varying degrees of mitosis. Binucleated cells were also observed. The underlying musculature was free of invasion by the neoplastic cells.

Thus, successful transplantation of ethmoid carcinoma cells was achieved for the first time. It was successfully repeated in a second attempt using adenocarcinoma cells

from a different donor under the same conditions establishing the transplantability of the tumour.

Karki and Rajan (1986) attributed various factors like type of host, age of host, immune status of the recipient, type of tissue preparation, type of tumour, inoculation rate, viability of the cells and route of inoculation which would determine the transplantability of tumour.

In the present study, the experimental lot consisted of young animals which were naturally at a lower immunological competency. Further, they were immunosuppressed with CS-A. The observation that the tumour could be successfully transplanted only in mice treated with CS-A supports the assumption that immunosuppression is a prerequisite for the development of neoplasms. Lowering of the immunological barrier of the host therefore appears to be an important event in establishing neoplastic growth.

It has been suggested that adenocarcinoma is a less differentiated neoplasm than a squamous cell carcinoma. This observation supports the view that transplantability of neoplastic cells is inversely proportional to its differentiation. The difference in transplantability could either be due to the difference in the trypsin-cell interaction or due to the absence of certain factors present in the original host but lacking in the recipient.

Price *et al.* (1990) observed that at high doses of  $10^6$  cells and  $5 \times 10^6$  cells tumour developed in all the experimental mice whereas the lower dose of  $10^5$  cells produced tumour in two out of five mice only. The results of the present study is in agreement with their observation that a dose rate of  $10^6$  cells was able to produce tumour growth in 8 out of 10 mice inoculated with trypsinised ethmoid tumour cells suspension.

Watanabe *et al.* (1980) suggested that subcutaneous route was more effective than I/P or I/V routes of inoculation probably due to better blood supply and presence of connective tissue framework for fixation and proliferation of the inoculum.

It has also been reported that some tumours fail to grow when inoculated subcutaneously (Al-Yamen and Willenborg, 1984) probably due to factors such as lack of proper vascularisation or to the lack of essential factors required for tumour growth, which is present in the original host but not in the recipient mice. The tumour transplant obtained in the present study revealed numerous capillaries indicating a moderate degree of vascularisation which might have facilitated in the proliferation and growth of the tumour cells.

The attempts for heterologous transplantation of bovine ethmoid carcinoma in rats and rabbits did not succeed. Histopathological examination of the site of inoculation of immunosuppressed rats and rabbits did not show any evidence of neovascularization or neoplastic cells.

The immune system of all experimental hosts might not have interfered with the growth of the inoculum since they were immunosuppressed. Microscopically, the periarteriolar lymphoid sheath of the spleen system of all immunosuppressed hosts contained only scanty lymphocytes when compared to that of the control. Red pulp was abundant than the white pulp. Germinal centers were not seen in immunosuppressed animals. The lack of inflammatory reactions as well as lymphoid aggregation at the site of inoculation, the low total leucocyte count and lymphocyte percentage and the extensive depletion of lymphocytes in periarteriolar region of the spleen of immunosuppressed animals support the suggestion of suppressed immune system.

The successful repeatable transplantation of bovine ethmoid carcinoma cells, subcutaneously into mice has established the transplantability of those cells and can be utilized as an experimental model for the detailed study of the biology and chemosensitivity of these cells.

## Summary

A study was undertaken to transplant bovine ethmoid tumour cells into different heterologous species of animals using CS-A. Transplanted tumours offer an excellent model system for evaluating immunoprophylactic measures, chemosensitivity and tumour metastasis. Trypsinized single cell

suspension of fresh bovine ethmoid tumour was inoculated subcutaneously into immuno-suppressed rats, mice and rabbits. The animals were observed for 30 days. At the end of the period the animals were sacrificed and site of inoculation was taken for histopathology. Transplantation was attained for the first time. The tumour growth was observed as an oval to round mass embedded in the subcutaneous fat. The haematological studies were also due to evaluate the immuno-competency of the animals.

## References

- Al-Yamen, F. and Willenborg, D.O. 1984. Heterotransplantation of ovine squamous cell carcinoma into nude mice. *Res. Vet. Sci.*, **36**: 339-344.
- Borel, J.F. and Merzados, J. 1980. Skin transplantation in mice and dogs. Effect of cyclosporin A and dihydrocyclosporin C. *Transplantation*, **29**: 161-162.
- Chakrabarthy, A., Nayak, M.C. and Maity, B. 1988. Tumours of the ethmoid region in cattle and West Bengal. *Indian. Vet. J.*, **65**: 1132-1133.
- Galfande, E.W., Chaung, R.K. and Mills, G.B. 1987. The cyclosporin inhibit lymphocyte activation at more than one site. *J. Immunol.*, **138**: 1115-1120.
- Gangadharan, B. 1992. As assessment of the biological characteristics of the neoplastic cells of ethmoid carcinoma in cattle. *M.V.Sc. thesis*, KAU, 93 p
- Gunn, H.C., Varey, A. and Cooke, A. 1981. Effects of cyclosporin A on the function of T cells. *Transplantation*, **32**: 338-340.
- Karki, M.S. and Rajan, A. 1986. Transplantation studies on the carcinoma of ethmoturbinate mucosa of cattle. *Kerala. J. Vet. Sci.*, **17**: 74-84.
- Muralimanohar, B. 1988. Studies on ethmoidal neoplasm in animals. *Ph.D. thesis*, Tamil Nadu Veterinary and Animal Sciences University, Chennai, 248 p.
- Price, J.E., Polyzos, A., Zhang, R.D. and Daniels, L.M. 1990. Tumerogenicity and metastasis of human breast carcinoma cell lines in nude mice. *Cancer. Res.*, **50**: 717-721.
- Watanabe, S., Shimosato, Y., Kuroki, M., Sato, Y. and Nakajima, T. 1980. Transplantability of human lymphoid cell line, lymphoma and leukemia in splenectamized and/or irradiated nude mice. *Cancer. Res.*, **40**: 2588-2593.
- White, D.J.G., Plumb, A.M., Paevelec, G. and Brons, C. 1979. Cyclosporin A: an immunosuppressive agent preferentially active against proliferating T cells. *Transplantation*, **27**: 55-58.

