EXPRESSION OF ALDEHYDE DEHYDROGENASE 1 AND TNF-ALPHA IN BUFFALO MAMMARY GLAND

Thesis

Submitted to the Guru Angad Dev Veterinary and Animal Sciences University in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE
in
BIOTECHNOLOGY
(Minor Subject: Veterinary Microbiology)

By

Sheetal Thakur
(L-2016-ABT-07-M)

School of Animal Biotechnology
Post Graduate Institute of Veterinary Education and Research

Ludhiana - 141004

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CERTIFICATE – I

This is to certify that the thesis entitled, "**EXPRESSION OF ALDEHYDE DEHYDROGENASE 1 AND TNF-ALPHA IN BUFFALO MAMMARY GLAND**" submitted for the degree of **M.Sc**. in the subject of **Biotechnology** (Minor subject: **Veterinary Microbiology**) of the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, is a bonafide research work carried out by **Sheetal Thakur (L-2016-ABT-07-M)** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

(Dr. Ratan K. Choudhary)
Major Advisor
Assistant Professor
School of Animal Biotechnology
Guru Angad Dev Veterinary and Animal Sciences University
Ludhiana – 141 004, Punjab

CERTIFICATE – II

This is to certify that the thesis entitled "**EXPRESSION OF ALDEHYDE DEHYDROGENASE 1 AND TNF-ALPHA IN BUFFALO MAMMARY GLAND**" submitted by **Sheetal Thakur (L-2016-ABT-07-M)** to the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, in the partial fulfillment of the requirements for the degree of **M.Sc.** in the subject of **Biotechnology** (Minor subject: **Veterinary Microbiology**) has been approved by student's Advisory Committee after an oral examination on the same, in collaboration with an external examiner.

(Dr. Ratan K. Choudhary)
Major Advisor

(Dr. A.K. Mohanty)
External Examiner
Principal Scientist
Animal Biotechnology Centre
NDRI, Karnal, Haryana-132 001

(Dr. Ramneek Verma)
Director
School of Animal Biotechnology

(Dr. Sanjeev Kumar Uppal)
Dean, Postgraduate Studies
Guru Angad Dev Veterinary and
Animal Sciences University, Ludhiana

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Place: Ludhiana

Date: ***Sheetal Thakur***

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ABSTRACT

Knowledge of cellular and molecular profile of buffalo mammary glands is limited in spite of buffalo being the most important dairy animals of India. This thesis explores the changes in cellular composition of buffalo mammary glands collected from abattoir and examines the protein expression and gene expression studies of aldehyde dehydrogenase 1 (ALDH1), hepatocyte nuclear factor 4A (HNF4A) and tissue necrosis factor alpha (TNFA). ALDH1 and HNF4A are the mammary stem cell markers and TNFA is the inflammatory marker. Immunohistological analyses and gene expression studies using real time quantitative PCR of buffalo mammary tissues revealed mastitis tissue has a high expression of ALDH1 and TNFA than the lactating tissue. Putative marker of buffalo mammary stem cell, HNF4A expression (protein plus gene level) was reduced in mastitis. Although, the level of expression of these genes may vary, depending upon the physiological stage of the animals, however high expression of ALDH1 and TNFA may relate to hyperplasia and chronic mastitis. We conclude that, ALDH1 and TNFA expression was increased and HNF4A expression was decreased during naturally infected buffalo mastitis. A systematic study taking large population of buffalo may be investigated to evaluate expression of these markers for detection of mastitis as a molecular diagnostic tool.

Keywords: Buffalo, Mastitis, ALDH1, TNFA, HNF4A

Signature of Major Advisor **Signature of the student**

CONTENTS

CHAPTER	TOPIC	PAGE NO.
I	INTRODUCTION	1 – 3
II	REVIEW OF LITERATURE	4 – 10
III	MATERIALS AND METHODS	11 – 19
IV	RESULTS AND DISCUSSION	20 – 30
V	SUMMARY	31 – 32
	REFERENCES	33– 36
	ANNEXURE	
	VITA	

LIST OF TABLES

Table No.	Title	Page No.
1	Composition of phosphate-buffered saline (PBS) (10X)	13
2	Composition of Tris-EDTA Buffer (10x)	13
3	Primary antibody used for immunostaining of buffalo mammary tissue and their working concentration	15
4	Gene-specific primers pair sequences for RT-qPCR amplification, their melting temperature, and product sizes	18
5	Two independent t-tests showing the difference in number of mammary epithelial cells (MEC) and myoepithelial cells (MYO) between non-lactating and lactating buffalo mammary glands	21
6	F-test showing significant effect of physiological stages of the animal on immunolocalization of ALDH1 in buffalo mammary tissue	23
7	Tukey's test showing multiple comparison among physiological stages of the animal on immunolocalization of ALDH1 in buffalo mammary tissue	24
8	F-test showing significant effect of physiological stages of the animal on immunolocalization of ALDH1 in buffalo mammary tissue	24
9	Tukey's test showing multiple comparison among physiological stages of the animal on immunolocalization of TNFA in buffalo mammary tissue	25
10	Two-way ANOVA table showing the effect of stage and markers on the dCt value	28
11	Two-way ANOVA multiple comparison table showing the effect markers on the dCt value	29

LIST OF FIGURES

Fig. No.	Title
1	Tree-like structure of the mammary glands of ruminant with developed alveolar structure
2	Electron micrograph of cultured murine pale-staining mammary epithelial cell
3	A working model of bovine mammary stem cell hierarchy
4	Quantification of immuno-positive signals of ALDH1 and TNFA staining in buffalo mammary tissue
5	A Real time thermocycler used in gene expression analysis
6	Hematoxylin and eosin (H&E) staining of buffalo mammary glands
7	Immunostaining of PCNA in lactating and mastitis glands of buffalo. a- mastitis gland; b –lactating gland
8	Quantification of cells and cellular components of buffalo mammary gland during non-lactation and lactation stage.
9	ALDH1 staining (red staining) of buffalo mammary glands
10	TNFA staining (red staining) of buffalo mammary glands
11	HNF4A staining (red staining) of buffalo mammary glands
12	Quantification of immuno-positive signals of ALDH1 and TNFA staining in buffalo mammary tissue
13	Ultrastructure of lactating buffalo mammary glands
14	Estimation of RNA quality and concentration using agarose gel electrophoresis and NanoDrop spectrophotometer
15	Amplification plot and melt curve analysis of expressed genes (RPS23, HNF4A, ALDH1 and TNFA) in buffalo mammary glands
16	Sample-wise relative quantification of ALDH1, HNF4A and TNFA in buffalo mammary glands
17	Estimated marginal means of delta Ct (dCt) value of ALDH1, HNF4A and TNFA in the buffalo mammary glands
18	Fold change (log 2) expression of ALDH1, HNF4A and TNFA in buffalo mammary tissue

LIST OF ABBREVIATIONS

AD	:	Alveolar debris
AL/DL	:	Alveolar lumen/ ductal lumen
ALDH1	:	Aldehyde dehydrogenase 1
ANOVA	:	Analysis of variance
DNA	:	Deoxyribounucleic acid
ECM	:	Extra cellular matrix
EDTA	:	Ethylene diamine tetra acetic acid
EMT	:	Epithelial-mesenchymal transition
FACS	:	Fluorescent activated cell sorting
HNF4A	:	Hepatocyte nuclear factor 4 alpha
IAEC	:	Institutional animal ethical committee
IGF-1	:	Insulin like growth factor -1
LYM	:	Lymphoctyes
MEC	:	Mammary epithelial cells
MFG	:	Milk fat globules
MYO	:	Myoepithelial cells
NCBI	:	National Center for Biotechnology Information
ND	:	NanoDrop
PBS	:	Phosphate buffer saline
PCNA	:	Proliferating cell nuclear antigen
PCR	:	Polymerase chain reaction
PMN	:	Polymorph nuclear
RER	:	Rough endoplasmic reticulum
RGB	:	Red, green and blue
RNA	:	Ribonucleic acid
RT	:	Room temperature
SD	:	Stromal debris
SE	:	Standard error
SPSS	:	Statistical Package for the Social Sciences
TEM	:	Transmission electron microscope
TNFA	:	Tissue necrosis factor alpha

CHAPTER I

INTRODUCTION

Milk is synthesized and secreted through mammary gland located in the udder. Udder is formed of teats, ducts and alveoli. Alveoli is the functional unit of mammary gland that secrete milk into the lumen. Alveoli (singular alveolus) contain two layers of mammary epithelial cells namely luminal epithelial layer and basal epithelial cells, surrounded by myoepithelial cells and stroma. The mammary epithelial cells extract the nutrients from the blood present in stroma and convert it into milk that finally released into alveolar lumen by the squeezing action of myoepithelial cells. The progress of lactation can signify by huge change in number and structure of mammary gland secretary cells (Capuco *et al* 1997). On the onset of lactation, mammary epithelial cells undergo differentiation to produce milk. In the rodents and cow, massive burst of proliferation during pregnancy is mediated by side branching and development of secretory alveoli.

Mammary development is driven by mammogenic hormones and growth factors however, the histological features differ among species. For example, in both ruminants and humans, stromal fat invasion and ductal branching are concomitant phenomena, whereas in the mouse, extensive branching takes place after primary ducts have invaded the fat pad (Prpar *et al* 2012). Growth of mammary epithelium is brought about by the specialized cells present therein throughout the life called mammary stem cells (MaSC). Conversion of stem cell to become specialized cell, called differentiation, which indicates important role of MaSC in mammary gland. The population of mammary stem cell is very rare (<1 % of total mammary epithelium).

Buffalo mammary gland epithelial cells have been used to establish cell lines (Anand *et al* 2012), to study the characteristics of buffalo mammary epithelial cells. Knowledge of MaSCs of buffalo mammary gland is rare. There could reasons to justify why characterization of buffalo MaSCs are very important. Buffalo MaSCs, like that of cow and goat MaSC, could be exploited in animal production to study lactation persistency for more milk production in lifetime of the animal. In addition to genetic selection program to increase milk yield in buffalo, manipulation of MaSC could be done using exogenous hormones or paracrine factors or even the use of

nucleosides like xanthosine and inosine (Capuco *et al* 2012; Choudhary 2014). These factors or compounds are likely to alter cell numbers of MEC which ultimately secrete more milk. Expression of factors namely stem cell markers, cell proliferation markers and cytokines like tissue necrosis factor-alpha (TNFA) are important in understanding development of normal and abnormal mammary gland. TNFA plays an important physiological role in development of normal mammary gland. Transcription of *TNFA* was increased during pregnancy but decreased during lactation and involution in rats (Ip *et al* 1992) suggesting its significant role in cell proliferation, morphological and functional differentiation of milk secreting cells. TNFA is also a marker of inflammation. Study of *TNFA* expression therefore, could be a key factor in understanding functional status and inflammation of the mammary glands. It has been realized that common characteristics of normal and abnormal mammary development is the excessive proliferation of affected cells and sometimes mutation of stem cells.

Considering the possibility that stem cell helps in tissue growth and regeneration like in the cow, it might help in growth and regeneration of buffalo mammary gland. Therefore, it is pertinent to identify prospective MaSC population in buffalo mammary glands at various physiological stages of the animal. It can also resolve the role of mammary stem cell in different breeds, animals in healthy and unhealthy condition. And can explains the reason by milk yield vary in different breed or within same breed when mammary glands of the animals appear to be completely healthy. The existence of MaSC/progenitor cells has been indicated by immunolocalization of hepatocyte nuclear factor 4 alpha (HNF4A), a liver stem cell marker in buffalo (Choudhary *et al* 2016). Presence of HNF4A-positive mammary epithelial cells in basal layer of mammary parenchyma was derived from a bovine study (Choudhary *et al* 2013). A population of aldehyde dehydrogenase 1 (ALDH1)-positive mammary epithelial cells was enriched for MaSC/progenitor cells in bovine (Martignani *et al* 2010) and goat (Prpar *et al* 2012) mammary glands. Ideas of testing hypotheses that ALDH1-positive mammary epithelial cells are buffalo MaSC/ progenitor cells and they are primary proliferative population at postnatal stages of mammary development, is novel.

The aim of this study was to identify *in vivo* MaSC/progenitor cells in the buffalo mammary glands during various physiological stages namely lactation, mastitis and non-lactation. We wanted to determine if the number of MaSC vary depending on the stages of the animal and how expression of TNFA vary with various stages. We hypothesize that 1) ALDH1 identifies MaSC/progenitor cells and or cancer stem cells in buffalo mammary glands, and 2) protein localization, cell number and mRNA expression of ALDH1 and TNFA (TNA-α) differ depending upon physiological stage of the animal. The objectives of the present study were:

1. **To quantify ALDH1-positive epithelial cells as MaSC/progenitor cells during various physiological stages**
2. **To analyze gene expression profile of *ALDH1* and *TNF-α***

CHAPTER II

REVIEW OF LITERATURE

2.1 Introduction of buffalo mammary glands

The first recognizable sign of mammary development, occurs during prenatal life, is the development of mammary line. In buffalo, mammary line develops at 34th day of gestation period (Challana *et al* 2014) that is comparable (35th day) to the development of mammary line in cow. The post-natal development of mammary gland, it involves a series of complex physiological process occurring during distinct physiological stages of the animal namely, prepubertal, pubertal, pregnancy, lactation and involution. These processes involved interactions of hormones, growth factors and suppressors act in orchestrated fashion to bring desired growth of the gland. Understanding cellular and molecular development of buffalo mammary gland is imperative because its gross structure and tissue architecture differ among species, as does endocrine and paracrine regulation of mammary gland growth and lactation (Capuco *et al* 2002). Mammary gland develops in tree-like fashion composed of hollow branches of ducts. At the end of the ductal branches, bunch of grape-like structures of mammary alveoli could be visualized. There are two types of cells namely, inner layer of alveolar or luminal epithelial cells (MEC) surrounded by an outer layer of myoepithelial cells. Myoepithelial cells secrete protein that forms the basal lamina of basement membrane which separates mammary parenchyma from the stroma (**Figure 1**).

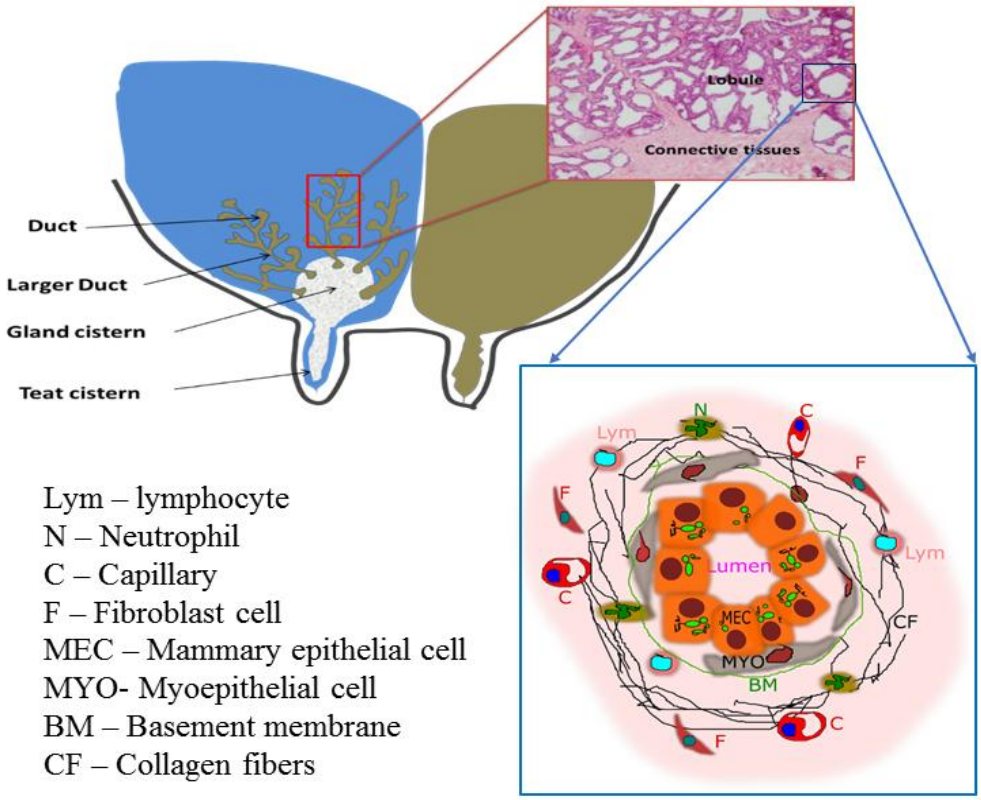

Photo credit: Choudhary, R. K. et al (2018). Xanthosine treatment: A novel strategy to increase milk production and mammary stem cells. Research Project no. BT/AAQ/01/AB-ITF-AP/2017, School of Animal Biotechnology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana

Figure 1: Tree-like structure of the mammary glands of ruminant with developed alveolar structure.

2.2 Challenges in buffalo milk production

Nearly 80% of the cattle breed and 60% of the buffalo breeds on India are non-descript types and hence are poor in milk production (Mathur 2000). Some of the important dairy breeds of buffalo known for high productivity, in Punjab and adjacent states are the Murrah and Nili-Ravi. The shape of buffalo udder, teat size, and milk let-down characteristics are significantly different in buffalo than the cow (Mathur 2000). That is he reason, machine milking was not possible in buffalo until recently. This suggests that an increased knowledge of buffalo mammary development and growth would be of practical importance to the dairy Indian industry. Efforts to rear buffalo at accelerated growth for an early start of milk production and continued milk production without the issues of repeat breeding, mastitis and agalactia, would

enhance lifetime milk production in animal. Therefore, knowledge about the different cell types and their characteristics like proliferation, number and their percentage composition can lead to management schemes that enhance animal's productivity (Capuco *et al* 2002). So far, the knowledge of characteristics of buffalo MEC and myoepithelial cells are limited.

2.3 Types of cells in mammary gland

In mammary gland epithelial cell is the main component and considered to be the cause of maximum activity of the mammary gland. It is basically present in two forms luminal and myoepithelial cells. Luminal cells are in abundence and present in lining of ducts and alveoli. Myoepithlial cells located near alveoli luminal cells and basement memberane. In duct system it is present continous patteren while near alveoli it is some what scattered. It help in milk release from the alveoli lumen. Myoepithelial cells are dependent on oxytocin hormon to cause contraction movement. During lactation alveoli cells become large in size and less compact. The luminal size is maximized and synthesised milk components in alveoli cells are pushed toward lumen. (Emeran and Pitelka 1977) said that epithelial cells are polarized into two parts basal and apical side. The basal part is close to basement membrane where as apical side face toward lumen. So, the basal cells help in extracting nutrients from capillaries. (Bauman *et al* 2006) explained that epithelial cells basically take nutrients from near by capillary network and process it into milk components.

2.4 Ultra-structure of buffalo mammary tissue

Ultra-structure investigates the various types at subcellular level, identify microenvironment of a cell and is one of the method of mammary stem cell identification. Large pale-staining cells of mouse mammary gland (Smith and Medina 1988) and bovine mammary glands (Capuco *et al* 2003) at all stages of development has been identified as mammary stem cells. Electron microscopic examinations of these pale-staining cells in mouse mammary gland showed that mammary stem cells may divide symmetric in culture and produce two pale-staining daughter cells (**Figure 2)**

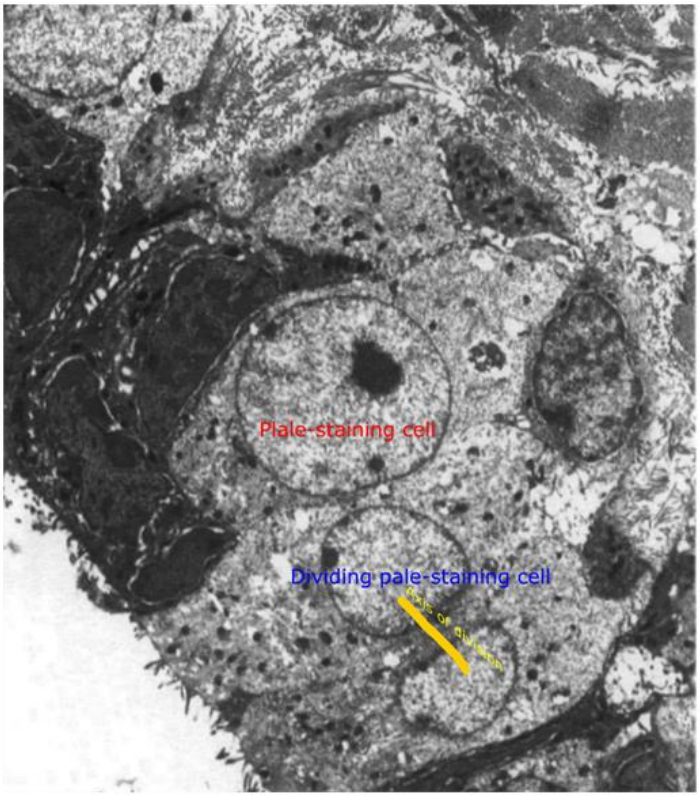

Figure 2: Electron micrograph of cultured murine pale-staining mammary epithelial cell.

The picture is showing symmetric division of pale-staining cell. Pale-staining cells are the mammary stem cells identified in mouse and cow mammary gland. (Photo modified from Smith and Medina, 1988).

2.5 Mammary stem cells

Among the methods that have been used for the isolation of mammary stem/progenitor cells, the use of cell surface markers is the most prevalent (Choudhary 2014). There are a number of reports on the methods of identification of mammary stem cells and progenitor cells of various species including cows (Rauner and Barash 2012), mice (Shackleton *et al* 2006), humans (Stingl *et al* 2006), 2006), goat (Prpar *et al* 2012), and sheep (McElroy and Bassett 2010). Other investigators have enriched the population of $CD24^{med}/CD49f^{pos}$ cells as the bovine mammary stem cells and the $CD24^{neg}/CD49f^{pos}$ cells as the bipotent progenitor cell population (Rauner and Barash 2012). In another study, MYO cells were shown to express Sca-$1^{neg}/CD24^{low}/CD49f^{low}$ and the basal MEC expresses Sca-$1^{neg}/CD24^{low}/CD49f^{high}$ (Meier-Abt *et al* 2013). Hierarchy of mammary stem cells in bovine (**Figure 3**) has

been presented. The very first existence of MaSC/progenitor cells has been indicated by immunolocalization of hepatocyte nuclear factor 4 alpha (HNF4A), a liver stem cell marker in buffalo (Choudhary *et al* 2016). Presence of HNF4A-positive mammary epithelial cells in basal layer of mammary parenchyma was derived from a bovine study (Choudhary *et al* 2013).

Ginestier *et al* (2007) showed that normal and cancer human mammary epithelial cells having increased aldehyde dehydrogenase activity (ALDH1) were stem/progenitor cells. Genetically engineered ALDH1A1 deficient mice, demonstrated that although ALDH1 is highly expressed in hematopoietic stem cells, but not a critical regular of adult stem cells (Levi *et al* 2009). This study indicated the possibility of other isoforms of ALDH1 rescue its function. Fluorescence activated cell sorting (FACS) analysis of mammary tissue from a heifer (pubertal virgin cow), according to their ALDH1 activity, revealed two subpopulations of cells- high and low ALDH1 activity (31% $ALDH1^{high}$ and 67% $ALDH1^{low}$) cells. *In vitro* analysis of these cells for clonogenic potentials revealed that $ALDH1^{high}$ cells had luminal features and $ALDH^{low}$ cells had basal features. This study concluded that high expression of ALDH1 are enriched in luminal progenitor cells (Martignani *et al* 2010). Similarly, Rauner and Barash (2012) showed high expression of ALDH1 was the characteristics of bovine luminal progenitor cells. However, in basal layer of bovine mammary epithelium, expression of ALDH1 was 3x higher in MaSC in comparison to basal epithelial cell. A population of aldehyde dehydrogenase 1 (ALDH1)-positive mammary epithelial cells were enriched for MaSC/progenitor cells in bovine (Martignani *et al* 2010) and goat (Prpar *et al* 2012) mammary glands. These specialized mammary stem/progenitor cells can give rise to the tissues of the adult mammary gland during development, allow the enormous tissue expansion and remodeling of the gland during pregnancy, lactation and involution; and serve as a reserve for tissue repair during the damage caused by the mastitis (Woodward *et al* 2005).

Recently, our group published the first report of identification of putative buffalo MaSC/ progenitor cells using immunolocalization of transcription factor-hepatocyte nuclear factor 4 A (HNF4A), in the nuclei of buffalo mammary epithelial cells. This study, preliminary indicated that HNF4A could identify buffalo

MaSC/progenitor cell population (Choudhary *et al* 2016b). Low expression of HNF4A in the nuclei of MaSC/progenitor cells poses difficulty in their identification and co-localization with other stem cell markers.

In this study, we sought to analyze the expression profile (RNA and protein level) of ALDH1 and HNF4A in buffalo mammary tissue obtained from the abattoirs.

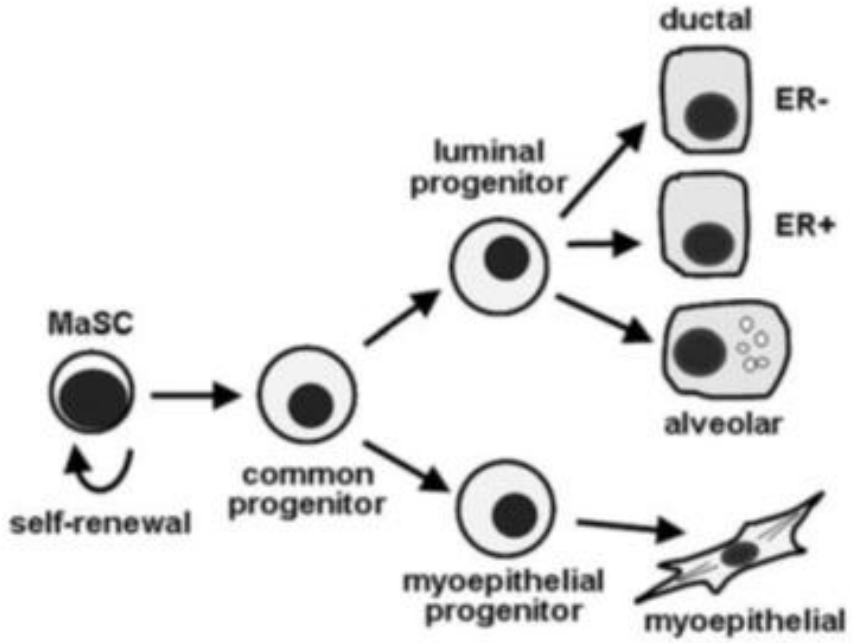

Figure 3: A working model of bovine mammary stem cell hierarchy.

Schematic representation of various types of mammary stem cell and progenitor cells ultimately giving rise to differentiated mammary epithelial cells. (Photo adapted from Capuco *et al* 2012).

2.6 Tumor necrosis factor alpha and Mastitis in mammary gland

Expression of factors namely stem cell markers, cell proliferation markers and cytokines like tissue necrosis factor-alpha (TNF-α) is important in the understanding development of normal and abnormal mammary gland. It has been realized that common characteristics of normal and abnormal mammary development is the excessive proliferation of affected cells and sometimes mutation of stem cells. Like other organs, mammary gland also contains stem cells called mammary stem cells (MaSC), which are the specialized undifferentiated epithelial cells responsible for growth and regeneration during various physiological stages of the animal. TNF-α plays an important physiological role in the development of normal mammary gland.

Transcription of TNF-α increased during pregnancy but decreased during lactation and involution in rats (Ip *et al* 1992) suggesting its significant role in cell proliferation, morphological and functional differentiation of milk secreting cells. Study of TNF-α expression, therefore, could be a key factor in understanding the functional status of mammary gland development in Buffalo. TNF-α is a pro-inflammatory cytokine and has been shown to induce a pro-apoptotic response in bovine mammary endothelial cells (Aitken *et al* 2011). Intramammary infusion of recombinant TNF-α into lactating bovine mammary gland resulted in suppression of lactogenic function evidenced by decreased concentration of caseins, α-lactalbumin, and β-lactoglobulin at 4-8 hours after infusion (Watanabe *et al* 2000). (Mingala *et al* 2009) studied the expression of TNF-α and TNF- γ in buffaloes and found that difference in cytokines expression could be associated with disease tolerance and susceptibility.

2.7 Incidence of mammary cancer in buffalo

Although, the incidence of mammary cancer in ruminant is rare, however our group reported some incidences of suspected cases of mammary cancer in buffalo. These observations were based on aberrant and high expression of some of the protein markers of cancer cell. Expression of cancer and mammary stem cell markers (FNDC3B and MSI1, respectively) was aberrantly high in buffalo mammary glands (Kaur *et al* 2016). Histological examinations of these mammary glands of buffalo revealed solidification of ducts with increased layers of epithelium. Abnormal shape and size of MEC were suggestive of mammary cancer. Additionally, elevated expression of estrogen receptor alpha (ESR1) was observed in these buffalo mammary glands.

CHAPTER III

MATERIALS AND METHODS

3.1 Animal tissue collection

Mammary tissue samples of postnatal stages (lactating, non-lactating, mastitis, mammary cancer) of buffalo were collected from buffalo slaughterhouse located at Derabassi, Chandigarh. Physiological stages of mammary glands (lactating, non-lactating, mastitis and cancer) were accessed using histopathology as per our earlier reports (Choudhary *et al* 2016b; Choudhary *et al* 2018b).

3.2 Mammary tissue processing for histochemical studies

1. The collected tissue samples were fixed in 10% neutral buffer formalin immediately.
2. The tissues were kept in tubes and washed under running tap water for overnight.
3. Then tissues were kept in 70%, 80%, 90% and100% ethanol for 1 hour each for dehydration.
4. Then samples were dipped in acetone for 15 min, then placed it in benzene for 15 min.
5. Dehydrated tissue samples in tubes were contained in melted paraplast (58-60˚C) and kept overnight.
6. Then cooling of paraplast was done at 4˚C.
7. Rough pre-trimming of blocks was done and then stored for further use.

Later blocks were used, 4-5µm thick tissue sections were cut with a microtome (Leica, Germany) and mounted over plain and positively charged glass slides (Globe scientific).

3.3 Hematoxylin and eosin (H&E) staining

Hematoxylin is a basic stain which binds to basophilic matter. So, it serves the purpose to stain nuclei of the cells by giving dark blue or violet stain to it. Whereas Eosin is used as a counterstain to color the cytoplasm as most of it is eosinophilic in nature.

Harris H&E staining was performed (Luna 1968) to study histomorphology of the tissue samples.

3.3.1 Protocol for H&E staining

1. Coated slides were dewaxed by immersing in 3 sets of xylene for 5 min each.
2. After dewaxing, rehydration is followed in descending order of alcohol from 100% ethanol for 2 min, repeat the 2nd set of 100% alcohol for 2 min.
3. Then slides were placed in 95% and 70% alcohol for 2 min each.
4. Now sections are enable to stain in aqueous hematoxylin solution.
5. Sections were washed with distilled water for 2 to 5 min.
6. Sections were stained with Harris hematoxylin for 15 min.
7. Then slides were washed under running tap water for 10 min to remove the excessive stain.
8. Sections were placed in acid alcohol for 1 min
9. Again slides were washed with distilled water for 1 min.
10. Slides were dipped in ammonia water for 1 min to develop stain colour.
11. The slides were washed under running tap water for 1 min.
12. Then for counterstaining sections were dipped in 2% eosin for 1 min 30 sec
13. Sections were washed under running tap water for 1 min
14. Then sections were dehydrated in 2 set of 100% alcohol for 2 min each.
15. Sections were washed with 2 set of xylene for 5 min each.
16. Slides were mounted with DPX (Sigma) to protect the sections physically.
17. Mounted sections were kept for overnight drying at room temperature.

3.3.2 Quantification of various cell types of buffalo mammary tissue

Haematoxylin and eosin stained sections of each animal were viewed under bright field microscope and 5-6 microscopic fields were randomly captured. Images were taken at 40X magnification and quantification of cells and other cellular components were conducted as done in our earlier study (Kaur *et al* 2016). Of eight components that were counted include, cells of mammary tissue namely, mammary epithelial cells (MEC), myoepithelial cells (MYO) and lymphocytes (LYM) and

cellular components of mammary tissue were stroma (S), spaces within the stroma (SS), stromal debris (SD), alveolar lumen/ductal lumen (AL/DL) and presence of debris within the alveolar lumen (AD). Upon capturing images were inserted into Microsoft PowerPoint and a 10x10 grid was overlaid. The cells or cellular components within 100 intersections of 10x10 ocular grid were counted. Quantification of various cell types and other cellular components were done and the data were expressed in terms of mean percentage composition of cells or cellular components. Student's t-test was applied for the statistical significant difference and P-values $\leq$ 0.05 were considered significant.

3.4 Immunostaining

3.4.1 Buffers used for immunostaining

Table 1: Composition of phosphate-buffered saline (PBS) (10X)

Reagents	Quantity (per 1 litre)	Final concentration
Disodium hydrogen phosphate (anhydrous)	10.9 g	77 mM
Sodium dihydrogen phosphate (anhydrous)	3.2 g	23 mM
NaCl	90 g	1.5 M

700 ml of distilled water was added to the reagents after mixing it well pH was adjusted to 7.2. Them more distilled water was added to make up the final volume of 1000 ml.

Table 2: Composition of Tris-EDTA Buffer (10x)

Reagents	Quantity (per 1 liter)	Final concentration
Tris-base	1.21 g	10 mM
EDTA	0.37 g	1 Mm

700 ml of distilled water was added to the reagents after mixing it well pH was adjusted to 9.0. Them more distilled water was added to make up the final volume of 1000 ml.

3.4.2 Details of kit used for immunostaining

The following reagents were supplied with the kit (ImmPRESS Excel staining kit; Universal Peroxidise kit; Vector lab, Burlingame, USA)

1. 10 ml Imm PACT DABEQV. Reagent 1 (chromogen)
2. 10 ml ImmPACT DAB Eqv. Reagent 2 (buffer)
3. ImmPACT anti-rabbit/anti-mouse antibody

3.4.3 Immunohistochemistry staining protocol

The reagents supplied in the kit were ready to use. Staining was performed as per suggested procedure, at room temperature

1. Tissue slides were deparaffinized by immersing in 3 set of Xylene for 5 min each.
2. After dewaxing, the sections were hydrated using a descending grade of alcohol washings. Starting from 2 set of absolute ethanol for 3 min, followed by 95% and 70% ethanol 3 min each.
3. The sections were given 2 items of washing of double distilled water for 2 min each.
4. To block the endogenous peroxidize activity, drops of 3% hydrogenase peroxidase diluted in distilled water was put over each section and kept for 20 min at room temperature.
5. Then the slides were washed with three changes of double distilled water for 3 min each.
6. Heat mediated antigen retrieval in Tris-EDTA buffer(pH 9.0) was done on a hot plate (5 min heat, 5min rest, 5min heat, 25 min cooling at room temperature -5 min cooling under running tap water) to achieve antigen unmasking.
7. Slides were washed in 3 change of double distilled water for 3 min each.
8. Then slides were washed in 3 change of PBS for 2 min each.
9. Protein blocking with 1X casein solution (10X casein solution; Vector lab) was done for 20 min to prevent nonspecific binding of primary antibodies.
10. The tissue sections were incubated with primary antibody for overnight at 4˚C in the moist chamber to avoid drying

11. Washed the section with 3 change of PBS 2 min each.
12. Incubated with Secondary antibodies-impress anti-rabbit/anti-mouse 30 min at room temperature.
13. Washed the section with 3 change of PBS 2 min each.
14. IMPACT VIP/NOVA RED is used and incubated at room temperature for 2 min
15. Slides were washed with three changes of double distilled water for 3 min each.
16. Counterstained with hematoxylin for(1 to 3 min)
17. Washed slides Under running tap water and put in PBS for 30 sec to develop color.
18. Washed with distilled water for 1 min.
19. Dehydrated in 70%, 95% ethanol for 2 min each and in 2 set of 100% ethanol for 5 min each.
20. Put in 2 set of xylene for 5 min each.
21. Mounted with DPX and allowed to dry.

Table 3: Primary antibody used for immunostaining of buffalo mammary tissue and their working concentration

Antibody	Name of protein	Vendor	Working dilution
ALDH1	Aldehyde dehydrogenase 1	BioRybt	1:500 dilution
TNFA	Tissue necrosis factor alpha	BioRybt	1:500 dilution
HNF4A	Hepatocyte nuclear factor 4 alpha	Santa Cruz	1:200 dilution

3.4.4 Quantification of ALDH1 and TNFA staining

Mammary epithelial cells positive for ALDH1 and TNFA were captured. At least 5-6 images per animal were photomicrographed at 400x magnification under bright field microscope (Eclipse 90i, Nikon, Japan). In each image, at least 100-200 cells were counted thus, a total of 500-1200 mammary epithelial cells (e.g. 100 cells/image X 5 images/animal) were counted from each animal and percentage of marker-positive cells were expressed in terms of mean+/- standard error (Choudhary *et al* 2016b). Images were processed and analyzed using Image J software (Schneider

et al 2012). Schematic representation of quantification of immune-positive signals using image J has been given (**Figure 4**).

3.4.5 Statistical analysis for quantification of ALDH1 and TNFA staining

The two-way ANOVA was applied to compare the difference in means pixel intensity of the immunostained area of ALDH1 and TNFA staining. Tukey's multiple multiple comparison tests has been applied for post-ANOVA comparison using the SPSS 22.0 (SPSS Inc. IL, USA).

3.5 Ultra-structure of buffalo mammary gland

The fixation of mammary tissue for the transmission electron microscopy study were done as per the literature (Pathak and Bansal 2011). Briefly, mammary tissue after washing with PBS, fixed in Karnovsky's fixative (2 h) followed by secondary fixation in 2% OsO4 for additional 2 h. Tissue were dehydrated in ascending grades of acetone and cleared in toluene. Upon embedding tissue in capsule blocks were prepared and semi-thin sections of 0.5–2.0 micron thickness were sectioned on positive charged slides. Ultra-thin sections of ~80 nm were sectioned and coated with a film of 50Å thickness carbon onto copper grids as normal standard procedures of Sophisticated Analytical Instrumentation Facility of All India Institute of Medical Science (AIIMS), New Delhi (http://www.saifaiims.com/). Finally, the grids were stained with uranyl acetate for 15 min and lead citrate for 10 min before examining under the transmission electron microscopy. Photomicrographs were taken at different magnification using Tecnai electron microsocope (Thermo Fisher Scientific, USA).

3.6 RNA isolation from buffalo mammary tissue

3.6.1 Sample collection for RNA isolation

Mammary tissue was collected from dead buffaloes immediately after the slaughter at an abattoir. Mammary parenchyma from the mid-region of the glands was collected and stored in RNAlater for nucleic acid isolation. Another piece of mammary tissue (5x5x5 mm^3) were fixed in 10% (w/v) neutral buffered formalin for overnight. Subsequently, fixed tissues were processed for embedding in paraffin as per routine and standard procedures.

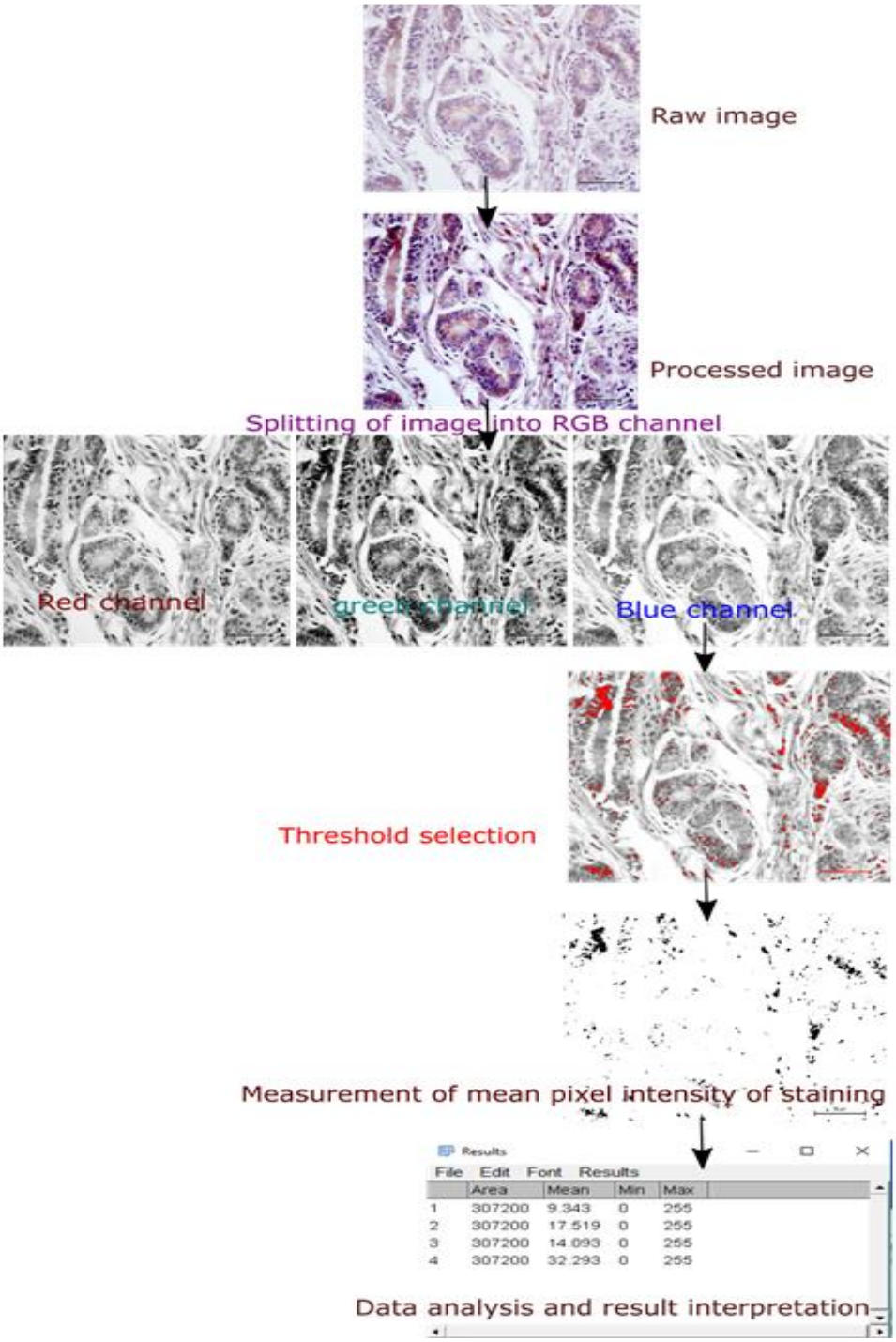

Figure 4: Quantification of immuno-positive signals of ALDH1 and TNFA staining in buffalo mammary tissue.

Photomicrographs of tissue sections were separated into RGB color into red (R), green (G) and blue (B) channels using Image J and quantification of pixel value (Mean + SE) after threshold correction were calculated.

Physiological stages of animals were accessed by gross morphological examination of mammary glands and histological examinations of hematoxylin and eosin (H&E) stained tissue sections as described elsewhere (Choudhary *et al* 2018b). Institutional Animal Ethical Committee (IAEC) approval was granted (reference number IAEC/2017/849-80) for this study.

3.6.2 Protocol for RNA isolation from mammary tissue

(as per the lab's protocol published earlier (Choudhary et al 2018a)

1. Tubes having tissue in RNA later were thaw at ice/RT for 5 min
2. 500 µL of RNAiso plus was taken into 2 ml nuclease-free tube
3. Weighed the tube and tare the weight of RNAiso plus then placed a small piece (50-100 mg) of tissue measure weight (recorded it).
4. Homogenized the tissue with a hand-held homogenizer (Qiagen) for 2-3 minutes on ice/cooler – pre-cleaned steel probe as per standard procedure before homogenizing next sample.
5. Added remaining 500 µL of RNAiso plus and added 250 µL of chloroform to it.
6. Centrifugation was done at 12000 X g for 5 min at 4 ˚C
7. Transferred supernatant to a new centrifuge tube and added 250 µL of chloroform
8. Vortex or mixed with shaking vigorously for 1 min
9. Incubated homogenate + Chloroform mixture at RT for 10 min
10. The centrifuged mixture at 12000 x g for 15 min at 4 ˚C**.**
11. Filtered through nucleospin filter (violet ring) and after filtration discarded the filter and centrifuged at 11000 x g for 1 min at 4˚c
12. Put 350 µL of 70% alcohol into nucleospin RNA column and placed in collection tube..
13. Centrifuged at 12000xg for 35 sec.

3.7 Synthesis cDNA and RT-qPCR

The integrity of isolated RNA was checked with 1% agarose. Quality and concentration of RNA were evaluated using Nanodrop (ND-1000). The concentration of RNA was also evaluated using Qubit spectrophotometer. DNase-treated RNA (~1000 ng) was reverse transcribed using the Affinity Script qPCR cDNA Synthesis Kit (Agilent). Transcript abundance was determined by Brilliant III Ultra-Fast SYBR QPCR master mix (Agilent Technologies Cat. no: 600882). A 10 µL of the RT reaction was used in the 0.20 ml PCR tube strips in duplicates. PCR amplifications were performed in a thermocycler (CFX 96, BioRad, CA, USA) (**Figure 5**).

Melt curve analysis was performed at the end of each reaction to confirm single PCR product. Genes of mammary stem cell markers (*ALDH1*, *HNF4A*) and inflammatory marker (*TNFA*) were designed using Primer-Blast of NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast) to evaluate abundance of transcripts during lactation, non-lactation and mastitis conditions. Ribosomal gene *RPS23* was used to normalize the gene expression data (**Table 4**) and $2^{-\Delta\Delta Ct}$ method (Pfaffl 2001) was employed taking lactation stage as calibrator. Statistical analysis for the difference in gene expression was performed at normalized threshold value (delta Ct) value.

Table 4: Gene-specific primers pair sequences for RT-qPCR amplification, their melting temperature, and product sizes

S. No.	Gene name	Primer pairs (5'------- 3')	Tm (°C)	Product size (bp)
1.	*ALDH1*	F: CTGGGCTGACAAGATCCAGG R: GACAACCACTGTGTTTCCGC	60	172
2.	*HNF4A*	F: GTCCTGTCCCAGCAGATCAC R: GGGATGTACTTGGCCCACTC	56	140
3.	*TNFA*	F: GAAGAGCAGTCCCCAGGTG R: GTTGATGTCGGCTACAACGTG	64	114
5.	*RPS23*	F: CCCAATGATGGTTGCTTGAA R: CGGACTCCAGGAATGTCACC	60	101

Figure 5: A Real time thermocycler used in gene expression analysis.

3.8 Statistical analysis

The two-way ANOVA was applied to compare the difference in means of delta Ct value of three markers among the three groups (lactation, non-lactation, and mastitis). Dunnett's multiple comparison tests were used for post-ANOVA comparison using the SPSS 22.0 (SPSS Inc. IL, USA). Data were analyzed by the relative expression level of mRNA and t-test or ANOVA test will be employed to test the significance of gene expression of ALDH1 and TNFA between different stages of the animal.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Estimation of physiological stages of buffalo

Animals were visualized and their glands were examined immediately after slaughter. Mammary glands of non-lactating (includes prepubertal, pubertal and non-pregnant animals), lactating and mastitis animals were examined by histomorphology of H&E stained sections (**Figure 6**). Presence of three layers of mammary epithelial cells are the characteristics of ruminant like bovine mammary glands (Meyer *et al* 2006; Choudhary *et al* 2018b). Non-lactating glands had regressed or poorly developed alveoli while lactating glands of buffalo had moderate to well-developed alveoli with evidence of milk secretions in the lumen. Loss of alveolar epithelial cells, an abundance of polymorphonuclear cells (PMN) and cellular debris in alveoli were the characteristics of buffalo mastitis (**Figure 6d, hollow arrow**). Additionally, aberrant expressions of cell proliferation marker, PCNA was observed in some of the mastitis glands in comparison to normal lactating gland (**Figure 7**).

4.2 Quantification of cells and cellular components of buffalo mammary glands

The alveoli were outlined by a single layer of cuboidal or columnar mammary epithelial cells (MEC). The number of MEC varies between non-lactating and lactating glands being high in non-lactating mammary gland (Mean $\pm$ SE; 46.5 $\pm$ 1.9% vs. 27.9 $\pm$ 1.2%; P = 1.09E-06). Myoepithelial cells were located along the basal lamina were significantly high in non-lactating gland than the lactating gland (Mean $\pm$ SE; 2.23 $\pm$ 0.64% vs. 0.26 $\pm$ 0.1 %; P = 0.0001). The mammary parenchyma of non-lactating buffalo had increased stromal tissue while the lactating gland has increased alveolar lumen (AL/DL) compared to ductal luminal area of non-lactating gland (**Figure 8**).

Stromal components, made up of cells of mesodermal origin (like adipocytes and fibroblast), extracellular matrix (ECM), blood vessels and lymphatics and inflammatory cells, in non-lactating animals are responsible for growth and development of mammary glands. Mammary parenchyma penetrates into the stroma. Myoepithelial cells (MYO) are located in the basal layer of epithelial compartment.

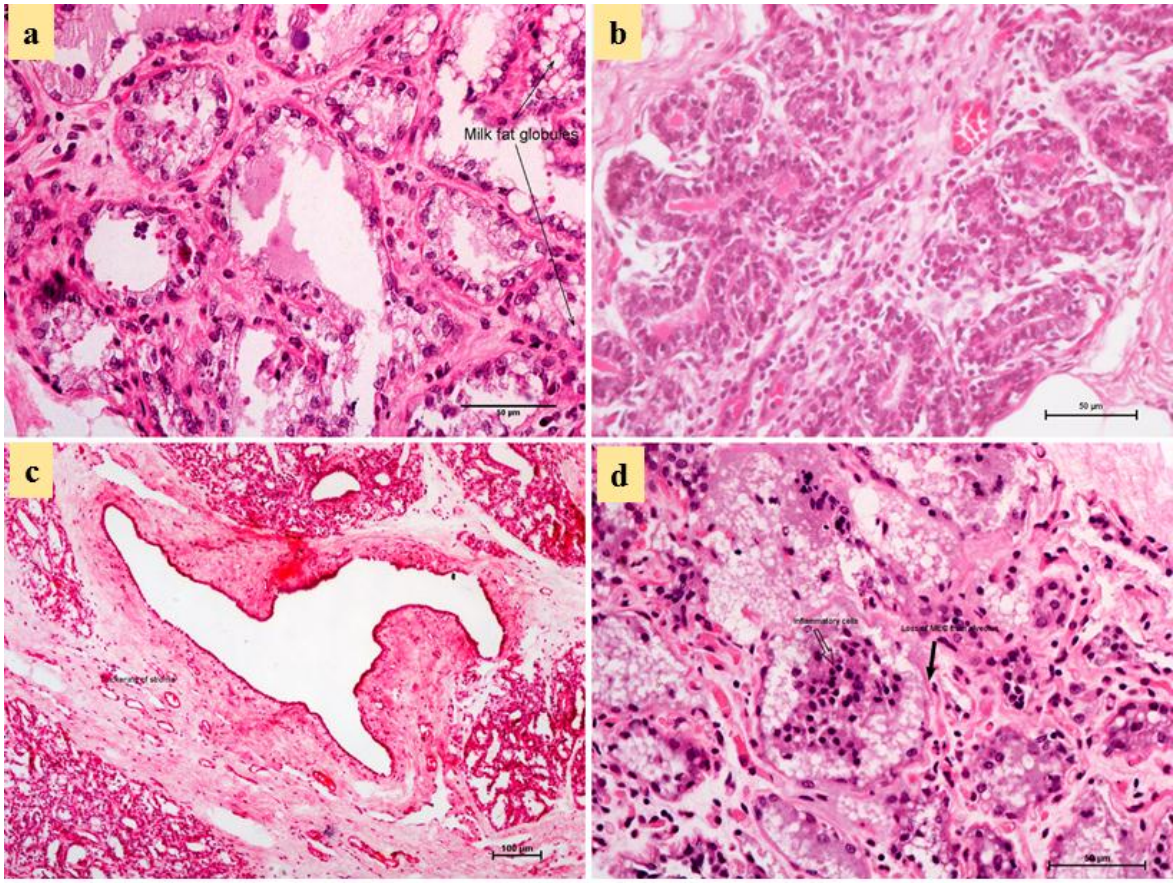

Figure 6: Hematoxylin and eosin (H&E) staining of buffalo mammary glands.

a- lactating gland; b- non-lactating gland and c-d – mastitic gland. Scale bar- a,b, d = 50 µm, c- 100 µm

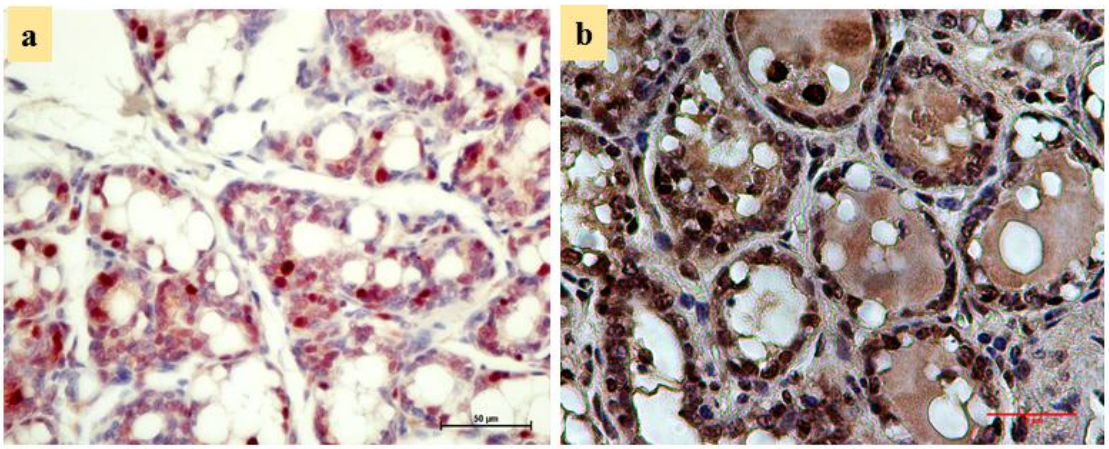

Figure 7: Immunostaining of PCNA in lactating and mastitis glands of buffalo. a- mastitis gland; b –lactating gland.

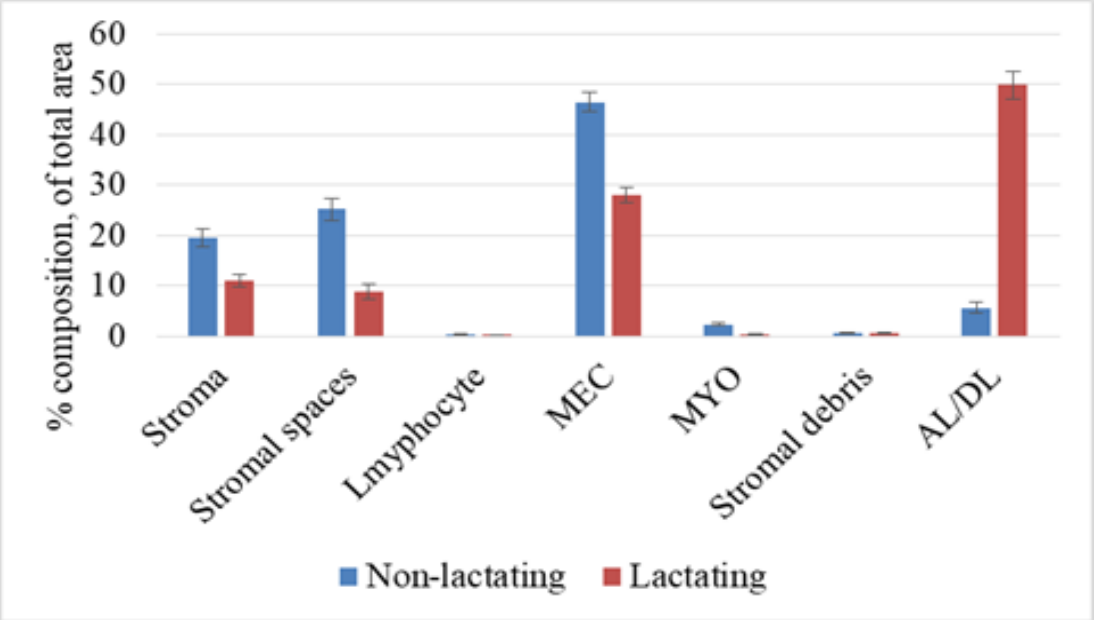

Figure 8: Quantification of cells and cellular components of buffalo mammary gland during non-lactation and lactation stage.

Non-lactation period include dry period, prepubertal buffalo calves. Data are presented in mean +/- standard error of percentage area occupied by the cells or cellular components. MEC – mammary epithelial cells. MYO – myoepithelial cells; AL/DL – alveolar lumen/ ductal lumen

Basal cells of mammary epithelium are enriched with undifferentiated mammary stem cells. More number of MYO in prepubertal gland indicate cells has a role in gland development. Mammary fat pad that contains MEC and MYO in non-lactating gland act under the influence of various hormones like IGF-1, estrogen, progesterone and influence growth of the gland.

Table 5: Two independent t-tests showing the difference in number of mammary epithelial cells (MEC) and myoepithelial cells (MYO) between non-lactating and lactating buffalo mammary glands

	Non-lactating	***Lactating***	***Non-lactating***	***Lactating***
	MEC		***MYO***	
Mean	46.46875	27.87692	2.2375	0.261538
Variance	27.41853	28.39026	0.647679	0.102564
Observations	8	13	8	13
df	15		15	
t Stat	7.848649		6.62905	
P(T<=t) two-tail	1.09E-06**[1]		0.000164**	

4.3 Immunolocalization of ALDH1, TNFA and HNF4A proteins

4.3.1 Tissue expression of ALDH1 in the buffalo mammary tissue

Immunohistological analysis of buffalo mammary parenchyma revealed varied expression of ALDH1 positivity in nucleus and cytoplasmic expression of MEC and stromal cells. Weak expressions of nuclear and cytoplasmic staining of ALDH1 observed in mammary alveolar cells of lactating glands. A strong cytoplasmic staining of epithelial cells and stromal cells were seen in mastitis glands. Weak to moderate nuclear staining of ALDH1 was observed in non-lactating glands (**Figure 9**).

[1] Highly significant difference between the groups

4.3.2 Tissue expression of TNFA in the buffalo mammary tissue

Immunohistological analysis of buffalo mammary parenchyma revealed varied expression of TNFA in lactating, mastitis and non-lactating glands of buffalo. Cytoplasmic expression of TNFA was observed in epithelial (solid arrow and hollow arrow) and stromal compartment (hollow arrowhead) of the mammary tissue. In stromal compartment, TNFA expression was seen to be associated with inflammatory cells (inflammatory cells are hyperchromic with round nucleus and morphologically distinct from the mammary epithelial cells and fibroblasts). Weak to moderate expression of TNFA was observed in the cytoplasm of MEC and stromal cells of lactating and non-lactating mammary tissues. A strong cytoplasmic expression of epithelial cells and stromal cells were seen in mastitis glands especially those of non-lactating with evident hyperplasia of MEC (**Figure 10**).

4.3.3 Tissue expression of HNF4A in the buffalo mammary tissue

HNF4A is nuclear stem cell markers of variety of tissue including mammary tissue. Immunolocalization of HNF4A in buffalo mammary tissue showed weak and nuclear expression (**Figure 11**). Very few HNF4A-positive MEC were observed in lactating glands whereas, during mastitis its expression was further reduced. Evident nuclear staining of HNF4A was observed in MEC of regressing alveoli during non-lactating period. Loss of stem cell markers during mastitis is consistent with our earlier publication with buffalo mastitis (Choudhary *et al* 2016b).

Using imageJ, quantification of immune-positive signals were done in terms of mean pixel area occupied by ALDH1 and TNFA staining (**Figure 12**). ALDH1-positive signals (in terms of mean pixel area intensity) were low in lactating glands followed by non-lactating glands (2.2 $\pm$ 1.0 % vs. 6.3 $\pm$ 1.2 %) of the total pixel intensity area of the image calculated. Highest ALDH1-positive signals were detected in mastitis glands (8.8 $\pm$ 2.0 %) (**Figure 12a**). TNFA-positive signals (in terms of mean pixel area intensity) were low in lactating glands followed by non-lactating glands (1.1 $\pm$ 0.17 % vs. 2.4 $\pm$ 0.61 %) of the total pixel intensity area of the image calculated. Highest TNFA-positive signals were detected in mastitis glands (3.4 $\pm$ 0.4 %) (**Figure 12b**).

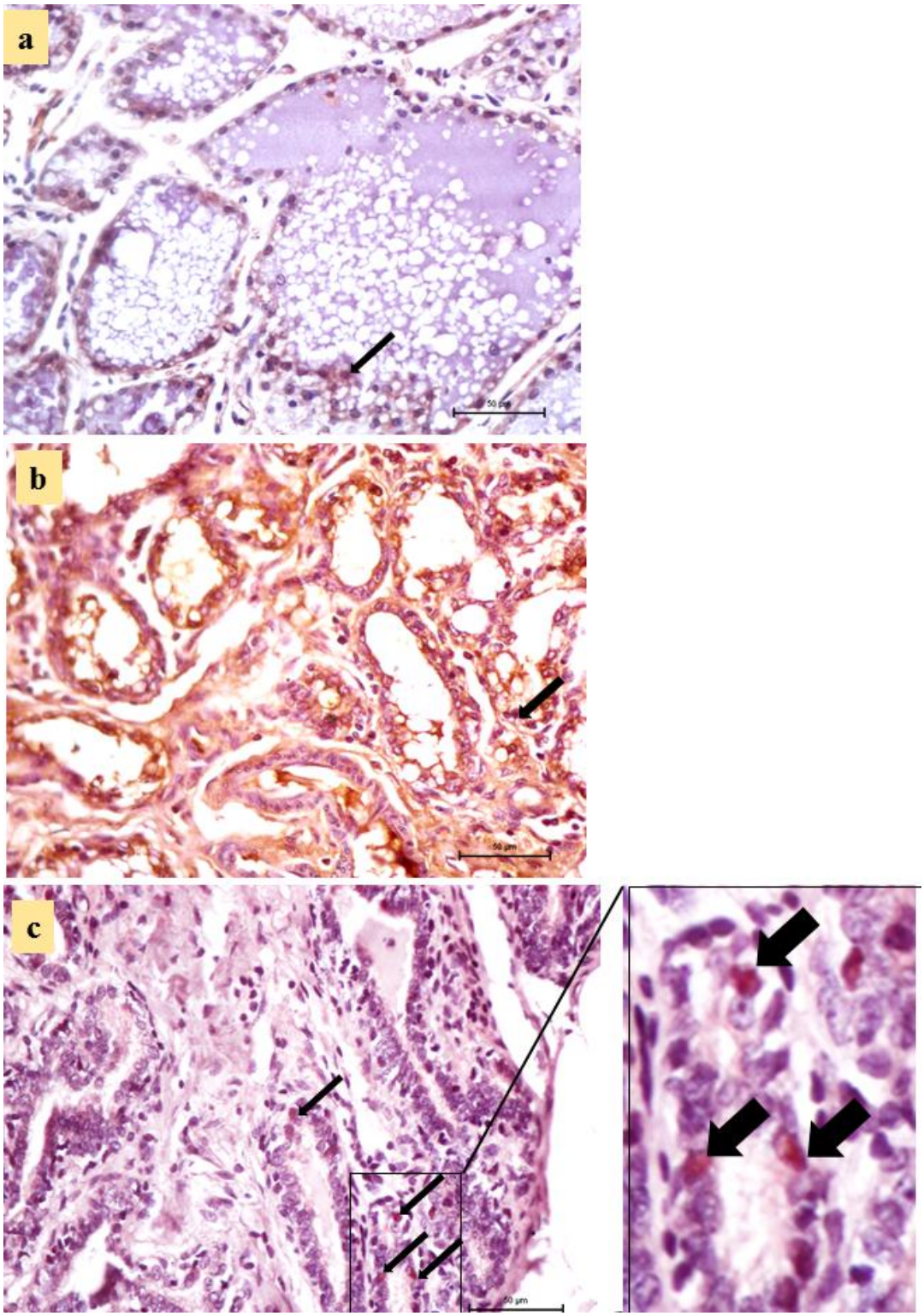

Figure 9: ALDH1 staining (red staining) of buffalo mammary glands.

Lactating gland; b- mastitis and c – non-lactating glands. Scale bar- a,b,c = 50 µm

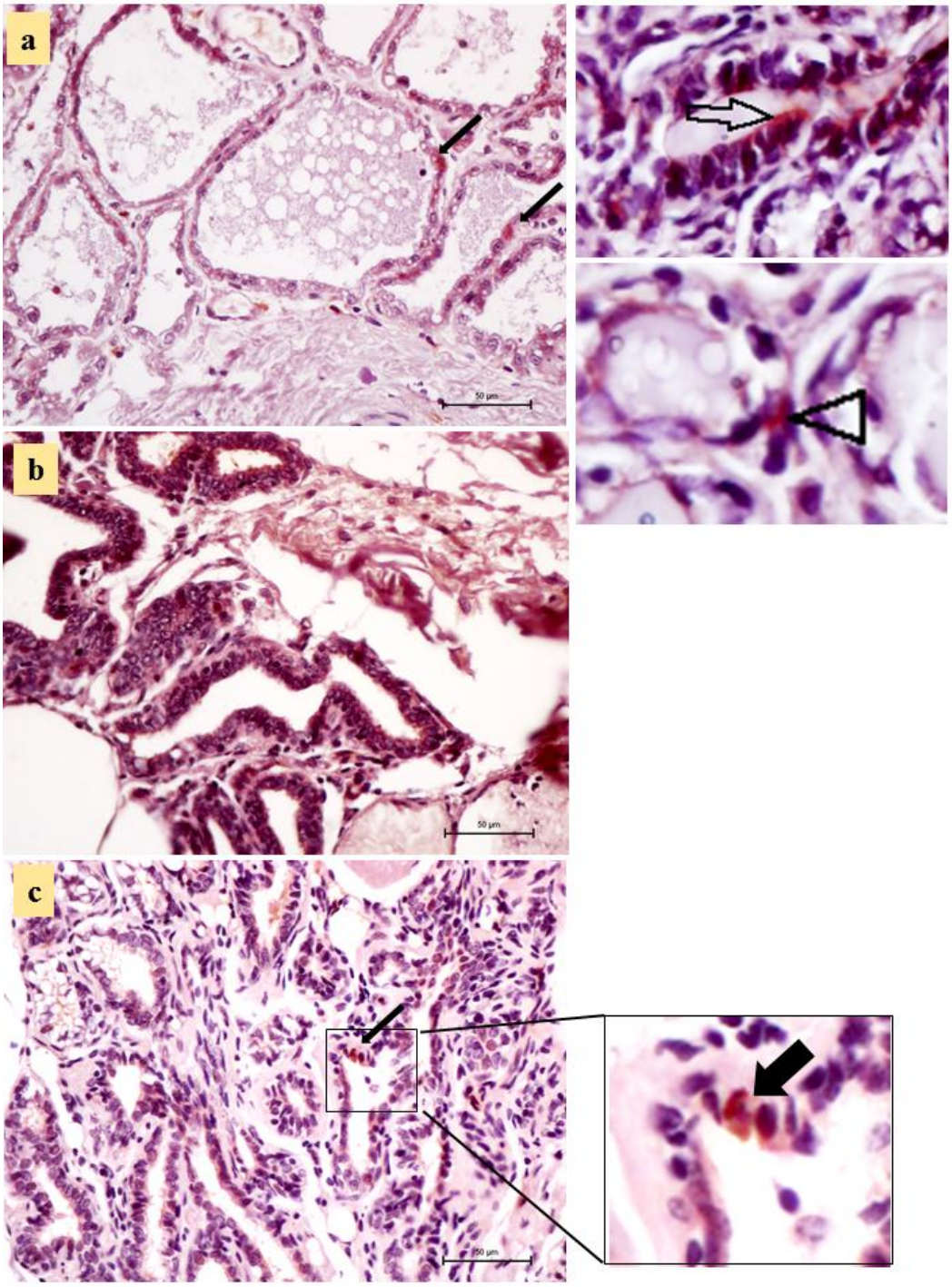

Figure 10: TNFA staining (red staining) of buffalo mammary glands.

Cytoplasmic staining of TNFA is shown in epithelial (hollow arrow and solid arrow) and stromal cells (hollow arrowhead).

a- lactating gland; b- mastitis and c – non-lactating glands. Scale bar- a,b,c = 50 µm

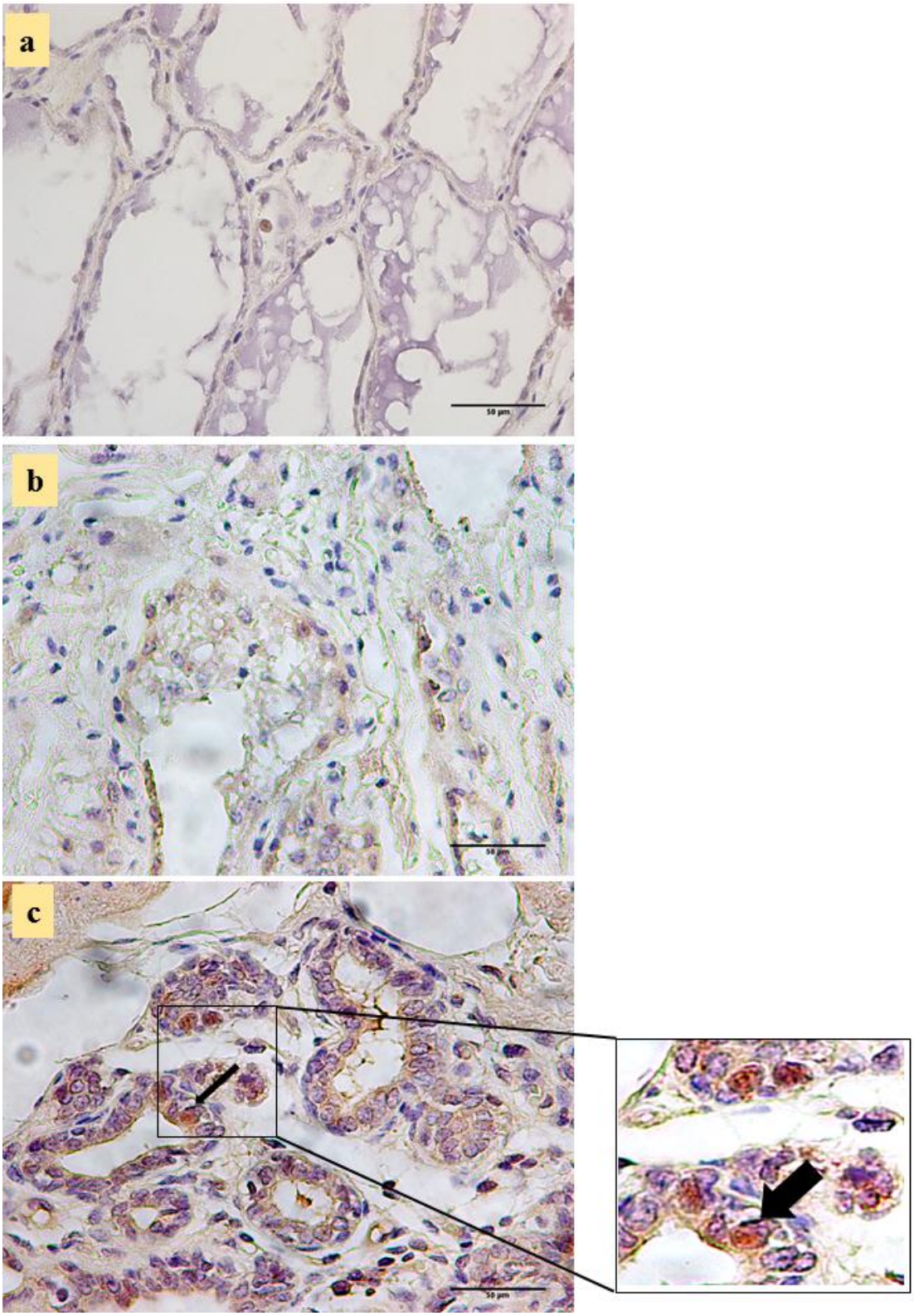

Figure 11: HNF4A staining (red staining) of buffalo mammary glands.

Evident nuclear localization of HNF4A was visible during non-lactating period. a- lactating gland; b- mastitis and c – non-lactating glands. Scale bar- a,b,c = 50 µm

One way ANOVA was conducted that examined the effects of stage on expression level of ALDH1. There was significant effect of physiological stage, F (4.9, 2), P =0.012 (**Table 6**). Multiple comparisons of the means using Tukey's test showed highly significant difference (P = 0.009) in ALDH1 staining between lactation and mastitis glands (**Table 7**). No difference (P = 0.16) in the expression of ALDH1 was observed between lactating and non-lactating mammary tissue.

One way ANOVA was conducted that examined the effects of stage on expression level of TNFA. There was significant effect of physiological stage, F (11.9, 2), P =0.0000 (**Table 8**). Multiple comparisons of the means using Tukey's test showed highly significant difference of TNFA between lactating and mastitis (P = 0.000) and significant difference (P = 0.04) between lactating vs non-lactating stage (**Table 9**).

Table 6: F-test showing significant effect of physiological stages of the animal on immunolocalization of ALDH1 in buffalo mammary tissue

Tests of Between-Subjects Effects

Dependent variable: ALDH1

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	354.848[a]	2	177.424	4.946	.012
Intercept	1545.409	1	1545.409	43.083	.000
Stage	**354.848**	**2**	**177.424**	**4.946**	**.012***
Error	1542.415	43	35.870		
Total	3437.033	46			
Corrected Total	1897.262	45			

Table 7: Tukey's test showing multiple comparison among physiological stages of the animal on immunolocalization of ALDH1 in buffalo mammary tissue.

Multiple Comparisons

Dependent variable: ALDH1

Tukey HSD

(I) Stage		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Lact	Mastitis	-6.61*	2.117	.009*	-11.75	-1.47
	NonLact	-4.06	2.192	.164	-9.39	1.26

Table 8: F-test showing significant effect of physiological stages of the animal on immunolocalization of ALDH1 in buffalo mammary tissue

Tests of Between-Subjects Effects

Dependent variable: TNFA

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	49.755[a]	2	24.877	11.987	.000
Intercept	257.688	1	257.688	124.169	.000
Stage	**49.755**	**2**	**24.877**	**11.987**	**.000**
Error	103.765	50	2.075		
Total	387.497	53			
Corrected Total	153.520	52			

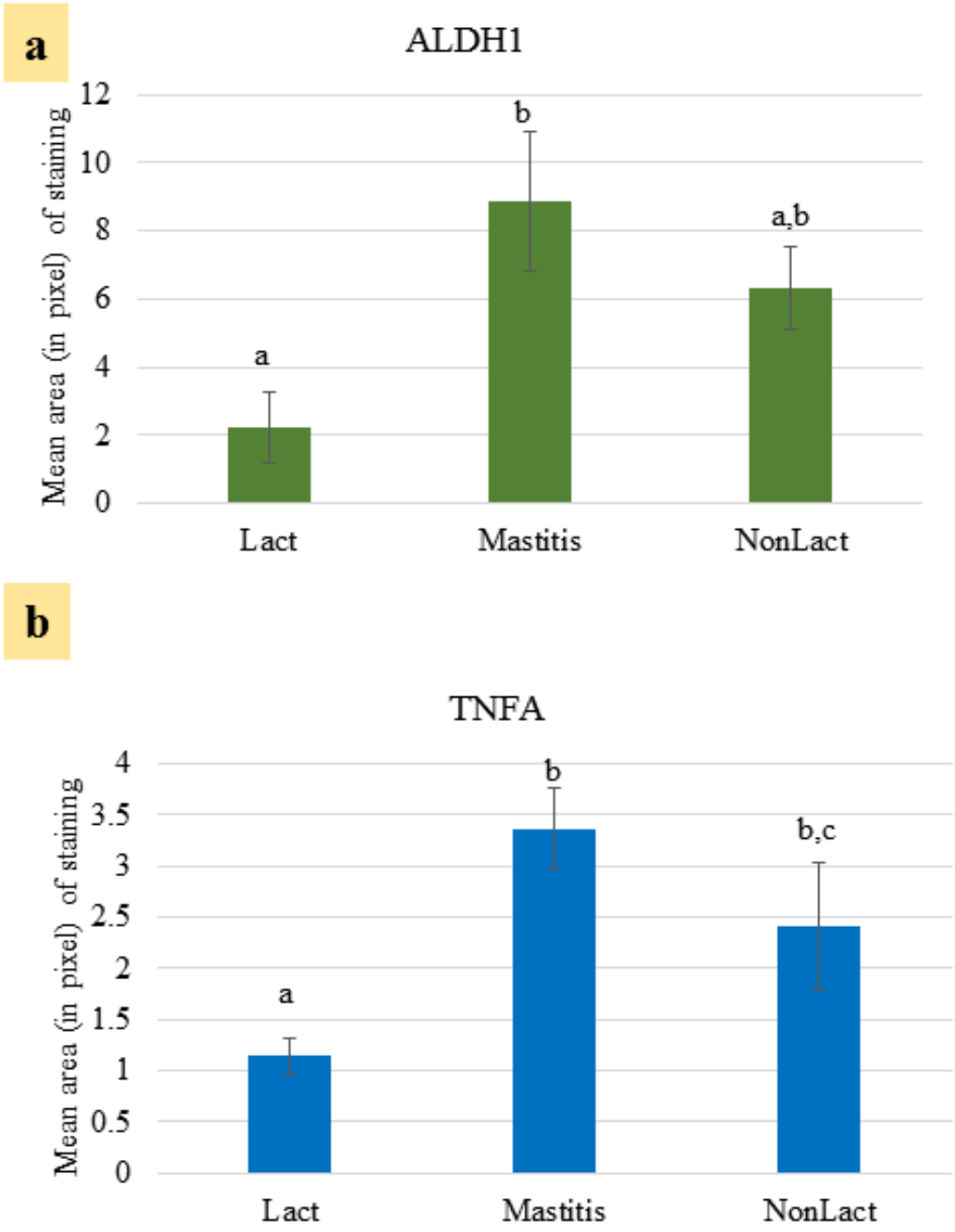

Figure 12: Quantification of immuno-positive signals of ALDH1 and TNFA staining in buffalo mammary tissue.

Photomicrographs of tissue sections were separated into RGB color into red (R), green (G) and blue (B) channels using Image J and quantification of pixel value (Mean + SE) after threshold corrections were calculated. a- Mean area of ALDH1 staining in lactating (Lact), mastitis and non-lactating (NonLact) glands of buffalo. b- Mean area of TNFA staining in lactating (Lact), mastitis and non-lactating (NonLact) glands of buffalo. Different letters on the top of the bars indicate significant difference.

Table 9: Tukey's test showing multiple comparison among physiological stages of the animal on immunolocalization of TNFA in buffalo mammary tissue

Dependent variable: TNFA

Tukey HSD

(I) Stage		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Lact	Mastitis	-2.22*	.461	.000	-3.34	-1.11
	NonLact	-1.27*	.506	.040	-2.49	-.05

ALDH1 is marker of mammary stem/progenitor cells as well as the cancer stem cells (Douville *et al* 2009). High expression of ALDH1 in mastitis gland, we speculate, not due to increased activity of stem/progenitor cells but, the other aspects involved in epithelial-mesenchymal transition and development towards mammary cancer. Stromal ALDH1 may be associated with mammary cancer development (Kunju *et al* 2011). In our earlier reports, we found incidences of aberrant expression of cancer cell markers in prepubertal and mastitis mammary glands in buffalo and goats (Choudhary *et al* 2016b; Choudhary *et al* 2016a).

Tumor necrosis factor-alpha (TNFA) is a marker of inflammation as well as plays important roles in mammary gland growth and development. Action of TNFA is mediated through its receptor TNF receptor (TNFR). In rats mammary gland, expression of TNFA is high during pregnancy and low during lactation (Varela and Ip 1996). During infection, TNFA is secreted through macrophages and involved in variety of cellular activities including cell proliferation, differentiation, apoptosis, lipid metabolism and cancer. In our study, increased expression of TNAF during mastitis indicates inflammation of the mammary gland. Concomitant increase in expression of ALDH1 and TNFA in naturally infected mastitis may be suspected for tumorigenesis, in some of our cases, especially non-lactating and prepubertal gland mastitis. Deficiency of TNFA inhibited growth of breast tumor in TNF $^{-/-}$ null mice

(Warren *et al* 2009). Our samples of naturally infected mastitis, which we suspect might be of chronic in nature, likely to have evidences of development of mammary cancer in buffalo.

We observed a positive correlation (r=0.76; P=3.9E-08) between the expression of ALDH1 and TNFA in buffalo mammary tissue. A high positive correlation indicates that both genes might be co-expressed in mammary glands.

4.4 Tranmsission electron microscopy of lactating buffalo mammary tissue

Tow lactating buffalo mammary tissue samples, evidenced by H&E staining tissue sections (**Figure 13a and 13c**), were selected for ultrastructural details under the transmission electron microscopy (TEM). TEM observations of lactating buffalo mammary tissues showed well developed rough endoplasmic reticulum (RER), nucleus of (N) of MEC, milk fat globules (MFG) and casein protein micelles in the lumen of alveoli (**Figure 13**). Myoepithelial cells (MYO) showed bundles of collagen fibers in the cytoplasm. The myoepithelial cell had a thin rim of cytoplasm. Presence of microvilli on luminal border of MEC was similar to the observations reported earlier in lactating goat (EL-Sayed *et al* 2013). Nucleus of MEC showed double layer of nuclear membrane. Patches of heterochromatin were found along the inner boundary of nuclear membrane as well as in the nucleoplasm.

4.5 Quality and quantity of total RNA isolated from buffalo mammary tissue

RNA quality and quantity isolated from buffalo mammary tissue was of good quality. Integrity of RNA evidenced on gel electrophoresis showed two ribosomal bands of 28S and 18S indicating intact RNA and electropherogram of spectrophotometer (**Figure 14**). Concentration of nucleic acid was measured through NanoDrop spectrophotometer and OD $_{260/280}$ ratio was 1.8 to 2.1. Concentration (mean $\pm$ SE) of RNA (eluted in 20 µL volume of elution buffer) was 1624.5 $\pm$ 246.4. Taken together the data of RNA integrity and electropherogram, data suggest that RNA was of good quality and suitable for cDNA synthesis.

4.6 Amplification of ALDH1, TNFA, and HNF4A genes

Four genes, 3 targets (*ALDH1, TNFA* and *HNF4A*) and one reference gene (*RPS23*) were amplified. Each gene amplification resulted in "normal" qPCR amplification as shown in **Figure 15a**. Melt curve analysis after PCR amplification

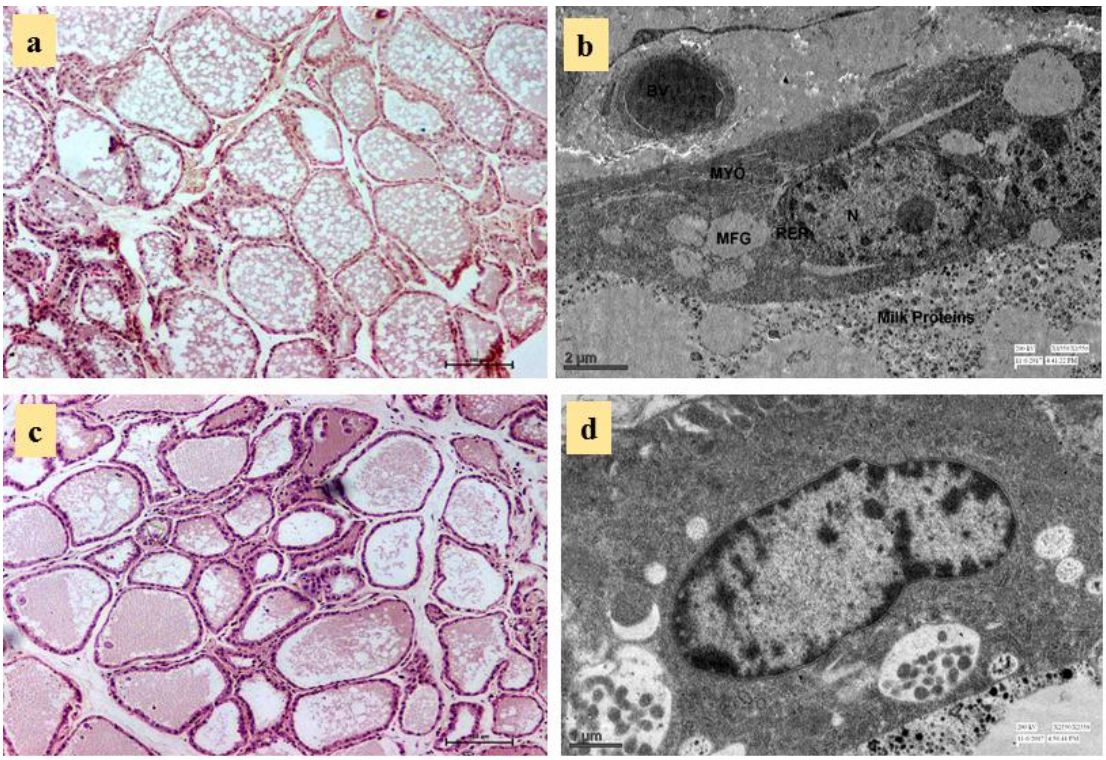

Figure 13: Ultrastructure of lactating buffalo mammary glands.

a, c- lactating mammary glands stained with Hematoxylin and eosin. b, d – transmission electron microscopic images of lactating gland respective to their H&E images in panel a and panel c. Scale bar- a,c = 100 µm; b = 2 µm; d = 1 µm.

MYO- myoepithelial cell; MFG – milk fat globule; RER – rough endoplasmic reticulum; N – nucleus.

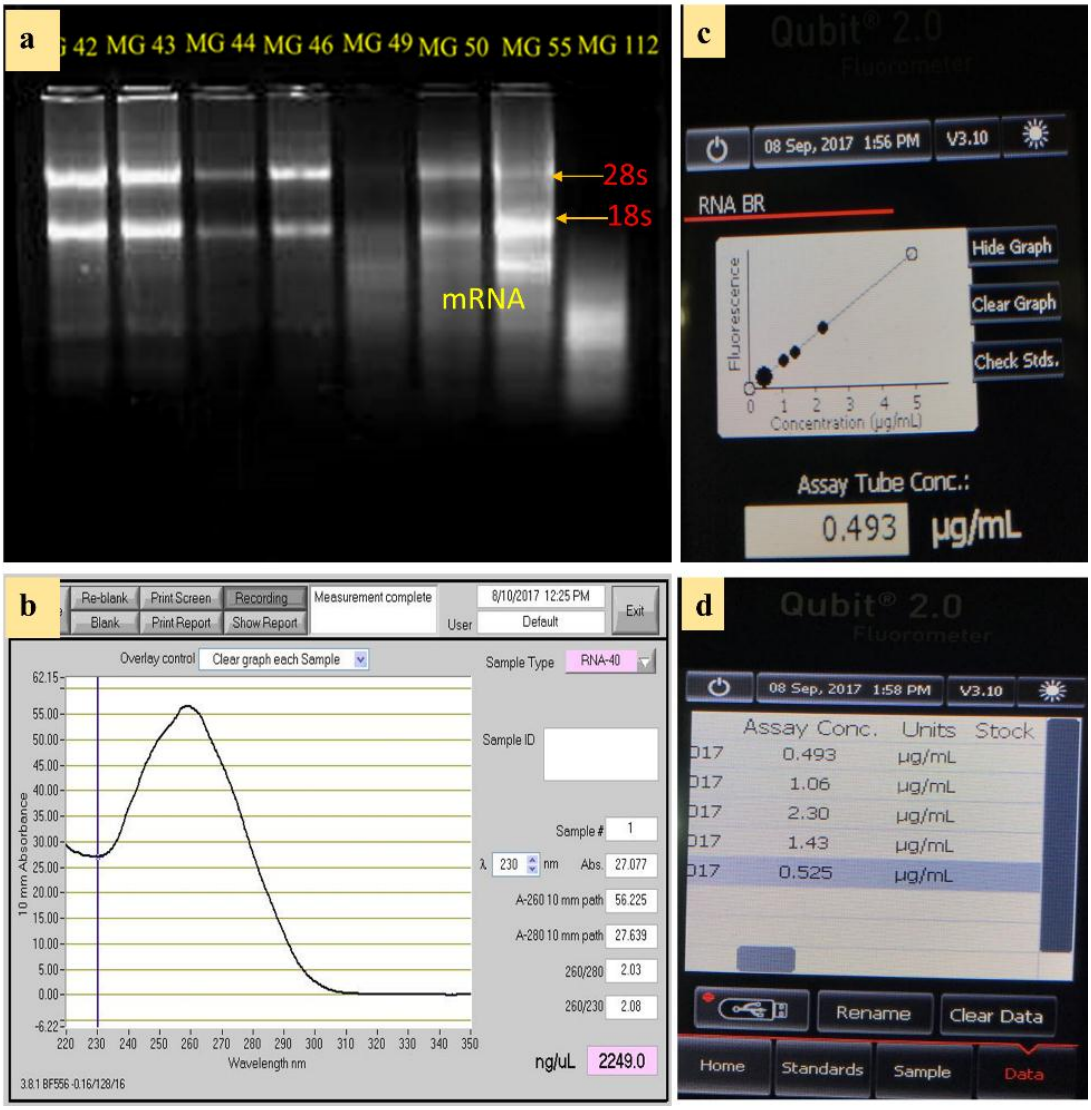

Figure 14: Estimation of RNA quality and concentration using agarose gel electrophoresis and NanoDrop spectrophotometer.

Integrity of RNA was evaluated by running 1% of agarose gel electrophoresis (a) and quantity of total RNA was evaluated by NanoDrop (b) showing electropherogram of one representative RNA sample. Quantity of total RNA of representative samples were also validated by Qubit spectrophotometer to measure absolute RNA quantity (c,d).

resulted single peak indicating the specificity of the product. All of our target genes amplification resulted single melt peak after PCR amlification. Melting analysis is performed to monitors duplex hybridization and thus indicates presence of non-specific amplicon in the reaction mixture. Additonally, we run PCR products on 2.5% agarose gel and found presence of single DNA band of desired product size (not shown here).

4.7 Relative expression of ALDH1, TNFA, and HNF4A genes

The focus of the present study was to evaluate the relative expression of mammary stem cell marker genes and inflammatory. All these genes were successfully amplified with the presence of a single peak in melt cure analysis and single and of PCR amplicon was visualized in agarose gel electrophoresis (not shown). Estimation of relative quantity of ALDH1, HNF4A and TNFA when analyzed sample wise, showed varied expressions of these three markers (**Figure 16**), indicating biological variations of gene expressions among the collected mammary samples.

Data were analyzed by two-way ANOVA keeping dCt as dependent variable and stages of animals as fixed factor. Higher the dCt value, lower is the gene expression.

These animals were grouped into three distinct groups depending on the histology and marker expressions as indicated in earlier section. Analysis of gene expression groupwise showed the mean relative expression (delta Ct) of genes vary among the different stages of the animal (**Figure 17**).

In comparison to lactation, relative fold change (log 2 value) of ALDH1 and TNFA was increased during mastitis by 2.98 and 4.71, respectively. However, the relative log 2 fold change of HNF4A, putative mammary stem cell marker, was decreased (-7.39) during the mastitis (**Figure 18**). Correlation analysis of normalized threshold value (delta Ct) of ALDH1 and TNFA revealed expression of both markers were positively correlated ($r = 0.76$; $P= 3.9E-08$) across the lactating, non-lactating and mastitis stage.

Data were analyzed in the non-lactating and mastitis mammary glands in comparison to lactating glands. Expression of ALDH1 and TNFA was increased whereas, HNF4A was decreased in mastitis gland.

To examine the effect on marker expression in the various physiological stage, 2-way ANOVA was conducted. A significant difference was observed in the expression of one of these three genes due to the physiological stage of the animal and expression of the marker, F (8, 53) = 30.4; P-value <0.01 (**Table 5**).

Table 10: Two-way ANOVA table showing the effect of stage and markers on the dCt value

Tests of Between-Subjects Effects					
Dependent Variable: dCt					
Source	**Type III Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Corrected Model	1032.23	8	129.029	43.936	.000
Intercept	4873.157	1	4873.157	1659.359	.000
Stage	15.692	2	7.846	2.672	.079^{NS}
Markers	673.500	2	336.750	114.667	.000**
Stage * Markers	369.563	4	92.391	31.460	.000**
Error	146.839	50	2.937		
*NS: non-significant; ** Highly significant*					

A two way ANOVA was conducted that examined the effects of markers and stage of the animal on the gene expression (in terms of relative threshold value or dCt) of markers. There was a signficiant interaction between the marker and stage of the animal on gene expression of those markers, F (4, 50) = 31.4, P = 0.000 (**Table 10**). There was no statistically significant difference in mean value of stage of the animal among the markers (P = 0.079), but there were statistically significant differences among the expression of these three markers (P = .0000) (**Table 11**).

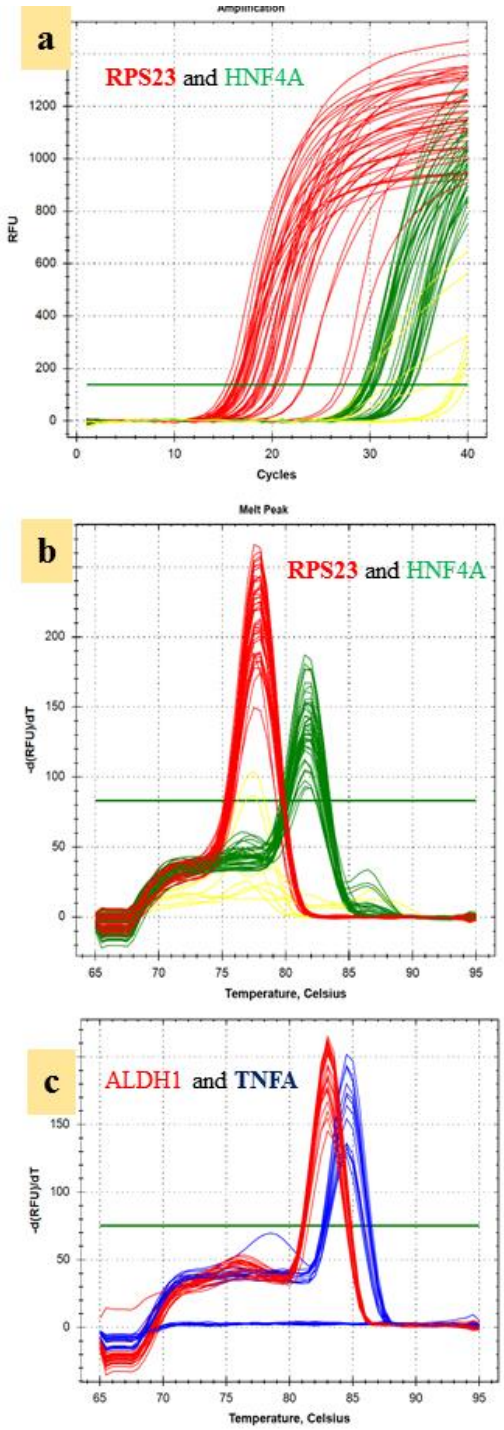

Figure 15: Amplification plot and melt curve analysis of expressed genes (RPS23, HNF4A, ALDH1 and TNFA) in buffalo mammary glands.

a-…… R T-qPCR amplification plot of endogenous gene RPS23 and mammary stem/progenitor cell marker HNF4A; b- Melt peak curve analysis of RPS23 and HNF4A; c – Melt peak analysis of mammary stem/progenitor cell and cancer stem cell marker ALDH1.

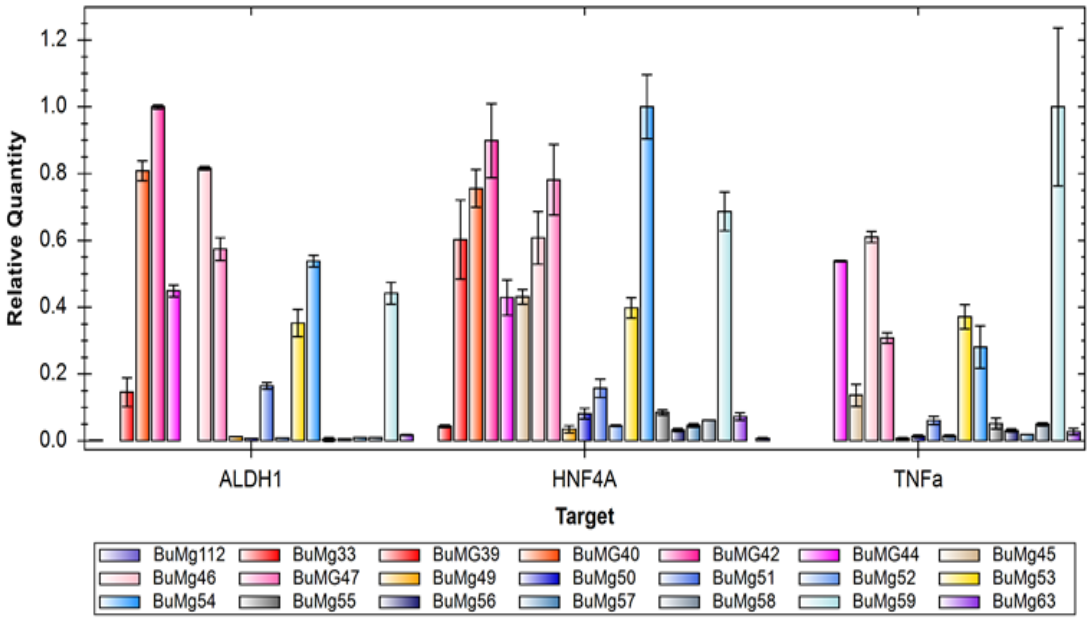

Figure 16: Sample-wise relative quantification of ALDH1, HNF4A and TNFA in buffalo mammary glands.

Table 11: Two-way ANOVA multiple comparison table showing the effect markers on the dCt value

Multiple Comparisons

Dependent Variable: dCt

Dunnett t (2-sided)[a]

(I) Markers	(J) Markers	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
HNF4A	ALDH1	3.9744*	.53543	.000	2.7552	5.1936
TNFA	ALDH1	8.2545*	.55677	.000	6.9867	9.5223

**. The mean difference is significant at the .05 level.*

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Mastitis can be defined as multi-etiological factor disease-causing inflammatory reactions to the mammary parenchyma of the gland. Mastitis in buffalo is not uncommon and annual incidence may reach up to 37% in Murrah buffalo (Jingar *et al* 2017). Acute clinical cases of mastitis are thought well characterized by the typical symptoms of blood and pus containing milk, hard and hot indurated udder, shreds of fibrin in milk and high somatic count. Chronic mastitis may arise as a sequela of acute infection if acute mastitis remains untreated. Chronic mastitis leads to progressive fibrosis of the gland showing udder asymmetry that may eventually lead to atrophy (Fagiolo and Lai 2007) and results in agalactia. Naturally infected mastitis, all of our samples, were collected from the slaughterhouse, indicating animals inability to produce milk. These affected animals were appeared to be chronically affected mammary gland and may have atrophy or hyperplasia of mammary epithelial or stromal cells.

ALDH1 is an enzyme and has been shown to be highly expressed by stem-like cells of the mammary gland (Ginestier *et al* 2007). High expression of ALDH1 is considered to be the marker of normal as well as cancer mammary stem cells (Douville *et al* 2009). High expression of ALDH1 in buffalo mastitis may be associated with increased expression of stem cells or cancer stem cell activity. TNFA stimulates EMT and generates breast cancer stem cells (Asiedu *et al* 2011), Thought

EMT is a natural process of gland development and tissue repair, however, it is also a hallmark of cancer development (Felipe Lima *et al* 2016).

Interestingly, down-regulation of HNF4A has been correlated with epithelial-mesenchymal transition (EMT) of hepatocytes (Delaforest *et al* 2011). However, expression of HNF4A, in comparison to lactation, was decreased during buffalo mastitis. HNF4A is a liver stem cell marker (Cicchini *et al* 2015) and has been suggested as bovine (Choudhary *et al* 2013) and Buffalo (Choudhary *et al* 2016b) MaSC/progenitor cell marker. The result of this study is consistent with our earlier study, where expression of HNF4A-positive MaSC/progenitor cells was decreased during buffalo mastitis (Choudhary *et al* 2016b).

To further delineate acute infection from chronic infection of the mammary gland, expression of TNFA was analyzed. Relative expression of TNFA did not differ between lactation and non-lactation stage, whereas, TNFA expression was significantly up-regulated during mastitis. Although, an elevated level of TNFA expression at lactation, parturient period and mastitis is necessary to display its indispensable role in the regulation of inflammatory cells. However, an elevated level of TNFA in serum and milk is found in experimentally induced (Hoeben *et al* 2000) and naturally infected coliform mastitis (Nakajima *et al* 1997). TNFA is one of the candidate markers to monitor the severity of the coliform mastitis and prognosis (Alluwaimi 2004). In bovine, subclinical mastitis, which is often unnoticed by the small farmers of the village, may leave untreated and developed into chronic mastitis. Subclinical mastitis, in bovine, is often developing into chronic mastitis (Gogoi-Tiwari *et al* 2017)**.** Study suggested that tumor necrosis factor receptor–associated factor 1 has potential direct or indirect regulations of cancer stem cells in oral squamous cell carcinoma (Wu *et al* 2017), indicating the role of TNFA in regulation of cancer stem cells.

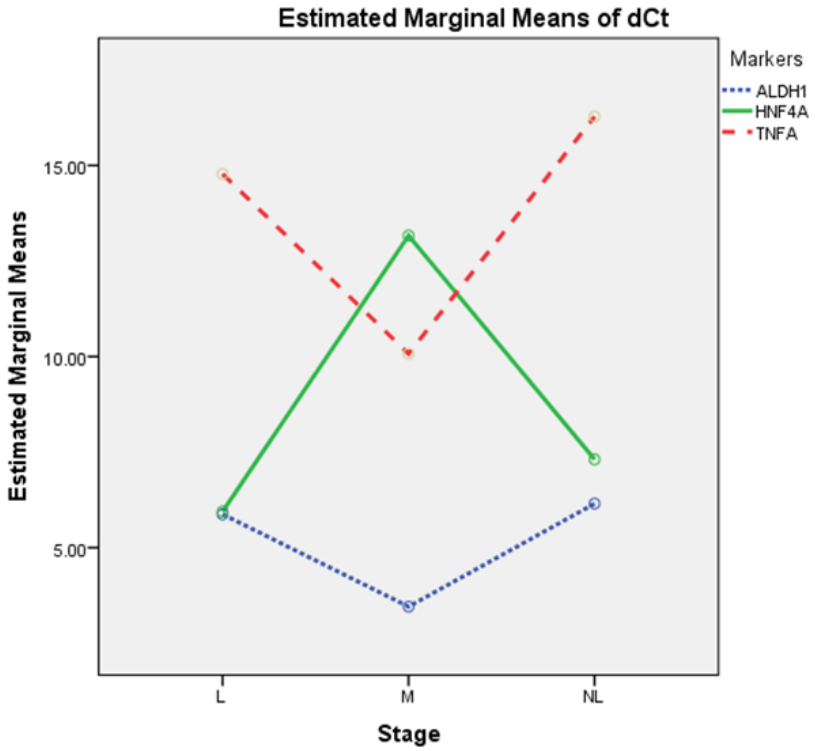

Figure 17: Estimated marginal means of delta Ct (dCt) value of ALDH1, HNF4A and TNFA in the buffalo mammary glands.

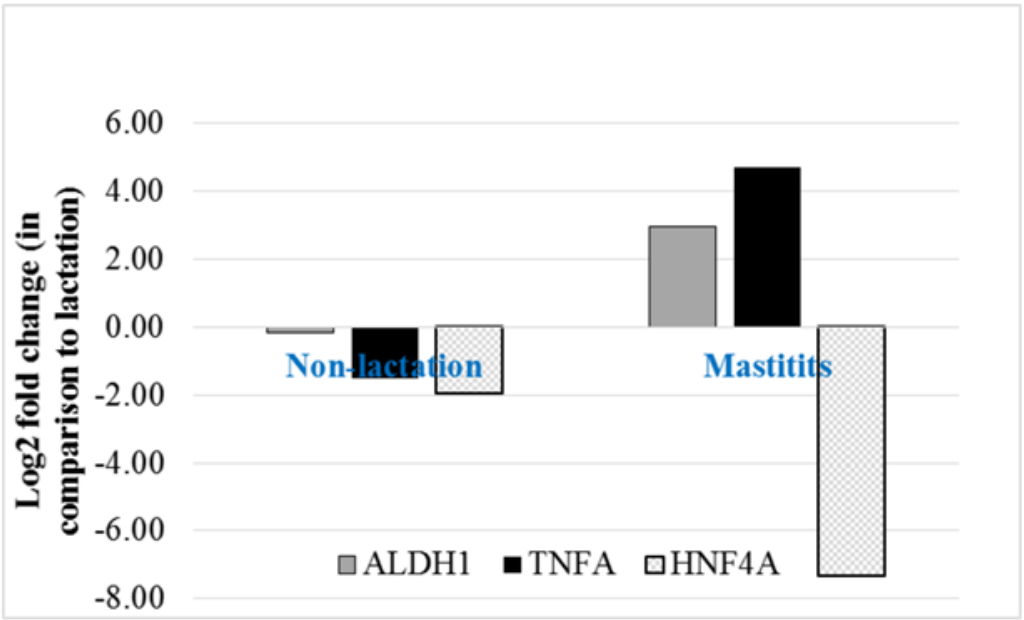

Figure 18: Fold change (log 2) expression of ALDH1, HNF4A and TNFA in buffalo mammary tissue.

CHAPTER V

SUMMARY AND CONCLUSIONS

Knowledge of buffalo mammary tissue and its gene expression pertaining to mammary epithelial cells and mammary stem cells are very limited. This investigation explores percentage composition of various cell types of buffalo mammary tissues obtained from abattoir and explore protein and gene expression of stem/cancer cell marker and inflammatory marker. Our objectives were to evaluate expressions of mammary stem cell marker, aldehyde dehydrogenase 1 (ALDH1) and inflammatory marker tissue necrosis factor alpha (TNFA) in the mammary tissue of buffalo. We hypothesized that 1) ALDH1 identifies the mammary stem/progenitor cells and mammary cancer stem cell population and 2) expressions of ALDH1 and TNFA differs among the various physiological stages of buffalo. Cell surface markers of one species have been used to identify presumptive mammary stem cell population of other species. This investigation has utilized ALDH1 to identify buffalo mammary stem/progenitor cells. The histological investigations exhibited binding of the ALDH1 in the cytoplasm and nucleus of MEC while HNF4A was exclusively present in the nuclei of the cells. The location of TNFA was in the MEC of epithelial compartment (prominent in mastitis tissue) and in the inflammatory cells of stromal compartment, indicated an active role of TNFA in the activities of the stroma and alveoli of the buffalo mammary gland. Additionally, gene expression of ALDH1, HNF4A and TNFA were investigated. It was found that transcripts of ALDH1 and TNFA was upregulated while, transcripts of HNF4A was down-regulated during mastitis (**Figure 18**). Interestingly, increased expression of TNFA was positively correlated with the expression of ALDH1. Since, the expression of HNF4A, a buffalo putative mammary stem/progenitor cell marker decreased during the mastitis, it is logical to suspect that ALDH1 identifies mammary cancer stem cells during mastitis. Thus, this study identifies the association of expression of ALDH1, TNFA and HNF4A in buffalo mastitis.

CONCLUSIONS

1. Protein expression of TNFA in mammary tissue varies among lactation, non-lactation and mastitis glands, being highest expression during the mastitis.
2. Protein expression of mammary stem/progenitor cell and cancer stem cell marker, ALDH1 in mammary tissue significantly differ between lactation and mastitis and no difference in ALDH1 expression was found between lactating and non-lactating animals.
3. Gene expression of *ALDH1*, *HNF4A* and TNFA in buffalo mammary tissue varied significantly among the animals.
4. There was a significant interaction between the markers (ALDH1, HNF4A and TNFA) and stages (lactating, non-lactating and mastitis) of the animal on gene expression.

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VITA

Name of the student	:	Sheetal Thakur
Father's name	:	S. Santosh kumar Rana
Mother's name	:	Mrs. Kushla Devi
Nationality	:	Indian
Date of Birth	:	24-12-1994
Permanent address	:	Vill. Bharin, P.O. Ropa, Distt. & Teh. Hamirpur (H.P.) Pin code-177001

EDUCATIONAL QUALIFICATIONS

Bachelor degree	:	B.Sc. Biotechnology
Institute/University	:	Punjab College Groups of Technical Education
Year of award	:	2016
OGPA/OCPA/%marks	:	70%
Masters degree	:	M.Sc. Biotechnology
OCPA	:	7.23/10.00