Isolation and Characterization of Genetic Variants of Beta-Casein Protein (A1/A2) and Study Their Impact on Early Precipitation of Osteoporosis

THESIS

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by

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CERTIFICATE

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List of Abbreviations and Symbols

AC	Adenylate cyclase
ACD	Acid citrate dextrose
ACTH	Adrenocorticotropic hormone
AMQW	Autoclaved milli-q water
ANOVA	Analysis of Variance
ATP	Adenosine triphosphate
BCM7	Beta-casomorphin 7
BMC	Bone mineral content
BMD	Bone mineral density
BMPs	Bone morphogenic proteins
AP	Anteroposterior
BS	Bone surface
BS/BV	Specific bone surface
BS/TV	Bone surface density
BV	bone volume
BV/TV	Bone volume fraction
Ca	Calcium
cAMP	cyclic Adenosine monophosphate
CCD	charge-coupled device
COPD	Chronic obstructive pulmonary disease
CPCSEA	*Committee for the purpose of control and supervision of experiments on animals*
CRH	Corticotropin-releasing hormone
CSN2	Beta-casein gene
CTX	Carboxyterminal telopeptide of type I collagen
2D	2 dimensional
3D	3 dimensional
DA	Degree of anisotropy
DEXA	Dual-energy X-ray absorptiometry
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulphate
DNA	Deoxyribonucleic acid
DPP-IV	Dipeptidyl peptidase IV
EDTA	Ethylene di amine tetra acetic acid
EIA	*Enzyme immunoassay*
ELISA	Enzyme-linked immunosorbent assay
Fig	Figure
FIS	Fixation index
FSH	Follicle stimulating hormone
GIT	*Gastrointestinal tract*
GnRH	Gonadotropin-releasing hormone
GPCR	G-protein coupled receptor
He	Heterozygosity
HF	Holstein friesians
Ho	Homozygosity
HPA	Hypothalamic pituitary adrenal
HPG	Hypothalamic pituitary gonadal
IAA	Isoamyl alcohol

IAEC	*Institutional animal ethics committee*
IFN-γ	Interferon gamma-γ
IL	Interleukin
IP	Intraperitoneal
ISBMR	Indian society of bone and mineral research
IST	*India Standard Time*
JNK	Jun N-terminal kinase
LH	Luteinizing hormone
LSC	Lower socioeconomic class
LTR	Likelihood ratio test
M-CSF	Macrophage colony stimulating factor
MALDI-MS/MS	Matrix-assisted laser desorption/ionization- Tandem mass Spectrometry
µCT	Microcomputed tomography
MIL	Mean intercept length
MQW	Milli-Q water
NFkB	Nuclear-factor kappa B
NaCl	Sodium chloride
NCBI	*National center for biotechnology information*
NPD	Normal pellet diet
Na	Sodium
NHP	National health priority
NBAGR	National Bureau of Animal Genetic Resources
OC	osteocalcin
OVX	*Ovariectomized*
OPG	osteoprotegerin
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PMF	Peptide mass fingerprinting
PMW-L	*Protein Molecular Weight Marker* - Lower Range
PO	Per oral
QUS	Quantitative ultrasonography
RANK	Receptor for activated nuclear factor kappa B
RANKL	Receptor for activated nuclear factor kappa B ligand
RGI	Registrar general and census commissioner of India
RTI	Respiratory tract infection
RT	Room temperature
SEM	Standard error of the mean
SDS	*Sodium dodecyl sulfate*
SIDS	sudden infant death syndrome
SMI	Structure model index
T1	Type-1 Diabetes Mellitus
Tb.N	Trabecular number
Tb.Sp	Trabecular separation
Tb.Sp.SD	Standard deviation of trabecular thickness
Tb.Th.SD	Standard deviation of trabecular separation
Tb.Th	Trabecular thickness
TGF-β	Transforming growth factor-β
TNFα	Tissue necrosis factor α
Tris	2- Hydroxyl methyl amino methane

TRAF-6	Tumor necrosis factor receptor-associated factor
TV	Total volume
USC	Upper socioeconomic class
UTM	Universal testing machine
VOMACs	volumetric marching cubes
WHO	World health organization
°C	Degree Celsius
cm	Centimeter
g	Gram
h	Hour
Kg	Kilogram
KD	Kilo Dalton
KN	kilo Newton
M	Meter
mg/dl	Milligram per deciliters
mg/kg	Milligram per kilogram
µCT	Micro–computed tomography
min	Minute
ml	Milliliter
mmol	Millimol
µl	Microlitre
µg	Microgram
µg/ml	Microgram per milliliter
mg	Milligram
mm	Millimeter
ng	Nanogram
pg	Picogram
pmol	Picomol
v/v	volume by volume

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Abstract

Caseins account for 80% of bovine natural milk and remaining 20% is serum, or whey protein. Caseins are encoded by a cluster of 4 genes, namely, α_{S1}-casein, α_{S2}-casein, β-casein and κ-casein, located on the 6th chromosome of cattle. Beta-casein has about 13 genetic variants, A1 and A2 variants have highest allelic frequency in dairy cattle. There is a single amino acid difference in A1 and A2 variants of beta-casein at position 67 of amino acid chain, A1 with histidine (CAT) and A2 with proline (CCT). A seven amino acid peptide, beta-casomorphin7 (BCM7) is released during the microbial fermentation or enzymatic digestion of A1 beta-casein, but it is not released by A2 beta-casein, this is the reason why A2 milk is believed to be much healthier than A1 milk. BCM7 is an important bioactive peptide having structural similarity with opioid peptides and have strong opioid activity, via μ-opioid receptors located on nervous, endocrine, immune systems and gastrointestinal tract as well as in the osteoblasts of mammals. Due to this activity, it is suggested to be associated as a risk in development of some important human diseases like Sudden Infant Death Syndrome (SIDS), Type-1 Diabetes Mellitus, and Schizophrenia, etc.

Genotype of cattle in Pilani region was estimated, by sequencing the exon VII followed by multiple alignment and chromatogram analysis. Cattle were then divided into A1A1, A1A2 and A2A2 genotypic groups and milk samples were collected. Female Wistar rats were randomly divided in 8 groups; test groups were either fed with beta-casein protein isolated from A1 milk or natural milk from A1A1 and A2A2 cows, in order to compare the effect of isolated protein and proteins present in natural milk. Synthetic BCM7 was given by both intraperitoneal *(IP) and oral route (PO) (1mg/kg), to compare effect* of enzymatic hydrolysis on BCM7 (during gastrointestinal digestion and absorption).

Dosing for all animals was done, for 4 months, along with normal pallet diet, to investigate the impact of BCM7 on precipitation of osteoporosis.

The overall genotype of HF cattle identified had 9% A1A1, 69.7% A1A2 and 21.3% A2A2 alleles. The allele frequency analysis of HF cows was 0.44 for A1 allele, 0.56 for A2 allele and 0.09, 0.70, 0.21 for genotypes A1A1, A1A2, and A2A2, respectively. The allele frequency analysis for Zebu cows was 0.1 for A1 allele, 0.9 for A2 allele and 0.00, 0.10, 0.9 for genotypes A1A1, A1A2, and A2A2, respectively. The value of gene homozygosity (Ho), gene heterozygosity (He) and fixation index (FIS) were found to be 0.40, 0.60, and - 0.20, respectively. Average milk yield in HF cattle was A1A1-16 L/day, A1A2-15.5L/day, A2A2 15.25L/day.

Low levels of serum osteocalcin, calcium and phosphorus, decreased bone tensile strength, high count of osteoclast cells, increase in body weight and decreased uterine weight in BCM7/A1 protein/A1 natural milk treated rats clearly indicated the adverse effect of BCM7, on bone formation and strength. Quantification of the trabecular bone and 3D images analysis obtained by Micro CT scans, revealed using measurements and 3D images analysis showed a significant decrease of trabecular bone volume, trabecular number, trabecular thickness and increased trabecular space, which strongly indicated degradation of bone in BCM7/isolated protein/A1 natural milk fed rats.

The studies conducted leads to an inference that people consuming milk of A1 variant cattle on a regular basis, may be at high risk of osteoporosis, or its early precipitation. Further specific studies, in a larger population and human trials, may be of importance to strengthen the findings and the hypothesis.

1. Introduction

1.1. Milk and Milk proteins:

Milk contains 3.3% total protein which is broadly defined in two 2 major categories (caseins and whey proteins), by their chemical composition and physical properties. Caseins account for 80% of bovine natural milk and remaining 20% is serum or whey protein. Caseins are encoded by a cluster of 4 genes, namely, alpha $_{S1}$-casein (α_{S1}-casein), alpha $_{S2}$-casein (α_{S2}-casein), beta-casein (β-casein) and kappa-casein (κ-casein), located on the 6th chromosome of cattle. Beta-casein proteins make up approximately 30% of the total protein of bovine milk (Phelan et al., 2009). Milk is undoubtedly the most favourite food, consumed for its nutritional aspects further which is processed to develop various dairy products such as yogurt, skim milk powder, cheese, etc. Casein is also incorporated in animal feed supplements, various industrial products such as pharmaceuticals, cosmetics, paint, glue, etc. Industrial production of casein, hence has become more important in the last few decades. Beta-casein does not form intermolecular disulfide bonds and contains a high number of proline residues, which do not interact. It has low molecular mass and is stable at large range of temperature (30-150 ºC) (T Huppertz et al., 2018; Sauer et al., 2012), **Table 1.1** describes important biochemical characteristics of milk proteins. In addition, beta-casein also has all the key characteristics of an excellent surface-active agent and polymeric stabilizer. Beta-caseins have excellent foaming and emulsification properties and can be used in various applications such as in paints, glues, protein supplements, cheese making, etc.

Table 1.1: Biochemical characteristics of milk proteins (EFSA, 2009; Eigel et al., 1979; Livney, 2010; Tay et al., 2011)**.**

Protein	Approx.% of skim milk protein	Isoelectric point	Molecular weight (KDa)
α-Casein	45-55	4.1	22.5, 24
κ-Casein	8-15	4.1	19
β-Casein	25-35	4.5	25.5
γ-Casein	1-7	5.8-6.0	--
α-Lactalbumin	2-5	5.1	14.4
β-Lactoglobulin	7-12	5.3	18
Blood serum albumin	0.7-1.3	4.7	68
Lactoferrin	0.2-0.8	--	87
Immunoglobulins:	--	--	--
IgG1	1-2	--	160
IgG2	0.2-0.5	--	160
IgM	0.1-0.2	--	~1,000
IgA	0.05-0.10	--	~400
Proteose peptone fraction	2-6	3.3-3.7	4.1 to 200

The share of global milk production of five largest milk producers is the European Union (20%), India (20%), USA (12%), Pakistan (6%), and China (5%). India with a global share of 25% in 2027, is poised to have the largest growth in milk production. Thus, India has witnessed remarkable growth in production and consumption of milk in recent years and this trend is almost certain to continue. India produces over 150 million tonnes of milk/year and per capita availability of over 300 grams per day. In the year 2015-16, the growth rate of milk production had been 6.28 % and total production reached 156 million tonnes. The per capita milk availability was 176 grams per day in 1990-91, increased to 337 grams per day by 2015-16, which is more than the world average of 294 grams per day. **Table1.2** provides details of per capita availability of milk in different states of India.

Table 1.2: State wise per capita availability of milk during 2015-16.

S. No.	States/UTs	Per capita availability (grams/day)
1	Andhra Pradesh#	475
2	Arunachal Pradesh	105
3	Assam	70
4	Bihar	219
5	Chhattisgarh	133
6	Goa	74
7	Gujarat	545
8	Haryana	877
9	Himachal Pradesh	505
10	Jammu & Kashmir	395
11	Jharkhand	152
12	Karnataka	282
13	Kerala	200
14	Madhya Pradesh	428
15	Maharashtra	239
16	Manipur	76
17	Meghalaya	83
18	Mizoram	57
19	Nagaland	89
20	Odisha	124
21	Punjab	1032
22	Rajasthan	704
23	Sikkim	282
24	Tamil Nadu	283
25	Tripura	109
26	Uttar Pradesh	335
27	Uttarakhand	434
28	West Bengal	145
29	A&N Islands	87
30	Chandigarh	93
31	Dadra & N. Haveli	72
32	Daman & Diu	10
33	Delhi	36
34	Lakshadweep	113
35	Puducherry	108
	All India	**337**

#Included Telangana, **Note:** Per capita availability is calculated based on State estimates of production and projected population as on 1st March, based on Census of India 2001 of Registrar General and Census Commissioner of India (RGI).

Information derived from- Press Information Bureau, Government of India, Ministry of Agriculture & Farmers Welfare 19-July-2016 16:23 IST.

In India, the vast majority of milk produced is consumed domestically as fresh milk products, instead of processed products, as mentioned in **Fig. 1.1.** Thus there is scope in the developing countries like India and Pakistan for milk processing industries, based on milk proteins. As a result of the globalization of diet and increase in income and population, more demand and consumption of dairy products are expected in developing countries. As per OECD/FAO (2018), per capita consumption in developed countries is projected to grow from 22.2 kg in 2015-17 to 23.1 kg in 2027 in milk solids, compared to an increase from 10.6 kg to 13.5 kg in developing countries

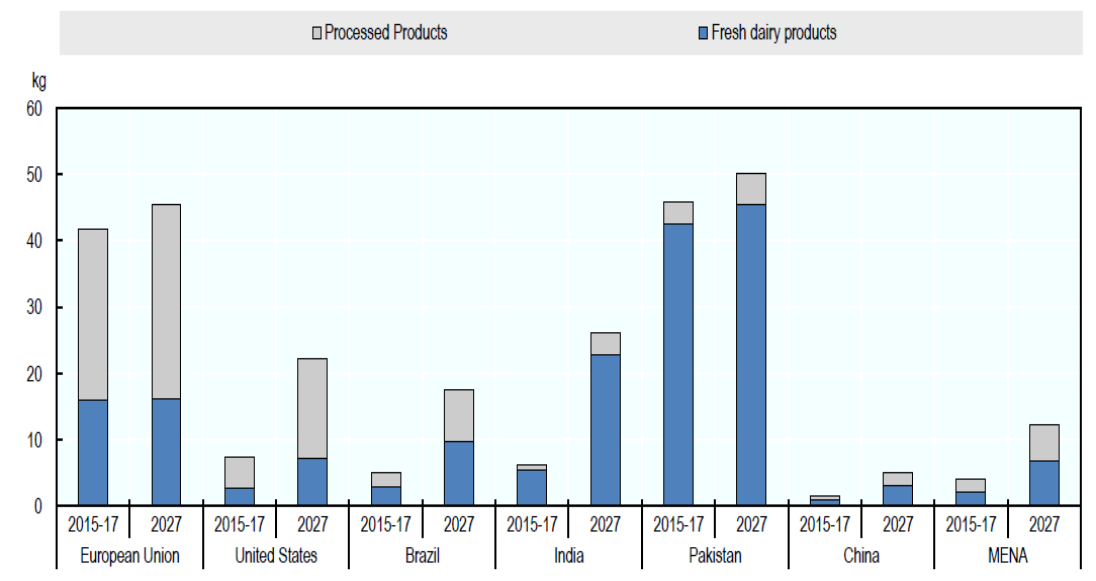


Fig. 1.1: Per capita consumption of processed and fresh dairy products in milk solids.
Note: Milk solids are calculated based on fat and non-fat solids (SNF) amount of for each product; Important processed products include are skim milk powder, butter cheese, and whole milk powder.
Source: OECD/FAO (2018), "OECD-FAO Agricultural Outlook", OECD Agriculture statistics (database), http://dx.doi.org/10.1787/agr-outl-data-en.

1.2. Beta-casomorphin 7 (BCM7):

European cattle have expressed a point mutation of cytosine to adenine at 201st position (exon VII) in the beta-casein encoding gene, few thousand years ago (Bradley et al., 1998). Based on this genetic variation, two alleles of beta-casein have been classified, namely A1 and A2 (Thom Huppertz et al., 2006). The A1 allele is the mutated protein having a Histidine (codon; CAT) at position 67, while the A2 allele is the regular protein having a Proline (codon; CCT) as mentioned in **Fig. 1.2** (Colameco et al., 2009; Kaminski et al., 2007; Pal et al., 2015; Woodford, 2009). The point mutation is believed to be the reason for the appearance of A1 beta-casein in some European herds (Thom Huppertz et al., 2006; Kaminski et al., 2007). A1 is most frequent in Holstein-Friesian (HF), Ayrshire and Red cattle (**Table 1.3**). In contrast, a high concentration of A2 is observed in Guernsey, Jersey, Indian Zebu cattle and Indian buffalo (Kaminski et al., 2007; Pal et al., 2015) (**Table 1.4**).

There are reports on the presence of both homogenous (A1A1or A2A2) alleles and heterozygous (A1A2) allele of beta-casein gene (CSN2), in a given population and is also associated with co-dominance of alleles (Eigel et al., 1984; Massella et al., 2017; Olenski et al., 2010) . There are other alleles present in population i.e. A3 E, D, H and I (**Fig. 1.3**), however, they are rare compared to A1 & A2 and very less information is available about the same (Formaggioni et al., 1999; Massella et al., 2017; Ng-Kwai-Hang et al., 2003).

This difference in amino acid sequence at position 67 of A1 beta-casein, suggests a conformational difference in the secondary structure of the expressed protein. It may possibly exert an influence on the physical properties of the respective casein micelles (Elliott et al., 1999; Groves, 1969). Casein peptides are not active within the parent protein (beta-casein) but can be released and activated during enzymatic hydrolysis,

microbial fermentation and during gastrointestinal digestion in humans (**Fig.1.4**). The A1 beta-casein protein, upon protease degradation, generate 7 amino acid long an opioid peptide, BCM7. Peptides other than BCM7, are also produced in very less amount, which are less stable and further degraded in the intestine as well as in the blood (Brooke-Taylor et al., 2017; Defilippi et al., 1995; Fiedorowicz et al., 2011; Phelan et al., 2009; Stefanucci et al., 2018). BCM7 resists further hydrolysis by intestinal brush border enzymes and gets absorbed intact, enters the systematic circulation, and acts through μ opioid receptors (Paroli, 1988; Teschemacher et al., 1997). BCM7 has been reported to be linked to numerous disorders and diseases, as heart disease, Type-1 Diabetes Mellitus (T1DM), Sudden Infant Death Syndrome (SIDF), etc. (Brooke-Taylor et al., 2017; Elliott et al., 1999; Fiedorowicz et al., 2011; Kaminski et al., 2007; Marcone et al., 2017; McLachlan, 2001; Phelan et al., 2009)

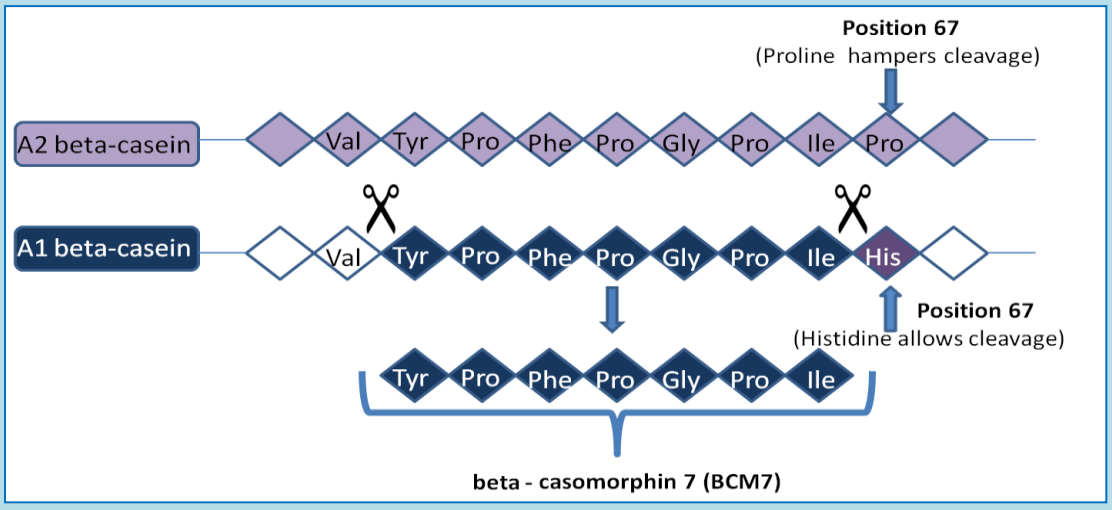


Fig. 1.2: A1 and A2 beta-casein proteins showing the amino acid difference at position 67. A1 variant has one histidine amino acid at position 67 that undergoes the enzymatic cleavage of the peptide bond, and BCM7 is released. The A2 variant does not cleave due to the presence of a proline amino acid at this position (Colameco et al., 2009; Woodford, 2009).

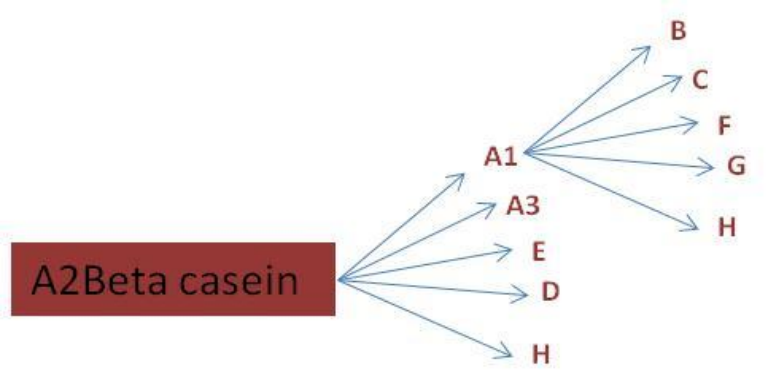


Fig. 1.3: More recent forms of beta-casein.

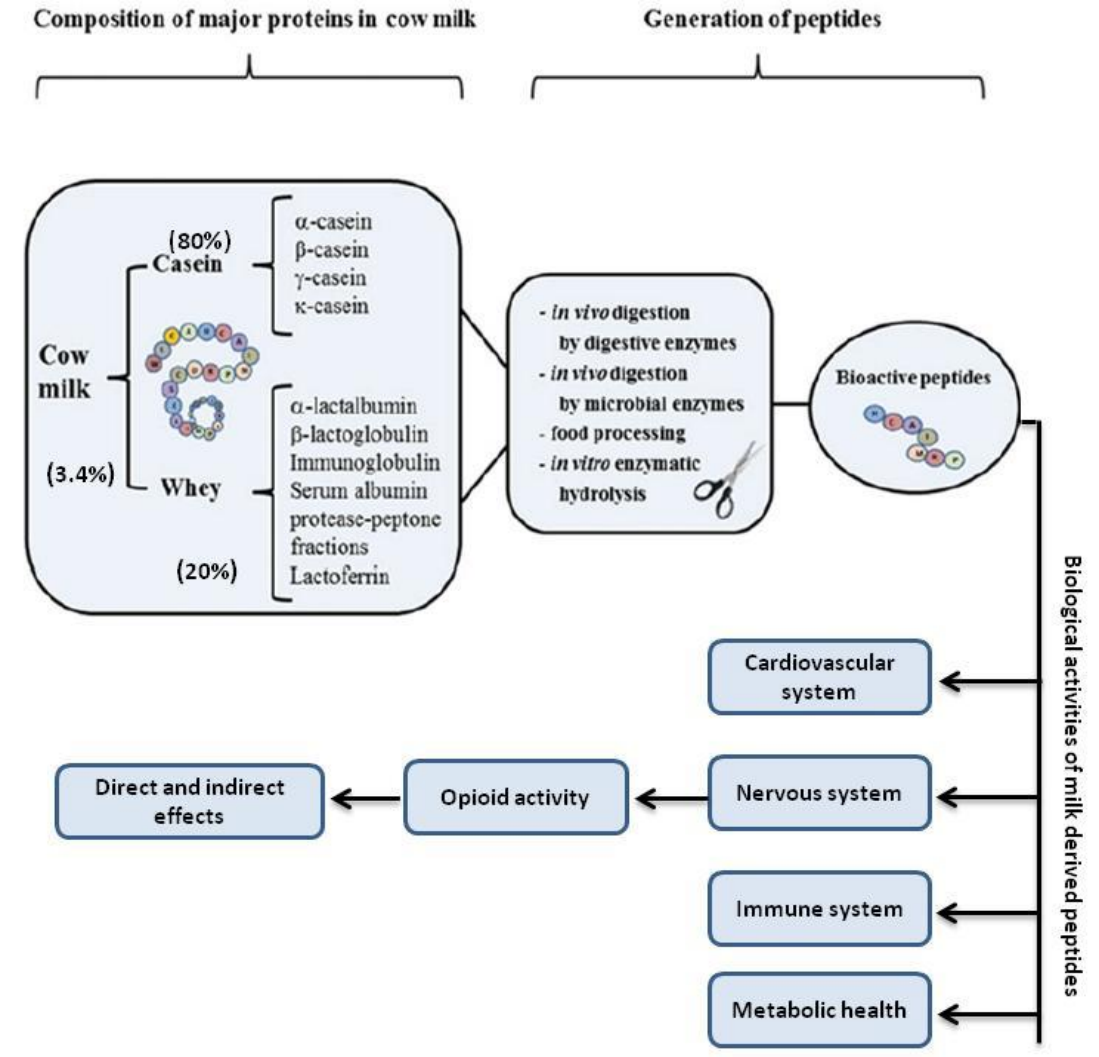


Fig. 1.4: Schematic illustrating generation of milk-derived bioactive peptides and their physiological functionalities (Marcone et al., 2017).

Table 1.3 Frequency of beta-casein alleles in various breeds of different countries (Kamiński et al., 2007).

Breed	Country	Frequency of beta-casein alleles	
		A1	A2
Jersey	Germany Denmark New Zealand	0.093 0.070 0.123	0.721 0.580-0.650 0.591
HF	USA USA Hungary Germany Polland Zew N Norway	0.310-0.660 0.310–0.490 0.418 0.472 0.402 0.465 0.400	0.240-0.620 0.490–0.620 0.470 0.496 0.598 0.510 0.490
Ayrshire	New Zealand Finland United Kingdom USA	0.432 0.509 0.600 0.720	0.527 0.490 0.400 0.280
Red	Denmark	0.710	0.230

Table 1.4 Frequency of beta-casein alleles in various breeds of India.

Breed	Frequency of beta-casein alleles		References
	A1	A2	
Vechur cattle	0.2	0.8	(Muhammed et al., 2012)
Ongole	0.06	0.94	(Ganguly, Gaur, et al., 2013)
Gir	0.02	0.98	(Rangel et al., 2017)

1.3. BCM7 and its similarity to opioids:

Metaphorically, BCM7, is considered as 'the devil in the milk'(Pal et al., 2015; Woodford, 2009, 2011). There are so many health benefits in consumption of milk proteins, but many diseases have been reported clinically, in individuals consuming exotic cow's milk

containing high proportion of A1variant of beta-casein in their milk e.g. heart disease, T1DM, SIDS, etc. (Elliott et al., 1999; McLachlan, 2001).

Recent studies have provided enough information, supporting the role of BCM7 in the regulation of certain functions of the nervous system, similar to opioid peptides (Defilippi et al., 1995; Fiedorowicz et al., 2011, 2014). Like opioids, BCM7 is characterized by distinct N-terminal sequence, where a tyrosine (Tyr) residue is coupled with the presence of another aromatic residue, e.g., phenylalanine (Phe) or Tyr, on the 3rd and 4th positions (Teschemacher et al., 1997) (**Fig. 1.5**). Thus, BCM7 can also act on μ-opioid receptors which are located in the nervous, endocrine, immune systems and gastrointestinal tract as well as in the osteoblasts of mammals. They also interact with their endogenous ligands, exogenous opioids and opioid antagonists, (Fiedorowicz et al., 2011, 2014; Pal et al., 2015) resulting in the induction of osteoporosis by direct or indirect mechanisms (Chang et al., 1981; Meisel, 1998; Pal et al., 2015) (**Fig. 1.6 & 1.7**).

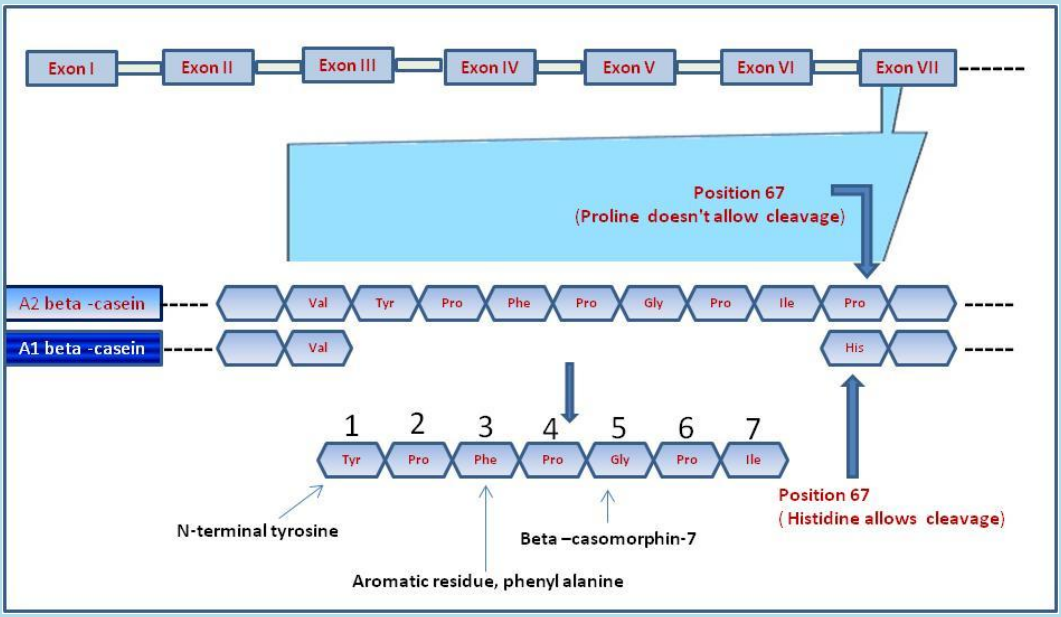


Fig. 1.5: Release of BCM7 and its structural similarity with opioids.

1.4. Opioids and other drug-induced osteoporosis:

Medication-induced imbalance of these cells is an emerging health problem related to a number of commonly used drugs such as opioids, selective serotonin receptor inhibitors (SSRIs), anticonvulsants and others. Polymedication and prolonged use of these drugs, increase the risk of reduced bone mineral density (BMD) and bone fractures (Ensrud et al., 2003; Mattia et al., 2012; Panday et al., 2014). Opioids have been recognized as a risk factor for the development of osteoporosis in the last decade (Bernabei et al., 2014; Prince et al., 2006; Rosen et al., 1994; Songpatanasilp et al., 2016).

1.5. Direct effect of opioid peptide:

In direct mechanism, the opioids increase the risk of fractures by acting on the μ-opioid receptor present on the human osteoblasts, as reported in osteoblast like cells; MG-63 (Perez-Castrillon et al., 2000). The direct effects of BCM7 on the ovaries and testes leads to the reduction of estrogen and testicular testosterone production, respectively, similar to that of opioids (Brennan, 2013; Delitala et al., 1983). Many researchers have also established a direct correlation between opioids and osteoporosis through induced hypogonadism (McLachlan, 2001; Shapses et al., 2017; Tegeder et al., 2004). It was observed that the synthesis of osteocalcin (a marker of osteoblast activity), was reduced when these cells were incubated with morphine. Naloxone (μ-opioid receptor competitive antagonist), was found to completely reverse this effect of morphine. In addition, low serum osteocalcin levels in pregnant women, addicted to heroin and cocaine, was reported by Rico et al., 1990 (Rico et al., 1990). All these findings suggest a direct toxic effect of these drugs, on osteoblasts. Both preclinical and clinical data suggest a direct mechanism by which opioids increase the risk of bone fracture (Rosen et al., 1994).

1.6. Indirect effect of opioid peptide:

The mechanism suggested is the suppression of hypothalamic pituitary gonadal (HPG) axis and hypothalamic pituitary adrenal (HPA) axis, where dysfunction of both, results in hypogonadism (Daniell, 2002; De Maddalena et al., 2012; Katz et al., 2009) (this is a complex mechanism that physiologically regulates bone turnover). Normally, gonadotropin-releasing hormone (GnRH), released by the hypothalamus, activates the anterior pituitary gland, to release luteinizing hormone (LH) and follicle stimulating hormone (FSH). These hormones, via systemic circulation, exhibit their effect on testes and ovaries, to produce testosterone or estrogen, respectively, which results in hypogonadism (Brennan, 2013; Delitala et al., 1983; Petraglia et al., 1986). In this condition, estrogen deficiency impairs the normal cycle, by increasing osteoclastic resorption activity, without a corresponding increase in osteoblastic formation activity. The amount of bone resorbed, therefore, is greater than the amount deposited, leading to a net loss of bone density. When estrogen levels drop after menopause, loss in bone density increases (Boyle et al., 2003; Ginaldi et al., 2005; McCormick, 2007; Pfeilschifter et al., 2002; Tella et al., 2014).

Another endocrine system that is affected by opioid peptides is the HPA axis. Cascade for this system is initiated with the release of corticotropin-releasing hormone (CRH) from the hypothalamus. This stimulates the pituitary to release adrenocorticotropic hormone (ACTH). This induces the adrenal glands to produce two hormones, cortisol and dehydroepiandrosterone (DHEA) (Allolio et al., 1987; Brennan, 2013; Palm et al., 1997. Cortisol is important for mounting stress responses, including responses to disease; DHEA is an important precursor for the synthesis of testosterone in men and estradiol in women (Katz et al., 2009).

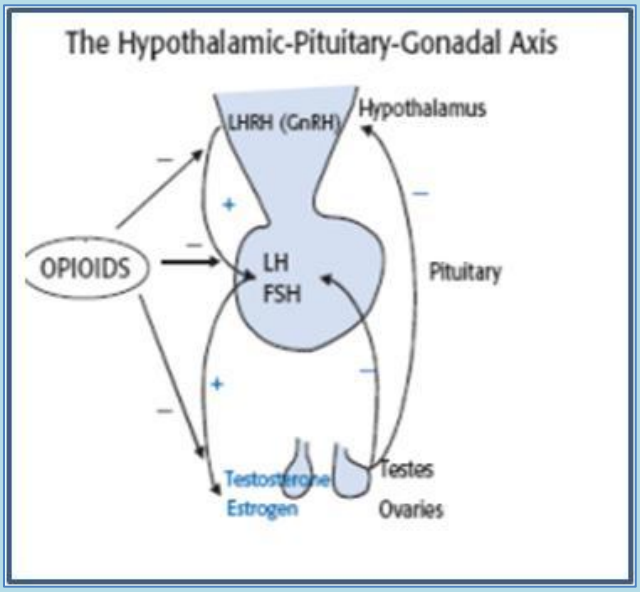


Fig. 1.6: Interactions between opioids and the endocrine system.

Figure adopted from Colameco et al., 2009; Katz et al., 2009.

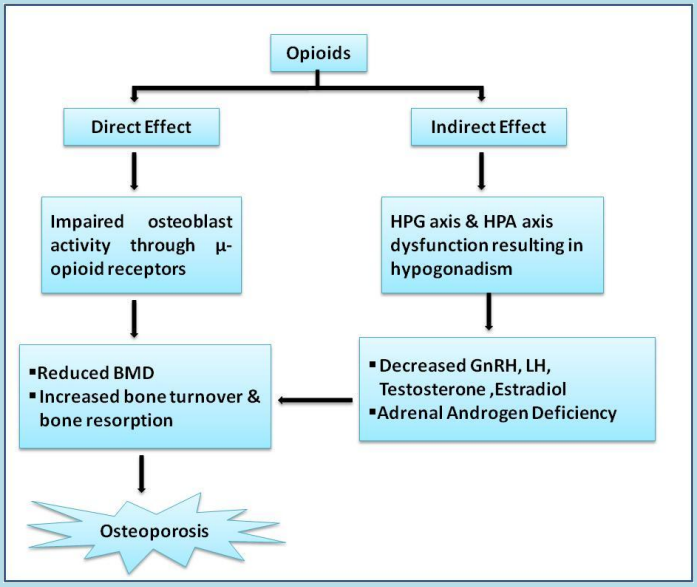


Fig. 1.7: Direct and indirect mechanisms by which opioids increase the risk of osteoporosis.

Abbreviations: LHRH-luteinizing hormone releasing hormone, GnRH- gonadotropin releasing hormone, LH-luteinizing hormone, FSH- follicle stimulating hormone.

In brief, this section provides selective information on: genetic variants of beta-casein gene, production of BCM7, its structural similarity with opioids and direct/indirect effect of opioid on precipitation of osteoporosis. However, impact of opioid peptide in osteoporosis is not very well explored, and related information available, is either based on epidemiological data taken from humans or is related to *in vitro* studies. Hence, an in-depth analysis is required to understand and delineate the exact role/mechanism of BCM7 in the precipitation of osteoporosis. The present study was aimed to explore the A1/A2 allelic variation in Hariana/HF cows and effect of BCM7 on the precipitation of osteoporosis.

Worldwide, a bone breaks due to osteoporosis every three seconds. Researchers estimated, that 1 in 3 women above the age of 50 will experience osteoporotic fractures, as well as 1 in 5 men (Kanis et al., 2000). In Europe, India, Japan and the USA alone, there are an estimated 125 million people with osteoporosis. As 10% of the Indian population (more than 100 million) is over 50 years of age, they are on high risk to experience osteoporotic fractures (Mithal et al., 2014). As per world health organization (WHO), 2012 report, nearly 300 million people suffer from osteoporosis in India and in the next decade it may increase as high as 50 percent of population. Another researcher study in Delhi, estimated the prevalence of osteoporosis as 24.6% in men and 42.5% in women above 50 years of age (Thulkar et al., 2015). Thus, considering the growing prevalence rate of osteoporosis, the proposed study on the association of osteoporosis with consumption of exotic cow milk is observed to be significantly important.

2. Literature review

2.1. Milk peptide induced diseases:

Milk has special significance in Indian mythology, culture, and diets. In 1992, a new dimension to the debate was added, when scientists in New Zealand established a correlation between the prevalence of T1DM and the type of milk consumed. The “Milk hypothesis” given by Scott et al., (1996), is about the direct correlation of T1DM and prolonged use of milk containing A1 beta-casein protein. Other researchers (Elliott et al., 1999, 1984; Meisel, 1998; Schrezenmeir et al., 2000) supported these findings and concluded that early exposure to cow milk protein may trigger T1DM. The intense interest generated by these researchers and public interest surrounding this issue, prompted the American Academy of Pediatrics to re-examine its guidelines for early infant feeding. Further work on milk protein was carried out to establish a relationship between T1DM and consumption of milk containing A1 beta-casein variant in infants as well as in adults (Elliott et al., 1984; Scott et al., 1996). So far different mechanisms have been proposed for the same i.e. suppression of gut-associated immune system or decreased oral tolerance, immunological cross reactivity (molecular mimicry) between cow’s milk proteins and autoantigens of the beta-cell (Harrison et al., 1999) etc. Hermann and Hubert proposed another hypothesis for mucosal immune suppression and risk of T1DM. [**Fig. 6** shows the different ways by which autoimmunity is enhanced (Wasmuth et al., 2000)]. The protective effect of BCM7, against oxidative stress in the pancreas of streptozotocin-induced diabetic rats, was also reported (Yin et al., 2012). Other properties of milk derived peptides like immunomodulatory and anti-inflammatory were also observed on oral administration (Russ et al., 2010). It was observed that food-derived BCM-7, might modulate the production of mucin, via a direct action on epithelial goblet cells (Martinez-Maqueda et al., 2012).

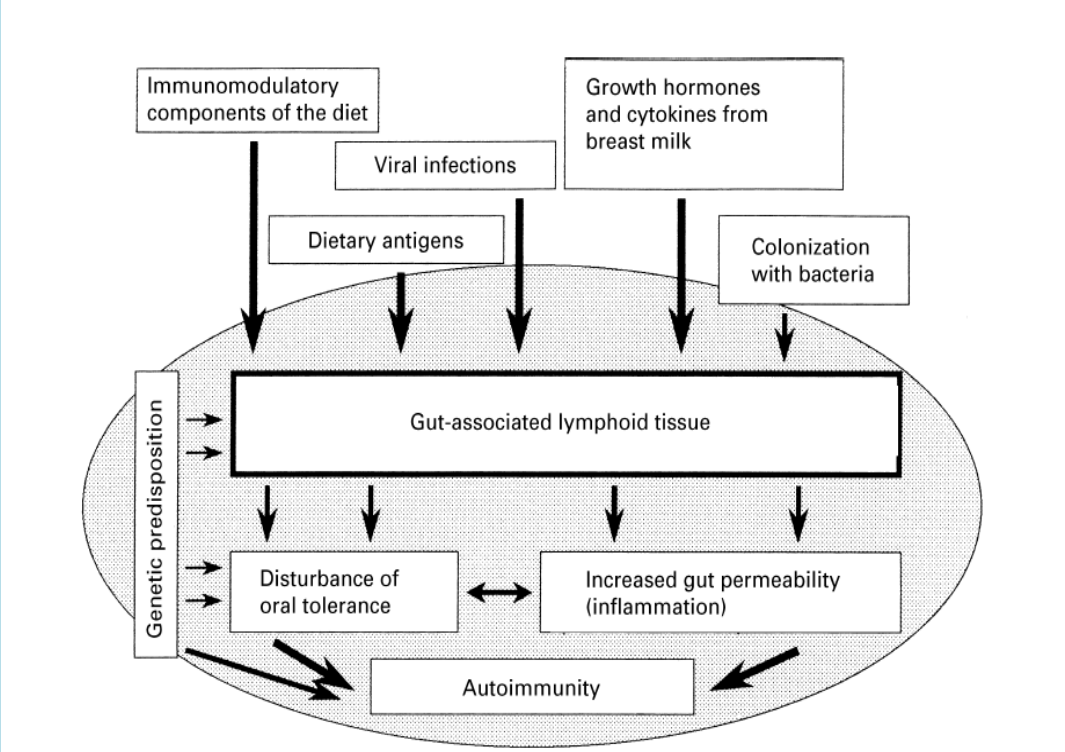


Fig. 2.1: Normal function of the gut immune system and factors controlling gut maturation. It is suggested that A1 cow's milk, promotes islet inflammation and development of the disorders like, Type-1 Diabetes Mellitus, deficient oral tolerance to dietary components, Figure adopted from Martinez-Maqueda et al., 2012.

2.2. Opioids and hypogonadism:

Opioids are considered to be the main drugs of misuse worldwide, and are increasingly prescribed worldwide, for all type of pain (acute and chronic, cancerous and non-cancerous) in every age group and are also used in managing people who have been addicted to heroin (Reddy et al., 2010). There are two forms of hypogonadism – primary hypogonadism resulting from problems of the testis or ovary and central hypogonadism caused by problems with the pituitary or hypothalamic glands. Opioids induced central hypogonadism leads to decreased levels of LH and FSH, released by the pituitary gland, opioids induce central hypogonadism. Prevalence of hypogonadism in the normal

population is < 10% for healthy men < 60 years old, 20% for men 60 –70 years old (Harman et al., 2001; Rajagopal et al., 2004). Hypogonadism developed by opioids, induce multiple deficiencies, affect body metabolism (**Fig.2.3**), including osteoporosis.

Table 2.1: Opioid induced deficiencies (Martinez-Maqueda et al., 2012).

Hypogonadism	**Symptoms**
Decreased Gonadotropin-releasing hormone(GnRH) Decreases Luteinizing hormone(LH Decreased Testosterone Adrenal Androgen Deficiency Decreased Dehydroepiandrosterone (DHEA) Decreased Dehydroepiandrosterone sulfate (DHEAS) Decreased Androstenedione	Anemia Decreased libido Decreased muscle mass Depression Erectile dysfunction Fatigue Hot flashes Menstrual irregularities Osteoporosis, sweating Weight gain

2.3. Opioid receptors:

Opioid receptors, Mu (μ), kappa (κ), delta (δ) and opioid-receptor like-1 (ORL-1) have been targeted for the treatment of pain and related disorders, by using analgesics in the clinic. **Table 2.2** describes different clinical use of receptor subtypes. All four receptors are G-protein coupled receptor (GPCR) and activate inhibitory G-proteins. (Al-Hasani et al., 2011; Bailey, 2008; Brooke-Taylor et al., 2017; De Maddalena et al., 2012; Koehl et al., 2018; Mattia et al., 2012; Pasternak, 2001). On activation of receptor by an agonist (endogenous or exogenous μ-opioid peptide), the Gα and Gβγ subunits dissociate from one another and intracellular effector pathways are activated (**Fig.2.2**). Opioids have been proposed to inhibit neurotransmitter release by inhibiting calcium entry by

enhancing the outward movement of potassium ions. They also inhibit neurotransmitter release by inhibiting adenylate cyclase (AC), the enzyme which converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) (**Fig.2.2**) (Al-Hasani et al., 2011; Bailey, 2008; De Maddalena et al., 2012; Mattia et al., 2012; Pasternak, 2001; Pathan et al., 2012). Presence of edogenous opioid peptides and their receptors (μ, δ, and κ), in the skeletal system was reported by many researchers (Baldock et al., 2012; Böhm et al., 2012; Spetea, 2013), but the role of endogenous opioid peptides in the regulation of bone remodeling processes and their effect on the skeletal system have not been fully clarified yet.

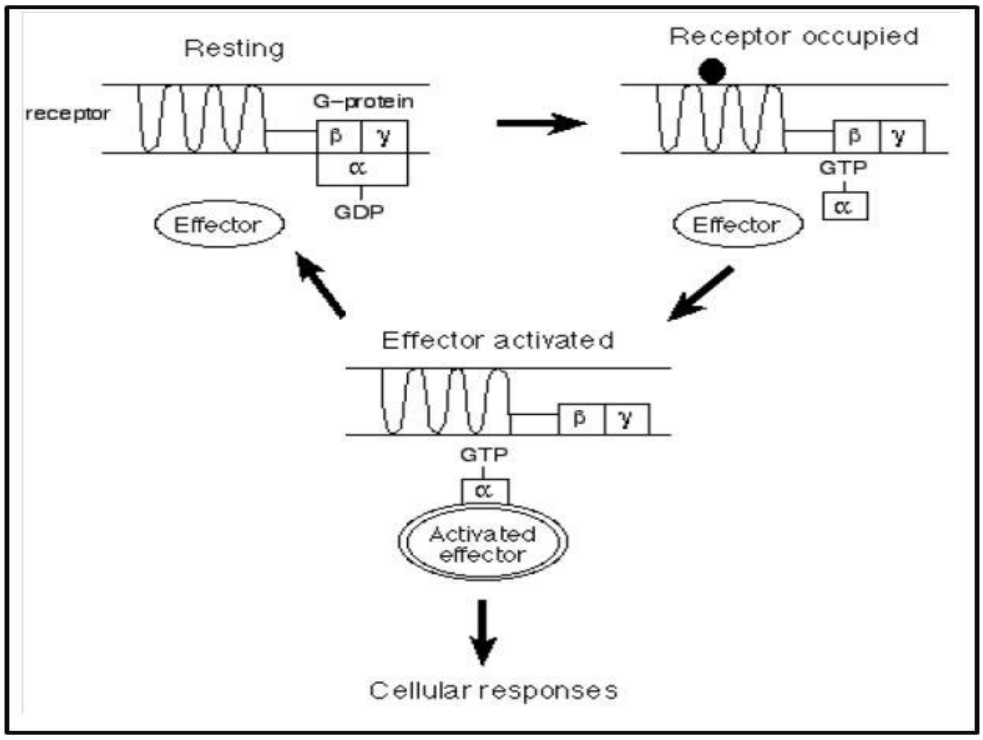


Fig. 2.2: Functioning of GPCR (Brooke-Taylor et al., 2017; Pathan et al., 2012).

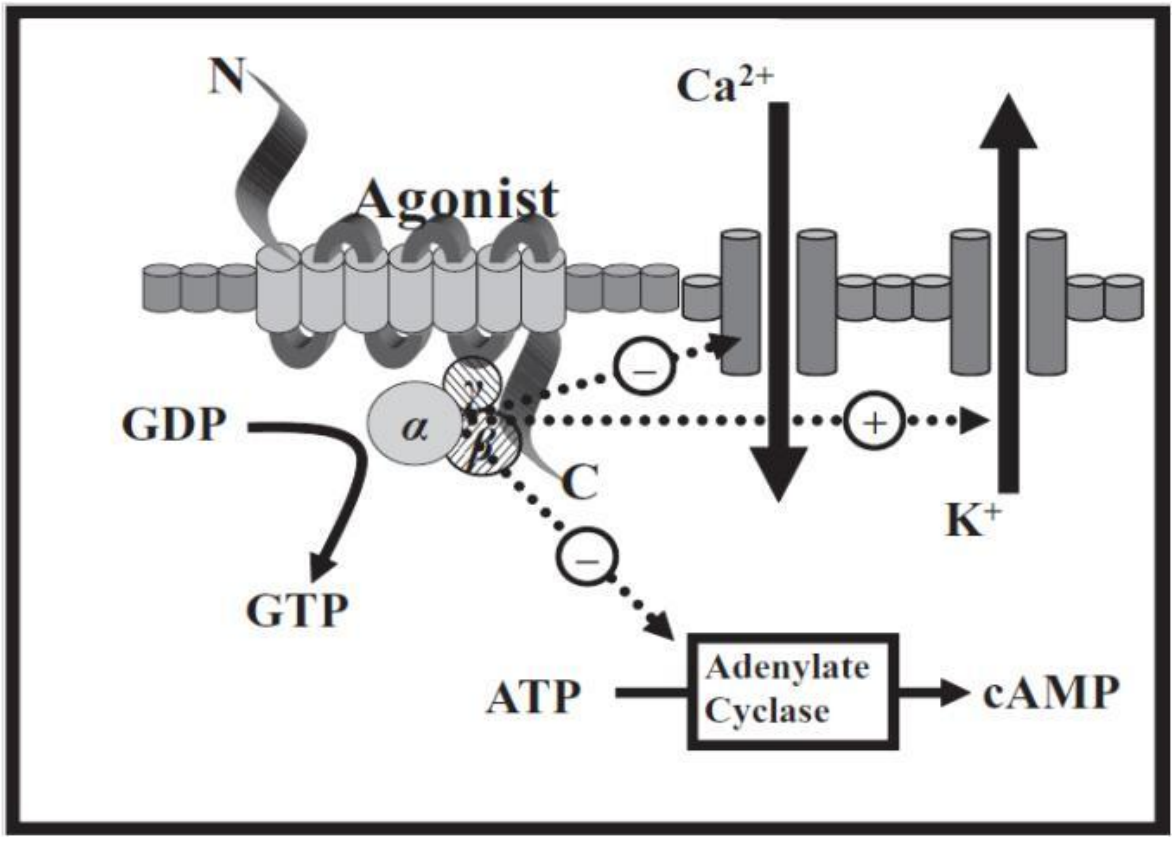


Fig. 2.3: Binding of an opioid agonist to a G-protein-coupled opioid receptor and intracellular changes. Figure adopted from Pathan et al., 2012.

Table 2.2: Clinical effects of individual opioid receptors (Drewes et al., 2013; Ensrud et al., 2003; Ramsin et al., 2008).

	Mu	**Delta**	**Kappa**	**ORL-1**
Clinical effects	Analgesia Sedation Depression Respiratory Euphoria dependence Physical	Analgesia Modulation of mu receptor Inhibit dopamine release	Analgesia Diuresis Dysphoria	Analgesia Sedation

2.4. Proline dipeptidyl peptidase-IV (DPP-IV):

Along with proline dipeptidyl peptidase-IV activity, the release from the protein precursors during food digestion, content in the food are some factors responsible for maintaining the concentration of milk-derived opioid peptides like casomorphins in the human body (Jarmołowska et al., 2019). DPP-IV, found on cell surfaces within mesenteric tissues (Lambeir et al., 2003), appears to be the primary degrading enzyme of BCM7cleaving BCMs at the

second proline on the opioid peptide chain). Moreover, DPP-IV is known to restrict the activity of proinflammatory peptides. It is responsible for cleaving BCM7, at the second proline on the opioid peptide chain (Fiedorowicz et al., 2011, 2014). In many people, the intestinal lining along with degrading activity of DPP-IV, prevent the BCM7 to inter into the bloodstream. However, people with leaky guts, provide a pass-through for BCM7 (Cade et al., 2000). There are many reasons for leaky guts; newborn babies have permeable intestines so that macromolecules in colostrum can pass through, In later life, permeability can arise due to coeliac disease, ulcerative colitis, Crohn's disease, stomach ulcers, etc. (Briggs et al., 1960). Lower enzymatic activity and/or deficiency of DPP-IV were suggested as possible causes for the higher level of milk derived peptides in human body (Cieslinska et al., 2017; Reichelt et al., 1991). Decreased activity of DPP-IV is generally associated with impaired immune status. It is also involved in immune response and nonspecific inflammatory processes (Eric-Nikolic et al., 2011).

2.5. Osteoporosis:

Osteoporosis, being a multifactorial systemic skeletal disease, is characterized by a decrease in bone mass and microarchitectural deterioration. WHO defines osteoporosis, based on standard deviations of BMD at the hip and/or the spine, compared with the

mean peak bone mass of young healthy adults, as determined by dual-energy X-ray absorptiometry (DXA) (Tella et al., 2014). WHO has established the following diagnostic guidelines:

- T score is + 2.5 to −1.0 ⟹ Normal
- T score is between −1.0 and −2.5 ⟹ Osteopenia
- T score is −2.5 or below ⟹ Osteoporosis.

Note: T score is BMD at the site when compared to the young normal reference mean (31yrs).

Normally, bone density accumulates during childhood and reaches a peak by around age 25 and is maintained for about 10 years (Hunter et al., 2000). After age 35, both men and women will normally lose 0.3% to 0.5% of their bone density per year, as part of the ageing process. Primary osteoporosis relates to decreased gonadal function and is associated with aging, occurs in older age > 75 years and seen in both females and males generally in the ratio of 2:1. Osteoporosis incidences are low in males because of the greater bone strength, in terms of both cortical bone expansion and trabecular bone volume. The male hormone, androgen, protects bone fracture due to its anabolic effect on muscle mass (Coluzzi et al., 2015; Mattia et al., 2012). In women, it has been postulated that accelerated bone loss starts the year before menopause and continues for another 3-4 years which is major cause of osteoporosis in women, referred to as **postmenopausal osteoporosis**. In subsequent 10 years post menopause, this increased rate of bone loss reaches equilibrium and then merges into a continuous age-related loss (Kadam et al., 2018). Secondary osteoporosis may occur at any age and affects both men and women, but is more frequent in men than women due to overuse of alcohol and corticoids, hypogonadism and idiopathic hypercalciuria

(Abrahamsen et al., 2009; Bernabei et al., 2014; Garg et al., 2012; Tella et al., 2014; Vanderschueren et al., 2014).

Regardless of the etiology, in all cases of osteoporosis, an imbalance exists between osteoblast and osteoclast activity (Järvinen et al., 2015; Malhotra et al., 2008). These situations lead to great societal costs, including direct medical costs and indirect costs resulting from the reduced quality of life, disability, and death. Osteoporotic fractures associated mortality ranges from 15 to 30%. Furthermore, 50% of women with osteoporotic hip fractures develop a disability, with great impact on the ability to live independently and in most cases, there is a requirement of institutionalization (Bernabei et al., 2014).

2.5.1. General risk factors for osteoporosis:

Calcium and vitamin D play a pivotal role in maintaining healthy bone. Large populations in developing countries have serum 25-hydroxycholecalciferol levels below 20 ng/mL (Shivane et al., 2012). Researchers have shown, that approximately 80-90% of hip fracture patients, are vitamin D deficient, due to low sun exposure, inadequate dietary vitamin D intake, lack of food fortification with vitamin D, pigmented skin, environmental pollution and traditional dress code (Dhanwal et al., 2013; R Khadgawat et al., 2013; Rajesh Khadgawat et al., 2010; Mithal et al., 2014). Calcium is needed to build up peak bone mass during pubertal years and the increased calcium requirement during pregnancy time, lactation, and postmenopausal years (Ohlsson et al., 1998). Habitual low intakes of calcium have been reported in toddlers, adolescents, pregnant and lactating mothers and postmenopausal women (Jha et al., 2010; Kadam et al., 2010; Tandon et al., 2014). It may also result due to endocrine and metabolic disorders (**Table 2.3**) (Bernabei et al., 2014; Gupta et al., 2014; Khadilkar et al., 2015; Prince et al., 2006; Sanwalka et al., 2010; Songpatanasilp et al., 2016; Warensjö et al., 2011).

Table 2.3: General risk factors for osteoporosis (Bernabei et al., 2014; Gupta et al., 2014; Khadilkar et al., 2015; Prince et al., 2006; Sanwalka et al., 2010; Songpatanasilp et al., 2016; Warensjö et al., 2011).

S. No.	Risk factor	Gender affected
1	Family history of hip fracture, delayed onset of puberty , prolonged immobility and lack of weight-bearing exercise, poor nutrition, advanced age, hyperthyroidism or hyperparathyroidism, medication history, particularly chronic glucocorticoid use and medications that decrease calcium, vitamin D absorption (anti-convulsants), rheumatoid arthritis, chronic liver disease, low femoral neck BMD, chronic obstructive pulmonary disease (COPD), organ transplantation,T1DM.	Male and female
2	Estrogen deficiency (primary or secondary) early menopause (<45 years), including surgical removal of ovaries.	Female
3	Current smoking, excessive alcohol (> 2 drinks per day intaker), caffeine, and tobacco use.	Male

2.5.2. National health policy on osteoporosis:

Osteoporosis is not a national health priority (NHP) in India. Out of NHPs, the one that will most closely impact osteoporosis is the nutritional program aimed at school children to provide vitamins and minerals including vitamin D and calcium. Although not formally recognized in health programs, vitamin D deficiency is increasingly becoming an important public-health issue. Government supplementation programs provide pregnant and lactating mothers with 500 mg/day of calcium through a serving of 165 g of

micronutrient fortified food per day; however, there are no national programs for supplementation for or promotion of bone health (Kadam et al., 2010; Khadilkar et al., 2015). The Indian Society of Bone and Mineral Research (ISBMR) runs structured programs to enhance awareness and set standards of care for health professionals, which have had a significant impact on osteoporosis care in India.

2.5.3. Pathophysiology of bone loss:

Bone is a connective tissue which continuously undergoes remodeling. Two types of cells are involved in this process: the mesenchymal-derived osteoblasts, which synthesize new bone matrix and the monocyte/macrophage-derived osteoclasts responsible for bone resorption. The normal bone remodeling process consists of four phases: activation, resorption, reversal, and formation (**Fig.2.4**) (Coluzzi et al., 2015; Tella et al., 2014).

1. **Activation phase**: osteoclasts (cells of resorption) are recruited to the surface of trabecular or cortical bone.
2. **Resorption phase**: Mononuclear osteoclasts cells generate an acidic microenvironment between the cell and the surface of the bone, dissolving or resorbing the mineral content of the bone.
3. **Reversal phase:** It is an intermediate period between osteoclast-mediated resorption and osteoblast-mediated formation, during which osteogenic signals released by osteoclasts activate the bone surface and bone marrow cells (they also guarantee the coupling process). Osteoclasts undergo apoptosis and osteoblasts are recruited to the bone surface.
4. **Formation phase:** Osteoblasts then deposit collagen (bone matrix), which is mineralized, to form the new bone.

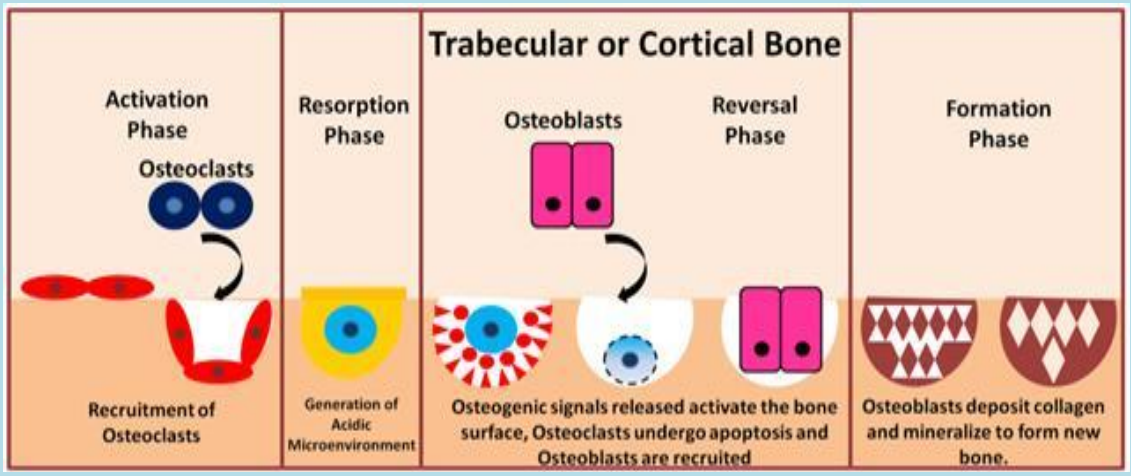


Fig. 2.4: Bone remodeling process.

Normally, bone density accumulates during childhood and reaches a peak by around the age of 25 and is maintained for the next 10 years. As a part of the ageing process, after the age of 35, both men and women normally lose 0.3% to 0.5% of their bone density. Bone loss more than the specified percentages are expected to increase risk of fractures.

2.5.4. RANK/RANKL/OPG system of bone homeostasis:

Bone-forming cells (osteoblasts) are of mesenchymal origin and share a common precursor cell with adipocytes and stromal cells. Bone resorbing cells (osteoclast) develop from myeloid precursor cells after stimulation by macrophage colony stimulating factor (M-CSF) and receptor for activated nuclear factor kappa B (RANK) ligand (RANKL) (**Fig.2.5**)

During normal bone remodeling, osteoblast cells produce receptor RANKL, which binds to the transmembrane receptor RANK, on preosteoclast cells and induces differentiation and activation of these cells. The transcription factor, nuclear-factor kappa B (NF-kB), plays an important role for activation of osteoclastogenesis and body's inflammatory

response and both processes are regulated via interleukin-6 (IL-6) (Boyle et al., 2003; McCormick, 2007; Walsh et al., 2006). Estrogen depicts its nuclear regulatory effects by inhibiting IL-6 activation of NFkB during bone remodeling (Ginaldi et al., 2005; Pfeilschifter et al., 2002). During normal bone remodeling, osteoblast cells also produce osteoprotegerin (OPG), a soluble decoy receptor that blocks RANKL binding to its cellular receptor RANK, thereby maintaining control of the remodeling process. OPG plays a key role in the success of the RANK/RANKL/OPG system of bone homeostasis (**Fig.2.6**) (McCormick, 2007). OPG is also produced by osteoblasts in response to anabolic agents such as estrogens and transforming growth factor-beta (TGF-β), related bone morphogenic proteins (BMPs). Activated T lymphocyte cells also produce RANKL. With reduced estrogen levels and/or chronic or recurrent immune activation from either systemic or gastrointestinal origin, there may be a reduction in the body's natural ability to limit the production of RANKL (Ginaldi et al., 2005). RANKL stimulates its receptor, RANK, which recruits tumor necrosis factor receptor-associated factor-6 (TRAF-6), a RANK adaptor protein that mediates NFkB activation. It acts as a key adaptor to assemble signaling proteins that direct osteoclast-specific gene expression leading to differentiation and activation of preosteoclast cells. The two most closely studied pathways are the activation of transcription factors NF-kB and Jun N-terminal kinase (JNK) (Wong et al., 1998). This signaling starts from RANK, culminates in the gene expression patterns, that characterize active multinucleated osteoclast (Boyle et al., 2003; Ginaldi et al., 2005; McCormick, 2007; Pfeilschifter et al., 2002; Takayanagi et al., 2000; Wong et al., 1998).

Estrogen deficiency, at menopause, impairs the normal cycle by increasing osteoclastic resorption activity, without a corresponding increase in osteoblastic formation activity. The amount of bone resorbed, therefore, is greater than the amount deposited leading to

a net loss of bone. When estrogen levels drop after menopause, loss of bone density accelerates (Akesson, 2003; Martin, 2004; Wong et al., 1998). Regardless of the etiology, in all cases of osteoporosis, an imbalance exists between osteoblast and osteoclast activity. The rate of bone formation is often normal, whereas resorption by osteoclasts, increases. The accelerated bone loss after menopause, is a major cause of osteoporosis in women, referred to as postmenopausal osteoporosis (Garg et al., 2012; Tella et al., 2014).

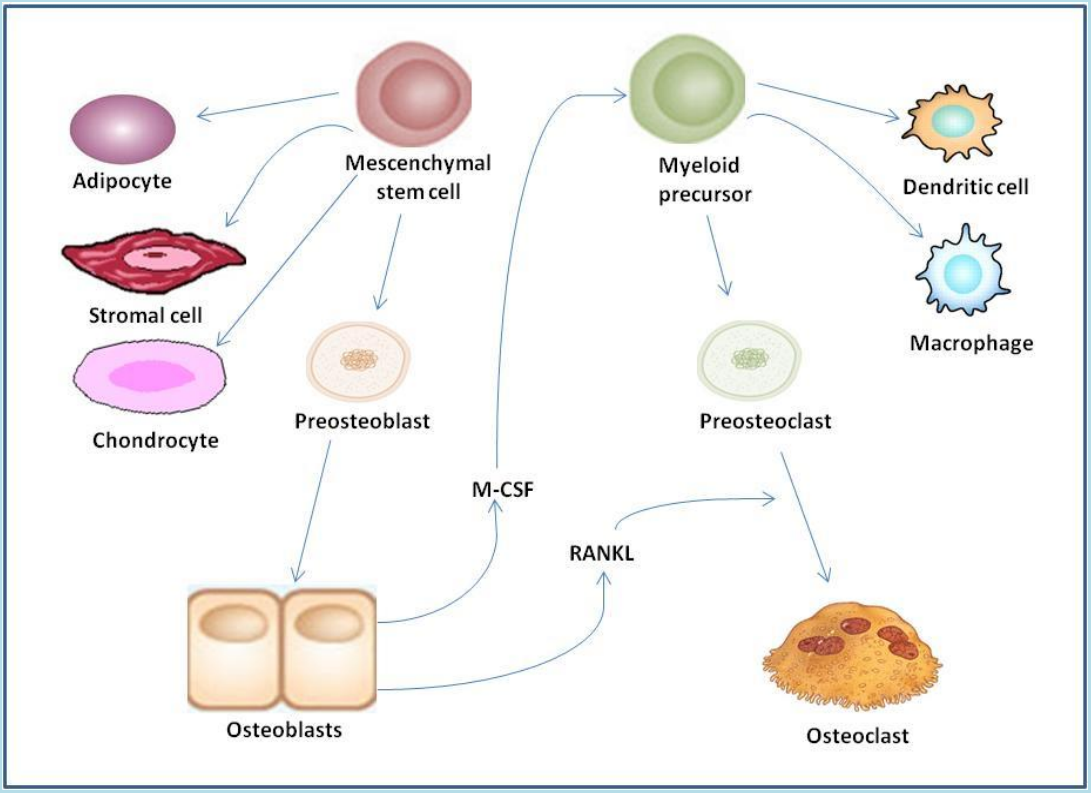


Fig. 2.5: Bone cell differentiation. Mesenchymal stem cells, which also give rise to adipocytes, chondrocytes and stromal cells, differentiate into preosteoblasts and then become osteoblasts on the bone surface. A common myeloid lineage precursor, which can also give rise to macrophages and dendritic cells, commits to becoming a preosteoclast and then fuses to become a mature, multinucleated osteoclast (Boyle et al., 2003; Walsh et al., 2006).

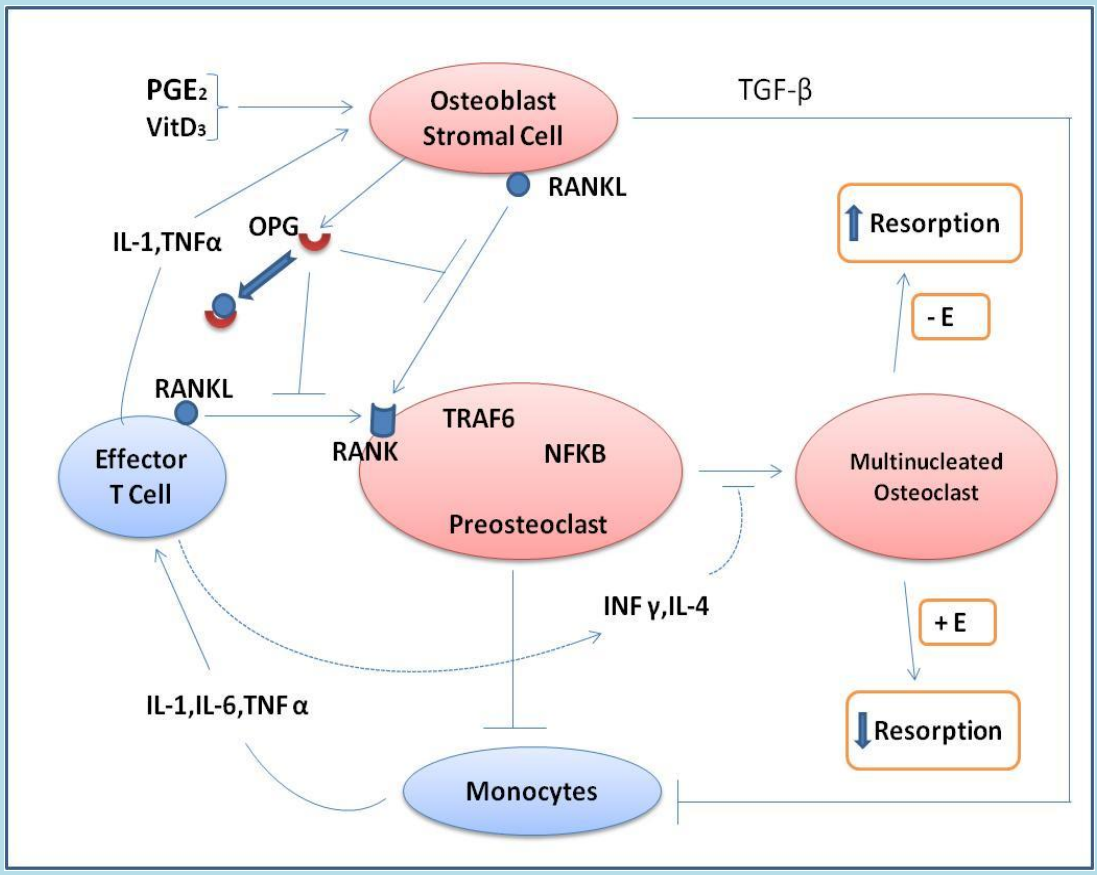


Fig. 2.6: Effect of estrogen on bone remodeling by RANK/RANKL/OPG system. +E end condition in presence of estrogen, -E end condition in absence of estrogen. PGE2 and VitD3 are calcitropic factors which induce RANKL expression. Estrogen normally preserves bone by increasing macrophage production of TGF-β and limiting CD4+ T-cell activation, IL-1 and tissue necrosis factor α (TNFα). It also increases the sensitivity of preosteoblasts to IL-1, thus suppressing M-CSF and RANKL. Estrogen increases vitamin D receptor activation and calcitonin release. It also increases osteoblast release of TGF-β, IGF-1, and OPG, which controls M-CSF and RANKL and increases osteoclast apoptosis. With low level of estrogen, TGF-β decreases, CD4+ T-cell activation increases the release of RANKL and TNFα, diverting progenitor cell differentiation toward osteoclastogenesis. Stimulators of bone resorption include IL-1, Tumor Necrosis Factor α (TNF-α), IL-6, IL-11, IL-15, and IL-17. Inhibitors of resorption include IL-4, IL-10, IL-13, IL-18, and interferon gamma-γ (IFN-γ). TGF-β and prostaglandins can have either stimulatory or inhibitory effects on resorption, depending on the conditions under which these factors are examined (Boyle et al., 2003; Hoegh-Andersen et al., 2004).

2.6. Estimation of parameters indicating osteoporosis:

2.6.1. Estimation of bone turnover:

Bone formation and bone resorption are two counteracting processes, responsible for continuous remodeling of bone and metabolically active. Bone physiology relies on the activity of osteoblasts (formation), osteoclasts (resorption) and osteocytes (maintenance). Under normal conditions, activity of osteoblasts and osteoclasts are tightly coupled to each other so that the amount of bone removed is always equal to the amount of newly formed bone. This balance is achieved and regulated through the action of various systemic hormones [e.g. vitamin D, parathyroid hormone (PTH), and other steroid hormones] and local mediators (e.g. growth factors, cytokines). In contrast, somatic growth, ageing, metabolic bone diseases, states of increased or decreased mobility, therapeutic interventions, and many other conditions are characterized by more or less pronounced imbalances in bone turnover. Thus, change in the balance between bone resorption and bone formation ultimately results in a net loss or gain of bone tissue. High bone turnover, with increased bone resorption, can compromise bone strength, leading to a thinning of the bone structure, resulting in abnormal bone micro-architecture and reduced bone mineralization (Deyhim et al., 2005; Garnero, 2007; Midtby et al., 2001; Seibel, 2005).

2.6.2. Marker of bone formation:

Osteocalcin (OC) specifically produced by osteoblast, is a hydroxyapatite-binding protein, with molecular weight 5.8 kDa. It had calcium binding properties due to presence of gamma-carboxyglutamic acid (Gla) residues (vitamin-K dependent). As a result of bone metabolism, after OC's release from osteoblasts, the largest part is incorporated into the extracellular bone matrix and a smaller fraction is released into the circulation where it can be detected by immunoassays. After release into blood, OC

interacts with other proteins, including cell surface receptors, which predispose OC as a molecule active in the organization of the extracellular matrix. Hence OC is considered as a specific marker of osteoblast function. Serum levels of immunoreactive OC have been shown to correlate well with the bone formation rate, as assessed by histomorphometry. Furthermore, since OC is incorporated into the bone matrix, some investigators have suggested that OC fragments may be released even during bone resorption. This may be particularly true for some smaller N-terminal fragments of OC, which are found in individuals with high bone turnover (Garnero, 2007; Hoegh-Andersen et al., 2004; Midtby et al., 2001; Seibel, 2005).

2.6.3. Markers of Bone Resorption:

The majority of bone resorption markers are degradation products of bone collagen. A major group of immunoassays involve carboxyterminal telopeptide of type I collagen, abbreviated as CTX (**Fig. 2.7**).

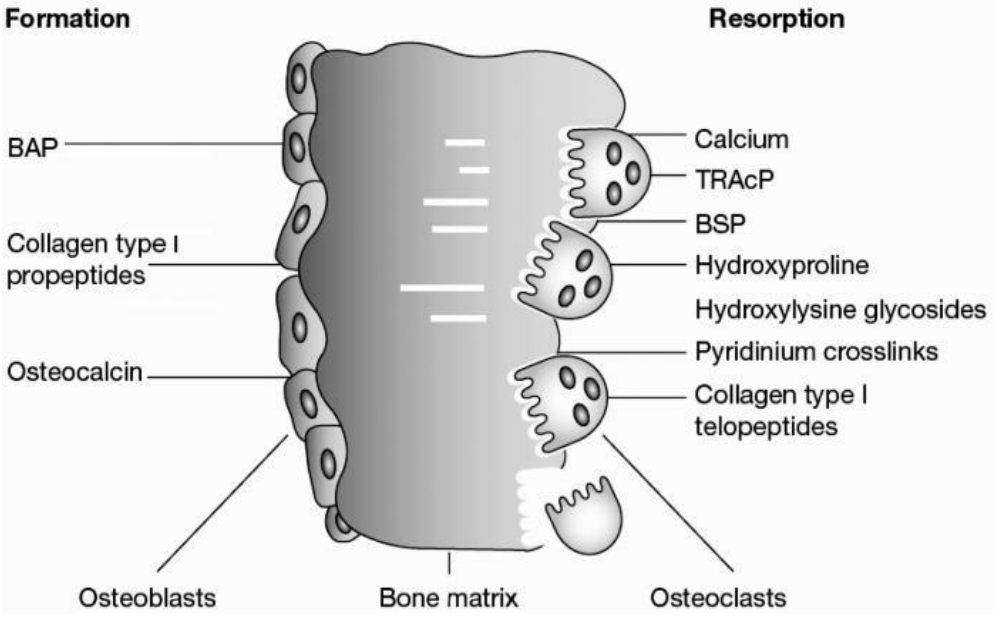


Fig. 2.7: Biochemical markers of bone remodeling. Figure adopted from Garnero, 2007.

2.6.4. Bone densitometry:

BMD is a medical term normally referring to the amount of mineral matter per square centimeter of bones and is used in clinical medicine as an indirect indicator of osteoporosis and fracture risk. The bone mineral content (BMC) and density (BMD) at femur neck and lumbar vertebrae (L4-L6) are measured ex-vivo, by dual-energy X-ray absorptiometry (DEXA) equipped with appropriate software for bone assessment in small animals. DEXA is the most accurate way to measure BMD and BMC (Deyhim et al., 2005).

2.6.5. Micro-computed tomography (µCT):

In recent years, µCT use has grown immensely for high-resolution imaging, to assess cortical and trabecular bone morphology in human and animal, for research as well as clinical purposes. First introduced by Feldkamp and colleagues in the late 1980s, µCT now has become the ‘‘gold standard’’ for evaluation of bone morphology and microarchitecture in rats and other small animal models in both *in vivo* & *in vitro* conditions. **Fig. 2.8** shows key components and basic operating principle involved in µCT (Genant et al., 2008; Wu et al., 2015).

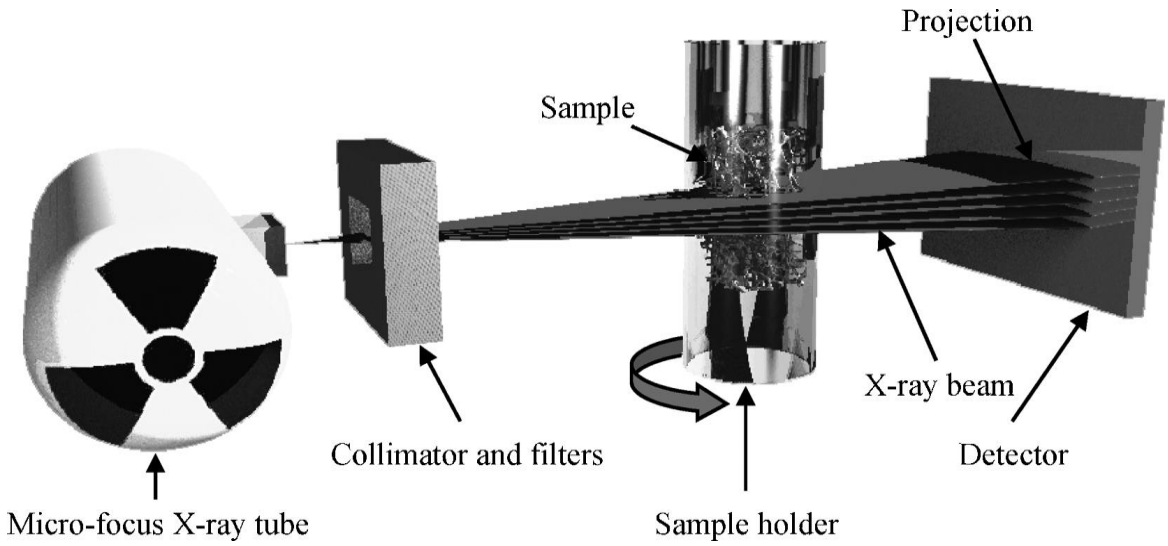


Fig. 2.8: Key components and operating principle for standard µCT scanner. X-rays emitted by microfocus X-ray tube are collimated and filtered to narrow the energy spectrum. The X-rays pass through the object and are recorded by a 2D charge-coupled device (CCD) array. A set of projections under different rotations of the object is practiced for a full scan. Figure adopted from Bouxsein et al., 2010.

2.6.5.1. Specifications of µCT and superiority over conventional 2D histology:

- Significantly larger volume of interest is analyzed, assessment of bone morphology by µCT scanning is nondestructive; thus samples can be used subsequently for other assays, such as histology or mechanical testing.
- **Easy sample preparation:** Specimen from neonates may be scanned intact, while those from older animals may be excised (soft tissues removed). A key concept is to orient the specimens consistently within the sample holder and scanner, mainly on vertical axis of the scanner, although alignment with the horizontal axis is possible as well.
- **Scanning medium:** Saline, ethanol, and neutral buffered formalin, as well as with no medium (in air).
- **X-ray energy:** µCT systems operate in the range of 20 to 100 kVp
- **Intensity:** The information content of a voxel depends on the signal-to noise ratio (SNR), which is governed by the number of incident photons and the sensitivity of

the charge-coupled device (CCD) detector. The total number of photons for each projection during a tomographic scan depends on the tube current (mA) and the integration time for each projection (ms), as well as the number of times each projection is repeated.

- **Voxel size and image resolution:** A voxel is the discrete unit of the scan volume that is the result of the tomographic reconstruction. It is a 3D volume representing two dimensions within the slice and the slice thickness. Differences in voxel size (10 to 20 mm) have little effect on the evaluation of structures with relatively high thickness (i.e., 100 to 200mm), but can have significant effects on smaller structures such as mouse or rat trabeculae, with approximate dimensions of 20 to 60mm (John et al., 2018; Kureel et al., 2017).

The minimal set of morphmetric indices includes the measurement of bone volume (BV) and the total volume of interest (TV). The ratio of these two indices is termed as bone volume fraction (BV/TV). These indices can be derived from either a simple voxel-counting method or a more advanced volume-rendering method, also referred to as volumetric marching cubes (VOMACs). Mean trabecular thickness (Tb.Th), mean trabecular separation (Tb.Sp) and mean trabecular number (Tb.N) are other important parameters **(Table 2.4)**, which all are based on 3D calculations, namely, a sphere fitting method **(Fig**.**2.9**), where spheres are fitted to the background of the trabecular separation, for thickness measurement. With this basic approach one can determine the diameter of the largest possible sphere that can be fitted through each voxel of the object. This approach yields a reasonable average thickness of the structure or the background, where the latter reflects the mean trabecular separation. The mean distance between the mid-axes of the structure, yields mean trabecular number (Bouxsein et al., 2010).

Table 2.4: **Definition and description of 3D outcomes for trabecular bone microarchitecture** (adopted from Bouxsein et al., 2010).

Abbreviation	Variable	Description	Standard unit
TV	Total volume	Volume of the entire region of interest	mm3
BV	Bone volume	Volume of the region segmented as bone	mm3
BS	Bone surface	Surface of the region segmented as bone	mm2
BV/TV	**Bone volume fraction**	Ratio of the segmented bone volume to the total volume of the region of interest	%
BS/TV	Bone surface density	Ratio of the segmented bone surface to the total volume of the region of interest	mm^2/mm^3
BS/BV	Specific bone surface	Ratio of the segmented bone surface to the segmented bone volume	mm^2/mm^3
SMI	Structure model index	An indicator of the structure of trabeculae; SMI will be 0 for parallel plates and 3 for cylindrical rods	
Tb.N	**Trabecular number**	Measure of the average number of trabeculae per unit length	1/mm
Tb.Th	**Trabecular thickness**	Mean thickness of trabeculae, assessed using direct 3D methods	mm
Tb.Sp	**Trabecular separation**	Mean distance between trabeculae, assessed using direct 3D methods	mm
Tb.Th.SD	Standard deviation of trabecular thickness	Measure of the homogeneity of trabecular thickness, assessed using direct 3D methods	mm
Tb.Sp.SD	Standard deviation of trabecular separation	Measure of the homogeneity of trabecular separation, assessed using direct 3D methods	mm
DA	Degree of anisotropy	1= isotropic, >1=anisotropic by definition; DA=length of longest divided by shortest mean intercept length vector	a
MIL	Mean intercept length	Measurements of structural anisotropy	a

Note: Variables in bold are the minimal set of variables that should be reported when describing trabecular bone morphology. [a] Dimensionless variable.

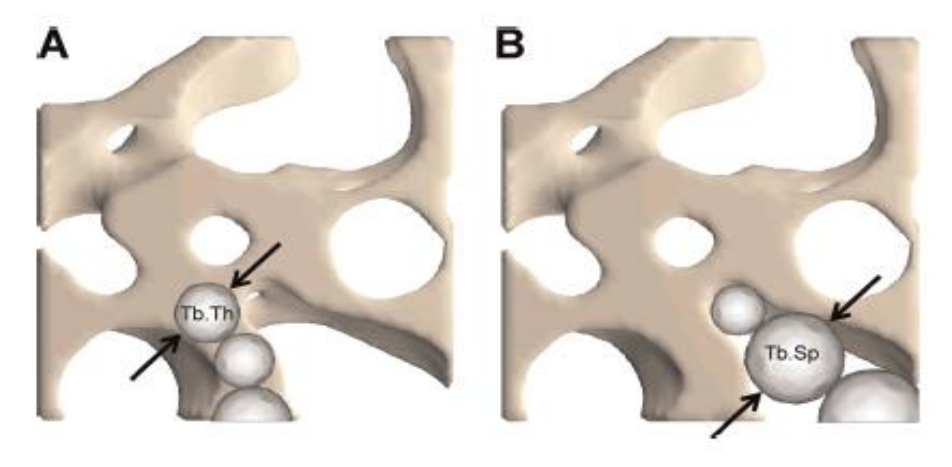


Fig. 2.9: 3D method for calculating trabecular thickness (A) and separation (B). 3D distances are computed by fitting spheres inside the structure i.e., to assess average trabecular thickness (sphere indicated by arrows written as '**Tb.Th'** is inside the trabecular structure) or inside the background/ marrow space, ie, to assess average trabecular separation (sphere indicated by arrows written as '**Tb.Sp**' is inside the marrow space). The average diameter of the spheres represents the object thickness, and the standard deviation of the diameter represents the variability in the object thickness. Figure adopted from Bouxsein et al., 2010.

2.7. Review of status:

2.7.1. International status:

Much research has been carried out on casein-derived bioactive peptides that are encrypted within the primary structures of intact casein rather than isolated BCM7. Conditions for release of bioactive peptide from parental casein have determined by many institutions like MTT Agrifood Research Finland, Food Research, FIN-31600 Jokioinen, Finland (Korhonen et al., 2006), Department of Food and Nutritional Sciences, University College Cork, Western Road, Cork, Ireland (Phelan et al., 2009). Industrial-scale technologies suitable for the commercial production of bioactive milk peptides have been developed and launched recently by the Faculty of Biology, University of Warmia and Mazury, Poland (Sienkiewicz-Szłapka et al., 2009). These

technologies are based on novel membrane separation and ion exchange chromatographic methods. Agricultural Management Group, Lincoln University, New Zealand (Woodford, 2011) is involved in the study of milk proteins and their effects on human health for a long time. Department of Paediatrics, School of Medicine, Auckland, New Zealand (Elliott et al., 1999) is involved in the study of T1DM precipitated by casein variant consumption in the different age group of children. Harrison et al., (1999) from Walter and Eliza Hall Institute of Medical Research, Royal Melbourne Hospital, Parkville, Australia, also study the effect of early exposure of infants to cow's milk or lack of breastfeeding. However, the data regarding the effect of A1 beta-casein on induction of osteoporosis, is negligible.

2.7.2. National status:

At the national level, very few organizations are working on milk peptides, the national bureau of animal genetic resources (NBAGR), the national dairy research institute and the Indian veterinary research institute started research on A1 and A2 milk in 2009 and again in 2012. But much of it involved a review of research carried out in other countries. Even these only referred to research papers and books from New Zealand, highlighting the harmful effects of A1 milk, while ignoring critical reports published (Joshi et al., 2012). Division of Dairy Microbiology and Division of Animal Biochemistry, National Dairy Research Institute, Karnal (Haque et al., 2008) is working on bio-functional properties of bioactive peptides of milk origin. Studies like polymorphism of beta–casein and kappa-casein gene in different livestock of India (Singh et al., 2015) and genotyping of allelic variants of beta-casein gene in native cattle of Ladakh (Sharma et al., 2018), are some efforts by investigators, to estimate the actual situation of genotype, of Indian cattle.

Many researchers have reported the prevalence of osteoporosis in India in different regions. **Table 2.5** provides details of studies conducted on prevalence of osteoporosis among women in India, by small groups spread across the country, Estimates from these studies suggest that of the 230 million Indians expected to be over the age of 50 years in 2015, 20% are osteoporotic women (Malhotra et al., 2008), prevalence of osteoporosis in Indian women of different age groups ranging from 8% to 62% (Khadilkar et al., 2015). N. S. Kadam et al., 2018, reported osteoporosis prevalence in women according to menopausal status versus men and observed low T-score in Indian men, as compared to women, which indicates higher susceptibility to osteoporosis, in man **(Fig.2.10**). No report(s) from Indian researchers, working on impact of beta-casein on precipitation of osteoporosis was found.

Table 2.5: **Studies describing the prevalence of osteoporosis among Indian women (**adopted from Khadilkar et al., 2015**).**

Sr no	Title of the study (year)	Study location	Subject details	Method of diagnosis	Prevalence of osteoporosis
1	Evaluation of BMD of women .40 years of age (Gandhi et al., 2005)	Mumbai	200 women attending well women clinic, >40 years	BMD proximal femur and spine-DXA	34% osteopenia and 8% osteoporosis
2	Bone status of Indian women from a low-income group and its relationship to the nutritional status (Shatrugna et al., 2005)	Hyderabad	289 slum-dwelling women, 30–60 years	Hologic DXA at AP lumbar spine, hip, and total body	52% osteopenia and 29% osteoporosis
3	Preliminary screening of osteoporosis and osteopenia in urban women from Jammu using calcaneal QUS (Sudhaa et al., 2006)	Jammu	158 women, 25–65 years	Calcaneal QUS	36.79% osteopenia and 20.25% osteoporosis
4	Prevalence of osteoporosis among elderly women living in Delhi and rural Haryana (Chhibber et al., 2007)	Delhi and rural Haryana	430 women, 60–80 years (125 rural Haryana, 250 affluent urban area Delhi, and 55 LSC Delhi)	Hologic DXA at hip and lumbar spine	29% osteopenia and 62% osteoporosis
5	Osteoporosis and osteopenia in India: a few more	Kerala	609 persons (52±12.8 years) 538 women	QUS distal radius	221 or 41.1% osteopenic women; 237 or 44.1% osteoporotic women

	observations (Babu et al., 2009)				
6	Bone mineral density in women above 40 years (Unni et al., 2010)	Pune	105 women (50.46±7.60 years, age range 40–72 years)	DXA lunar vertebral measurements lumbar spine	31.4% osteopenia and 14.3% osteoporosis
7	Low bone mass in urban Indian women above 40 years of age: prevalence and risk factors (Kadam et al., 2010)	Pune	172 (80 pre- and 92 postmenopausal) healthy women (40–75 years) attending a routine health check at Jehangir hospital in Pune city	Lunar DPX-PRO DXA BMD lumbar spine (L2–L4) and dual femurs	Lumbar spine (L2–L4): Osteopenia: 48.4% postmenopausal and 44.3% premenopausal Total hip: Osteopenia: 45.3% postmenopausal and 26.7% premenopausal women Osteoporosis: 2.3% postmenopausal women
8	Prevalence and related risk factors of osteoporosis in peri- and postmenopausal Indian women (Aggarwal et al., 2011)	Chandigarh	200 peri- and postmenopausal women aged older than 45 years	Lunar DPX-PRO DXA BMD at lumbar spine	53% had low BMD (osteopenia or osteoporosis)
9	Bone health in healthy Indian population aged 50 years and above (Marwaha et al., 2011)	Delhi	1,600 healthy subjects .50 years of age (792 males and 808 females) mean age of 57.67±9.46 years	DXA	44.9% osteopenia and 42.5% osteoporosis in women
10	Bone status of women over 40 years of age from two socioeconomic strata (Vaidya et al., 2012)	Pune	58 and 54 (112) women (mean age 49.5±7.2 years) from USC and LSC, respectively	Lunar DPX-PRO DXA BMD at lumbar spine and total femur	Osteoporosis in the USC women: 12% lumbar spine and 0% at femur Osteoporosis in the LSC women: 33% at lumbar spine and 11% at femur
11	Cross sectional study of osteoporosis among women (Agrawal et al., 2013)		158 women, older than 35 years of age (42.5±3.4 years; wives of staff)	Calcaneal quantitative ultrasonography	48.1%±7.79% osteopenia 13.3%±5.29% osteoporosis

Abbreviations: BMD-bone mineral density; DXA-dual energy X-ray absorptiometry; AP-anteroposterior; QUS-quantitative ultrasonography; LSC-lower socioeconomic class; USC-upper socioeconomic class.

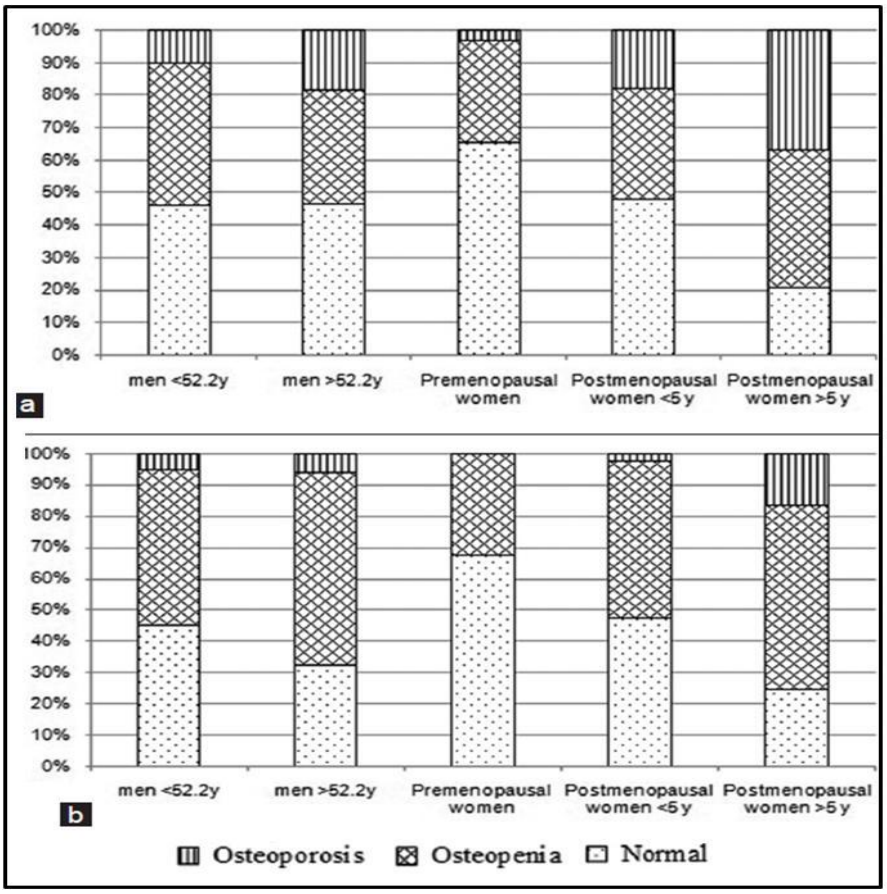


Fig. 2.10: (a) Prevalence of osteoporosis at lumbar spine in women according to menopausal status versus men. (b) Prevalence of osteoporosis at femoral neck in women according to menopausal status versus men. Adopted from (Kadam et al., 2018).

2.8. Gaps in existing research:

There have been reports implicating negative health effects of A1 protein/BCM-7 like Ischaemic Heart Disease, T1DM, Autism, inflammatory response and digestive discomfort, etc., but the scientific community believes the evidence is insufficient and inconclusive. The overall evidence for gastrointestinal effects from A1 and BCM-7 in animal and in vitro studies is conclusive, but the evidence from human studies is still limited.

Administration of opioid analgesics may adversely affect the skeletal system, many researchers established direct correlation between drug addiction and osteoporosis

(McLachlan, 2001; Keiler et al., 2012; McLachlan, 2001; Shapses et al., 2017; Tegeder et al., 2004). Rico et al., 1990, reported about the level of osteocalcin in pregnant women addicted to heroin and cocaine, Pérez-Castrillón et al., 2000 also observed impact of opioids on cells (MG-63) and suggested, a direct toxic effect of these drugs on osteoblasts. However, the results of in vitro and in vivo experiments as well as epidemiological and clinical studies, published so far are inconsistent (Bailey, 2008; Duarte et al., 2013; Dürsteler-Macfarland et al., 2011; Grey et al., 2011; Janas et al., 2017; R Khadgawat et al., 2013; Kim et al., 2006; Pedrazzoni et al., 1993). Reports about the impact of BCM7 on precipitation of osteoporosis are negligible.

The hypotheses of BCM7 induced suppression of the HPG axis (associated with osteoporosis, T1DM, depression and other gonadal hormones deficiency disorders) needs to be explored. The exact mechanism involved in these disorder(s), is still unclear. Only a few reports of T1DM due to consumption of milk with a higher amount of A1 beta -casein variant are available, which are not sufficient to understand the actual physiological, psychological and metabolic risks associated with consumption of a particular type cow's milk. Most of the studies are *in vitro* or clinical reports. *In vivo* effects of the BCM7, are not well reported. A suitable animal model for these deficiencies is not standardized, so far, in Indian conditions and very less abroad. Standard technologies for isolation of two variant at laboratory level as well as industry level are not available. Gene expression pattern for variant of beta- casein is not clear, more exploring is required to understand the actual mechanism.

Most of the available methods (Atamer et al., 2017; Lamothe et al., 2007) for isolation of pure beta-casein are costly, small scale and produce more impurities along with beta-casein protein. Thus there is requirement for development of a nonenzymatic method, which can yield more quantity and pure beta-casein/ml of milk.

3. Objectives and plan of work:

1. To identify genotypes of HF and Hariana cows in and around Pilani (100Km).
2. To develop a technique for nonenzymatic isolation of beta-casein from milk.
3. To perform comparative analysis of osteoporosis induction in rats using surgical method and by feeding of milk protein / natural milk.
4. To analyse the correlation between consumption of beta-casein variants of milk and early precipitation of osteoporosis.

3.1 Background and broad objectives:

3.1.1 To identify genotypes of HF and Hariana cows in and around Pilani:

In the last few decades, India has become the highest milk producer in the world and exotic cattle played a major role in this achievement. The exotic/crossbred milch cattle increased up to 34.78%, from 14.4 million to 19.42 million, whereas the indigenous milch cattle increased marginally from 48.04 million to 48.12 million, an increase of merely 0.17% (19th Livestock Census; http://dahd.nic.in). In this process, indigenous zebu cattle is replaced or mixed in germplasm with the exotic cattle due to unorganized breeding practices and willing of high milk production.

India has common marketing channels for milk and milk product of both A1 and A2 type cattle. New Zealand, US, and Canada have their separate set up of dairies/market for A1 and A2 type cattle milk or milk products, while the same is not possible in India due to unorganized farms, financial restrictions, emphasis on more milk production and lack of health awareness. Most of the Indian cattle are in unorganized farms, which is a major problem in the implication of proper breeding policies. There are chances that A1variant of beta-casein is also present in zebu cattle. Thus, there is a great requirement of investigation of germplasm to know the actual status of cattle genotype. First objective of

the present study was to determine the genotype of the cattle with similar breeds and environmental conditions, in and around Pilani region, as a trial study.

3.1.2. To develop a technique for nonenzymatic isolation of beta-casein from milk:

Milk broadly contains 2 major protein groups: caseins and whey proteins (lactalbumins and lactoglobulins). Since last century, industrial production of casein has become more important as an ingredient in food, pharmaceuticals, and paint industries often. During the last 5 decades, to meet the increasing industrial demand of beta-casein, several methods have been developed for large scale production of pure beta-casein. Important among them are differential precipitation (Hipp et al., 1952; Y Igarashi, 1999; Yasuo Igarashi, 1989, 1995), gel chromatography (Nakahori et al., 1972; Nakai et al., 1972), ion-exchange chromatography (Cayot et al., 1992; Christensen, 1989; Leaver et al., 1992) or chromatography on hydroxyapatite (Allen et al., 1985; Donnelly, 1977). However, these methods were effective for small scale production and pure beta-casein was not obtained in most cases. Besides it often required additional steps for purification, prior to its use as a food ingredient, which would add up to the cost of production.

In later half of 20th Century, potentially large-scale methods were developed to isolate beta-casein from caseinate at low temperature, e.g., from sodium caseinate by filtration through filter paper (Famelart et al., 1989), microfiltration (Famelart et al., 1994) or ultrafiltration (Murphy et al., 1991) or from cold-renneted calcium caseinate (Allen et al., 1985). Huppertz et al. (2006) reported that extensive hydrolysis of beta-casein occurs by this method and proposed another large scale method for beta-casein isolation. Protein yield reported in this method is still very low, and in addition, rennet used for coagulation, is expensive and its activity is temperature sensitive requiring higher temperature for its deactivation. However, most of the available methods are costly, small scale and

produce more impurities along with beta-casein protein. Thus, there is a requirement for development of possibly a nonenzymatic method, which can yield more quantity and pure beta-casein / ml of milk. The objective was set to address this issue first in the present study.

3.1.3. To perform comparative analysis of osteoporosis induction in rats using surgical method and by feeding of milk protein / natural milk:

Female Wistar rats were chosen as an animal model for the study of the impact of BCM7 on precipitation of osteoporosis. Several experimental interventions are used to induce osteopenia and osteoporosis in the rat. The rate of loss of bone mass in male and female rats is highly dependent on the method used to induce osteoporosis and the site evaluated. There are many ways to induce osteoporosis in rats, but it was observed that ovariectomy was much suitable to our laboratory conditions and with available expertise and resources. The objectives were set to standardize ovariectomy as an animal model for osteoporosis. Rat osteoporosis due to ovariectomy (postmenopausal osteoporosis) and immobilization bears a strong resemblance to human osteopenia, both in its anatomical features, as well as, in bone dynamics, and hence considered significant. This was followed by the feeding of earmarked rats with milk/milk proteins obtained from identified cows.

3.1.4. To analyse the correlation between consumption of beta-casein variants of milk and early precipitation of osteoporosis:

Most of the rural people in India depend on milk or milk product for their protein and fat requirements. Inspite of consuming a significant proportion of milk/milk products (a rich source of calcium and phosphorus), clinically, it is observed that the occurrence of

osteoporosis is more, in rural India. Literature survey indicated structural similarities between opioids and the BCM7 and hence it was proposed to investigate the correlation between consumption of A1 or A2 cow milk and precipitation of osteoporosis. If the current investigation progress brings to light such correlation, it may create awareness about these variants among people and may be helpful in decreasing the occurrence of osteoporosis.

3.2 Work plan:

Complete research work was targeted in 4 phases to achieve all the aforementioned objectives, as mentioned flow chart (**Fig. 3.1.)**

Phase –I: - Identification of cattle and determination of their genotypes.

Phase –II: - Isolation of beta-casein from the milk of identified cows.

Phase –III: - Standardization of animal model and animal study.

Phase –IV: - Study the impact of beta-casein variants on precipitation of osteoporosis.

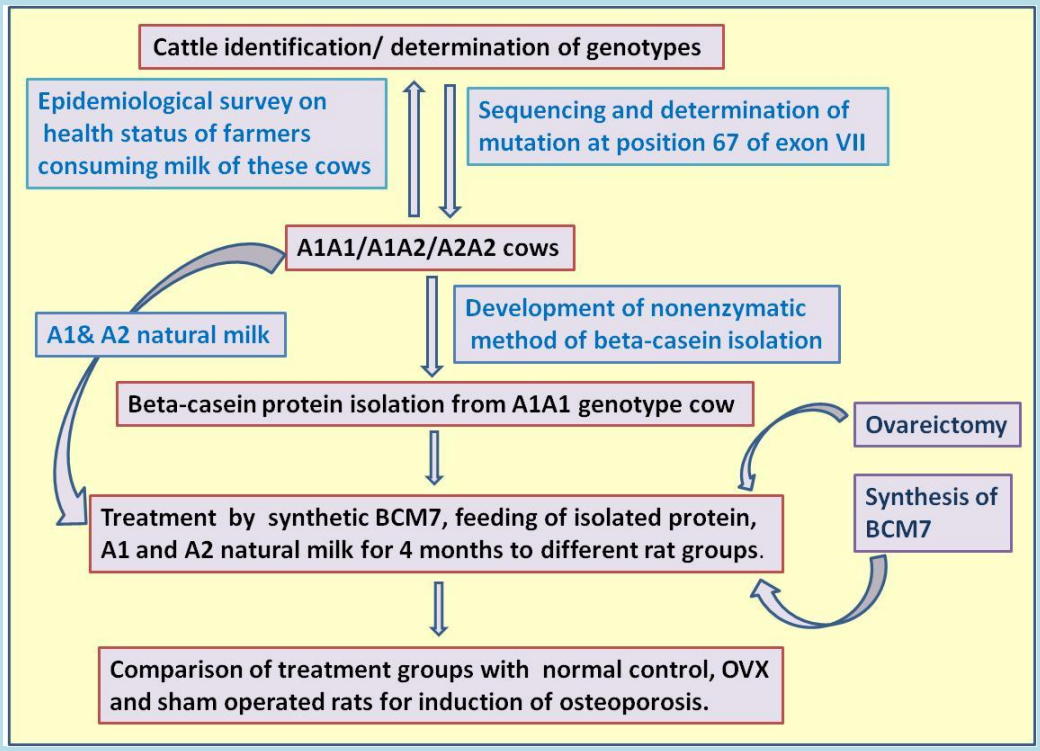


Fig. 3.1: Flow chart showing the work plan.

4. Materials and methods

4.1. Identification of cattle for study:

Cattle were identified on the basis of their Physical characters, breeding history, production of milk, etc. Hariana (**Fig. 4.1)**, Mewati, Nagori, Sahiwal, Gir, etc. are common zebu (*Bos indicus*) and HF (**Fig. 4.2**), Jersey are common exotic cattle breeds (*Bos taurus*) in Pilani region. The cattle blood samples were collected from different population, all located in similar climatic conditions receiving green and dry feed in 1:2 ratio along with concentrate. During rainy season there was availability of green *Pennisetum glaucum* and/or *Sorghum bicolor as fodder while* in dry season stalks of the same and seasonal green grasses are offered to animals. Milking is usually carried out twice a day at 5:00 am and at 5:00 pm. These cattle were reared by different small dairy owners near Pilani town, district Jhunjhunu, Rajasthan (state), located in the northwest region of India, with similar breeding and husbandry practices.

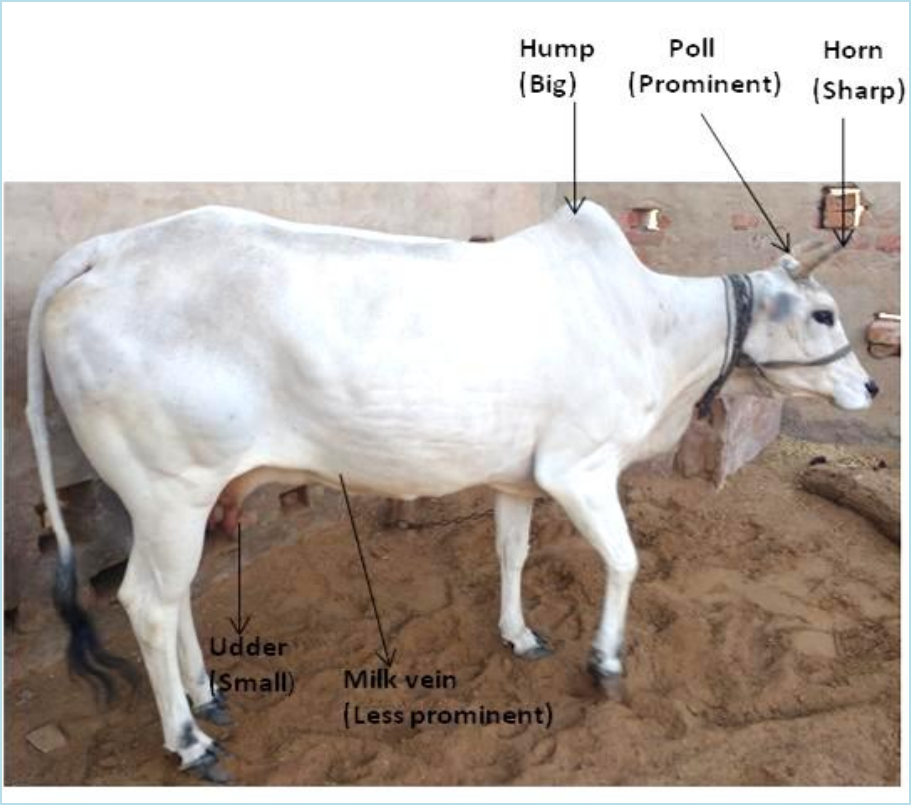


Fig. 4.1: Indian zebu (Hariana) cow.

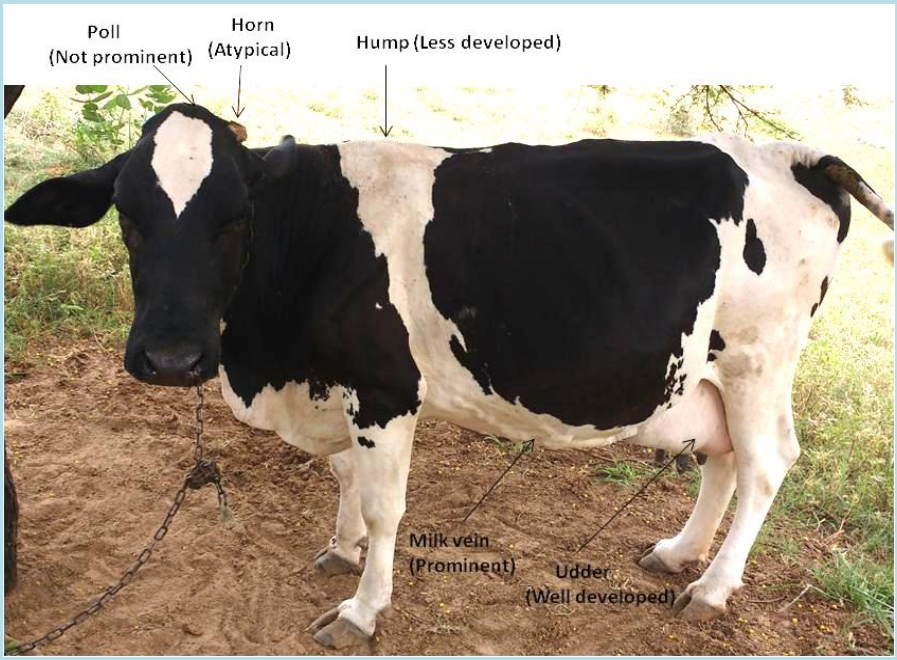


Fig. 4.2: Exotic (HF) cow.

Table 4.1: Difference of physical characters, between Hariana and HF cows (Banerjee, 1998)**.**

S. No.	Character	Desi cow	HF
1	Hump	Prominent large developed	No hump
2	Milk vein	Less prominent with small udder	Clearly visible with a large udder
3	Body and appearance	Proportionate, compact and graceful appearance	Ruggedly built and posses large feeding capacity
4	Horns	Upright horns and boney prominence in the center of the poll	Not in a typical manner
5	Face	Long and narrow	Wide
6	Color	White to grey	Black and white
7	Milk production	16 L/day	6 L/day
8	Dry period	1-2 months	4-5 months

4.2. Collection of cattle blood and DNA isolation:

About 0.5ml blood was collected from the clinical samples (remaining) from local veterinary clinics, with the assistance of registered veterinarians. Total 129 samples of both HF (89) and local zebu (40) were collected in acid citrate dextrose (ACD) solution, transported to labs at BITS, Pilani in cold (4ºC) condition, and stored at -20ºC until DNA extraction. The genomic DNA was isolated using standard protocols (Green et al., 2012) with minor modifications. 250µl of buffer A (NaCl, Tris, EDTA) and 250µl of buffer B (buffer A + SDS) were added to 500 µl blood, incubated at 37ºC for 45 minutes, followed by proteinase K (100µg/ml) and again incubated at 50°C (water bath) for 2hrs. The sample was then treated by equal volumes of Tris-saturated phenol, extracted upper aqueous phase, again treated with equal volume of phenol: chloroform: isoamyl alcohol

(IAA) (25:24:1) added and finally washed with chloroform: IAA with intermittent centrifugation at 10,000 rpm, for 10 min at 4°C. After overnight incubation with alcohol and 3M sodium acetate, the pellet was washed with 70% alcohol and dried. The pellet obtained was resuspended in 30-70µl of TE buffer and stored at -20°C freeze, till used. DNA quantity was estimated using SimpliNano (29061711, Biochrom Ltd., UK) and quality of DNA was also checked using DNA agarose gel electrophoresis (**Fig. 4.3**).

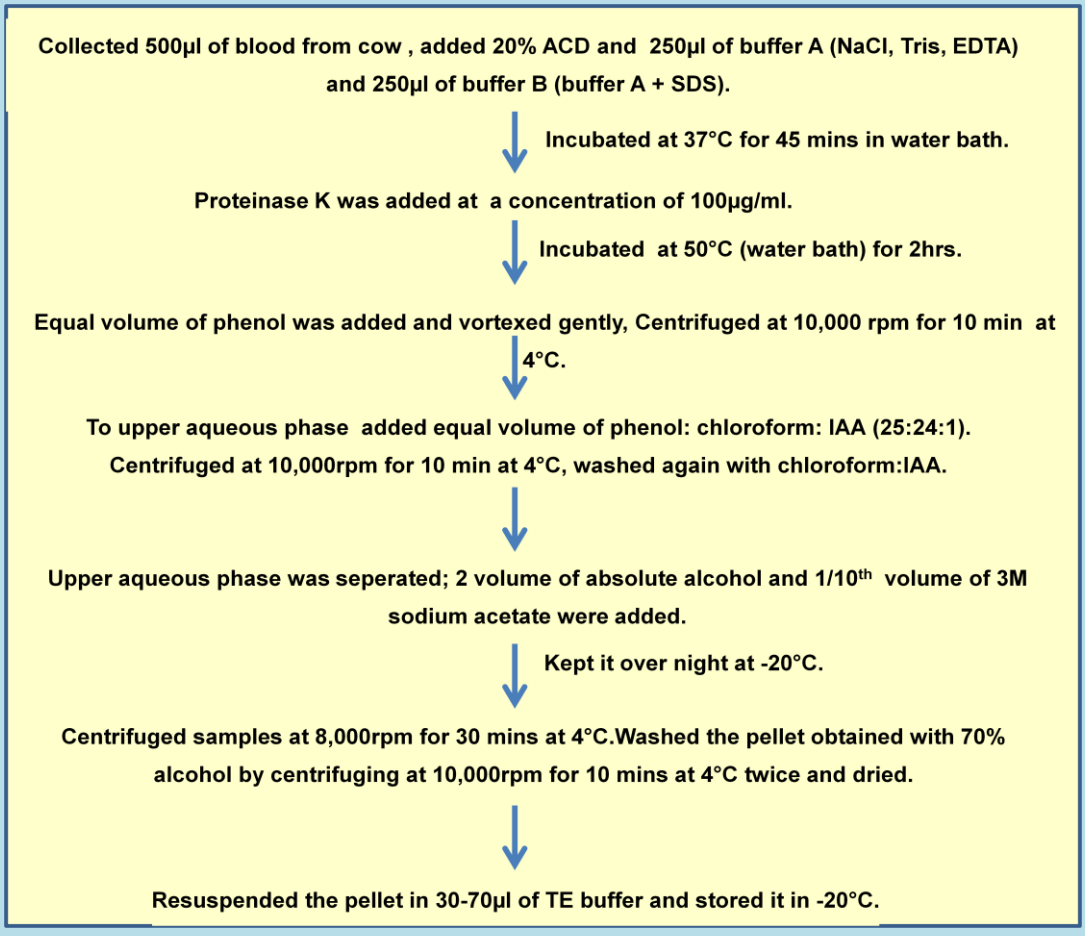


Fig. 4.3: Procedure for DNA isolation from cattle venous blood.

4.2.1. Beta-casein gene amplification:

The complete full-length DNA sequence (M55158) of *Bos taurus* isolates were obtained from NCBI database. The exon and intron regions were identified from the shortlisted sequence. Primers were designed following standard parameters using Primer 3 (NCBI) and Gene Runner software and cross-checked for their specificity using Primer-Blast (NCBI) (Untergasser et al., 2012). The VIIth exon partial region was amplified using forward primer 5ˈGATGAACTCCAGGATAAAATC3' and reverse primer 5ˈTACAATAATAGGGAAGGG TC C 3ˈ. For PCR, 200 ng of whole blood DNA was subjected to initial denaturation at 94°C for 3 min followed by 35 cycles of 94°C for 45 sec, 59°C for 45sec and 72°C for 1.0 min and followed by post extension at 72°C for 5 min. PCR products were checked on 1% agarose gel and purified using DNA Gel extraction kit, QIAquick (Qiagen, Germany). The amplicons were sequenced commercially on Genetic Analyzer (UDSC-3730XL-17121-005, Applied Biosystem) and further obtained sequences were analyzed using BLASTn (Madden, 2013) and compared with other reported sequences from the database using Clustal Omega (Sievers et al., 2011) to identify mutations. Chromatograms of all the sequenced samples were specifically analyzed for nucleotide mutations at position 67 of VIIth exon.

4.2.2. Estimation of cattle allelic and genotypic frequencies:

The obtained sequences were segregated on the basis of A1, A2 and A1A2 alleles. The population genetic indices i.e., gene heterozygosity (He), gene homozygosity (Ho) (Ganguly, Kumar, et al., 2013), and fixation indices (FIS) were calculated by Popgene 32 software version 1.32 (Yeh et al., 1999).

For beta-casein alleles, "k" allele frequencies (x_k) and genotypic frequencies for the genotype (kk), were obtained using the equations:

$$x_k = \frac{2n_{kk}+\sum n_{kl}}{2n:x_{kl}} = \frac{n_{kl}}{n}$$ (Rangel et al., 2017)

Where -n_{kk} = the number of homozygotes, n_{kl} = the number of heterozygotes; observed in the k allele, n = the number of cattle analyzed.

4.3. Natural selection analysis:

Codon analysis methods were used to identify natural selection force on *Bos taurus* CSN2 gene, by analyzing non-synonymous (Ka) to synonymous (Ks) changes in protein coding sequences. The *Bos taurus* coding DNA sequences (CDS) required for selection analysis were obtained from NCBI for A2 variant (XM_010806178.1) and from 'The Bovine Genome Database', for B (BT11954-RB) variant. The details of beta-casein amino acid variations for A1, A3 and I variants were extracted from Dai et al., 2016. The *B.taurus* CDS was then compared with 12 different closely related mammals.

4.3.1. Gene specific selection analysis:

To examine the selection pressure on different variants of *Bos taurus* gene, non-synonymous (Ka) to synonymous (Ks) nucleotide substitution rates, were calculated. Parsimony method using Discrete_ Grantham Matrix was applied to determine the Ka/Ks ratio for *Bos taurus* CSN2 gene variants with orthologous from other mammals. Ka/Ks ratio is the numerical indication of selection pressure [it will be greater than 1 in positive selection, while less than 1 for purifying selection (Doron-Faigenboim et al., 2005)].

4.3.2. Site specific selection analysis:

Selecton (Doron-Faigenboim et al., 2005), an online server was used to identify site specific selection pressure on different variants of *Bos taurus* CSN2 gene with closely related mammals' homolog, using codon biased multiple sequence alignment "Positive

Selection enabled M8 evolutionary model" and Jones–Taylor–Thornton (JTT) matrix. The M8 model is an advantageous model, that allows all types of selection i.e. positive, purifying and neutral selection and works on identification of modified sites based on beta distribution (defined within the interval [0,1]), likelihood test ratio (LTR) was applied to test the significance of Ka/Ks scores generated by Selecton (Doron-Faigenboim et al., 2005; Stern et al., 2007)

4.4. Isolation of A1 beta-casein protein:

A1 and A2 cattle (cows) were selected by estimating their genotype, and cows having A1A1 and A2A2 genotype, were identified for milk collection. A1 beta-casein protein from milk of A1A1 cattle was isolated, by the method described in our **patent application no. 201811018301, dated: 16/05/2018**. In brief, the procedure is as follows: Whole milk was obtained from Indian Zebu cows (Haryana, Mewati, Nagori and Sahiwal) and exotic cows (Holstein Friesian and jersey) from different locations in and around Pilani and transported to labs in BITS, Pilani maintaining cold chain (4 ºC) condition. Milk cream was removed by cream remover. The method for isolation of beta-casein is summarized as a flow diagram in **Fig. 4.4**. Initially, 50 ml raw milk was taken and cream was removed. To this, 1% glacial acetic acid was added and held for 15 minutes at room temperature (25 ºC) and later centrifuged at 7500 rpm for 10 minutes at RT. Supernatant was discarded, coagulum was mixed in an equal volume of Milli-Q water (MQW). After 5 minute incubation at RT, centrifuged the solution again under same conditions. Coagulum obtained after this washing, was again mixed in an equal volume of MQW and incubated at 4 ºC for up to 24 hours. The supernatant was filtered through Whatman No. 40 filter paper and stored at -20 ºC. All experiments were performed in triplicate for individual milk samples.

Beta-casein was isolated from whole milk using both rennin method described by (Thom Huppertz et al., 2006) and acetic acid method as mentioned in **Fig. 4.4**. For determination of the yield of beta-casein, the protein content of the filtered supernatant after cold-incubation was estimated by Bradford assay in triplicates (Kruger, 2009). The yield of the isolated fraction was expressed as µg/ml of casein in milk. Further, the protein bands were analyzed using MALDI-MS/MS, to confirm the presence of beta-casein.

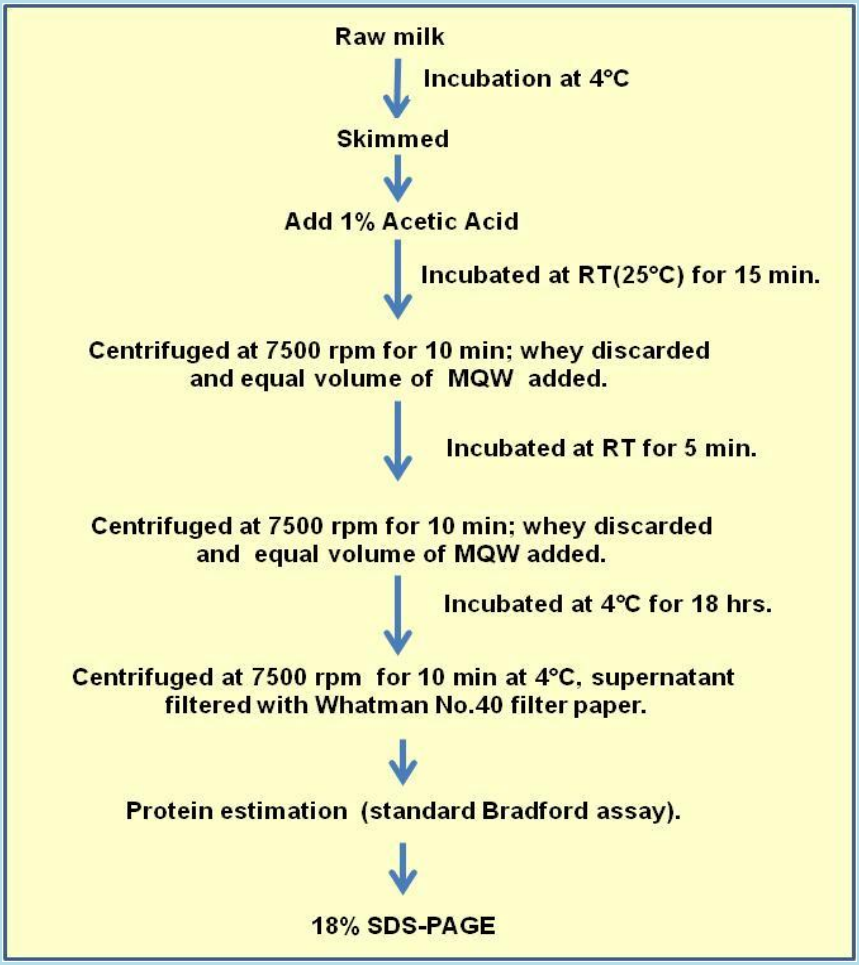


Fig. 4.4: Flow chart of the method for isolation of beta-casein from milk.

4.5. Animal study

4.5.1. Ethical approval:

All the experimental procedures performed on animals were in compliance with the protocols, approved by Institutional Animal Ethics Committee (IAEC), of Birla Institute of Technology & Science, Pilani, India (Protocol No. IAEC/RES/18/16, IAEC/RES/18/16/ Rev-1 /19/32 and IAEC/RES/19/32/ Rev-1/23/28).

4.5.2. Induction of osteoporosis:

Osteoporosis was induced by ovariectomy in 2.5 months old female Wistar rats. Animals were anesthetized with a combination of xylazine (10 mg/kg) and ketamine (75 mg/ kg) given intraperitoneally (IP). Following anesthesia, aseptic surgical site was prepared at left flank of the rats (landmark the ovaries: caudal end of the ribs on left lateral side of the animal). A 2 cm incision was made on the dorso-lateral area from second to either fifth lumbar vertebrae or to middle part of abdomen using a scalpel blade. Incision was of minimum length to allow the extrusion of ovaries. It is easy to locate left ovary and associated fat, by gentle retraction. Oviduct was ligated and ovary along with excess fat was removed. The same procedure was repeated for right ovary. After removal of ovaries, peritoneal cavity and muscles were closed with absorbable sutures (catgut no. 4-0) by '*lock-stitch suturing' technique.* Skin was sutured using nonabsorbable (silk no. 3-0) thread by **'***horizontal mattress suturing' technique*. High degree of the aseptic procedure was maintained throughout the surgical process (Kanis, 1994; Olson et al., 1986; Shen et al., 1997; Turner et al., 2001)(**Fig. 4.5**).

Postoperative care: Animals were housed and maintained in maximum possible clean and hygienic environment. Wound was dressed daily for 7 days with Povidone Iodine and Lorexane spray. Injection of long acting antibiotics e.g. Enrofloxacin (fortiver) was

given and sutures removed on the 10th day. Animals were maintained in standard conditions for 4 months.

4.5.3. Milk source

A1 and A2 cattle (cows) were selected by estimating their genotype, by sequencing of exon VII of the beta-casein gene (CSN2) and cows having A1A1 and A2A2 genotype, were identified for milk collection. The test groups were either fed with beta-casein protein isolated from A1 milk, or natural milk from A1A1 and A2A2 cows, in order to compare the effect of isolated protein and proteins present in natural milk. Synthetic BCM7 was obtained from Biolinkk (India) and given by both intraperitoneal (IP) and oral (PO) routes as aqueous solution of peptide with dose of 1mg/kg (Dubynin et al., 2008; Yin et al., 2010; Zhang et al., 2012; Zoghbi et al., 2006; Zong et al., 2007), to compare the effects of enzymatic hydrolysis on BCM7 (during gastrointestinal digestion and absorption). Dosing for all animals was done, for 4 months along with the normal pallet diet (NPD).

Rats were divided into 8 different groups, feeding/ dosing was done for four months, along with normal pellet diet (NPD) as mentioned in **Table 4.2** followed by estimation of different parameters.

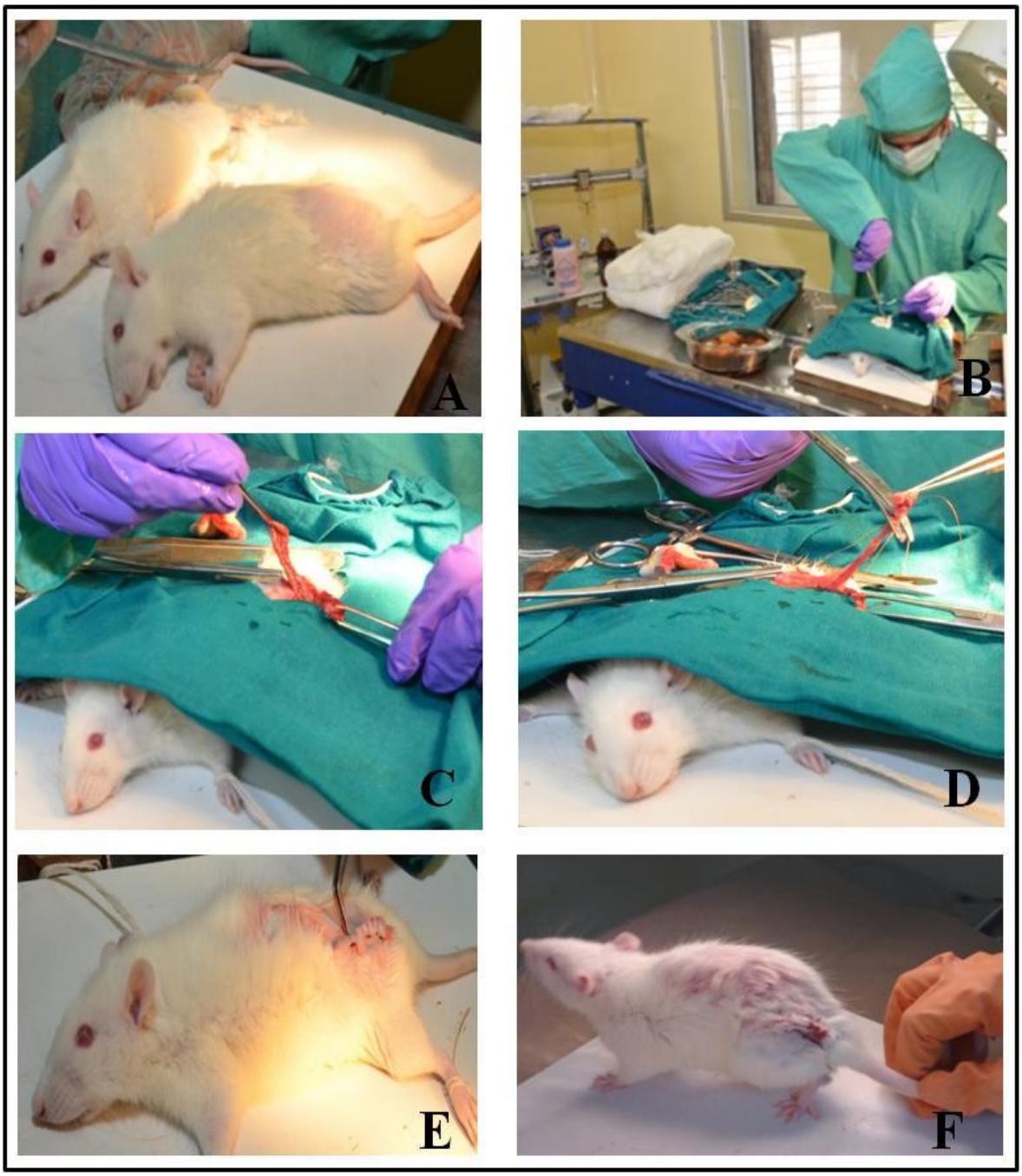


Fig. 4.5: Different stages of ovariectomy. A=aseptic surgical site, B= incision, C= retraction of uterine horns, D=ligation of oviduct and removal of ovary, E=suturing, F=recovered surgical wound.

Table 4.2: Animal groups, their dosing and feeding.

S. No.	Group name	No. of animals	Description of animals	Dosing /feeding (for 4 months)
1	Normal control	6	Natural / normal rats of same age group	NPD
2	OVX	6	Ovariectomized rats	NPD
3	Sham operated	6	Rats with incision only in skin	NPD
4	BCM7 (PO)	6	Normal rats + BCM7	BCM7 @1mg/ kg PO; + NPD
5	BCM7 (IP)	6	Normal rats + BCM7	BCM7 @1mg/ kg IP;+ NPD
6	A1 beta-casein protein	6	Normal rats + BCM7	Isolated beta-casein protein @1mg/ kg PO;+ NPD
7	A1 natural milk	6	Normal rats + A1 natural milk	A1 natural milk @ 7.8ml/ kg* (equivalent to 1mg/kg) PO;+ NPD
8	A2 natural milk	6	Normal rats + A2 natural milk	A2 natural milk @ 7.8ml//kg* (equivalent to 1mg/ml) PO;+ NPD

Dose of BCM7 was decided based on available literature (Dubynin et al., 2008; Yin et al., 2010; Zhang et al., 2012; Zoghbi et al., 2006; Zong et al., 2007). * Beta-casein in natural milk was calculated by Bradford assay of isolated protein and an equivalent volume of milk was fed.

4.5.4. Estimation of parameters indicating changes in bone mass and quality:

For the estimation of osteoporosis development, blood samples (0.5 ml) were collected using the retro-orbital method, after 4 months of dosing/feeding and serum separated, was used for the biochemical estimations. Further, the bones were assessed for their strength and articular surface erosion, for confirming the histological/ mechanical changes.

4.5.5. Biochemical analysis:

The level of serum osteocalcin (a marker of bone formation) was measured using Rat – Glutamate-Osteocalcin (Rat-Glu-OC) high sensitive EIA Kit. (Cat. #MK146, Takara Bio Inc, Nojihigasi 7-4-38, Japan) following manufacturer's protocol. The standard curve was plotted as suggested. The concentrations of Rat-Glu-OC (ng/ml) from all the study groups were determined by extrapolating the absorbance obtained at 450 nm, for the sera samples on this standard curve (Christgau et al., 2001; Garvican et al., 2010). Serum calcium and phosphorus contents were estimated, using commercially available kits (Sprseact Ref: 1001060 and 1001155), as per instructions given by manufacturer.

4.5.6. Histology:

For histology, knees (femur and tibia) were decalcified for 3 to 4 weeks in 10% formic acid and 2% formaldehyde followed by dehydration in graded alcohol. The decalcified knee joints were cleaved along the medial collateral ligament (lower femoral and upper tibial condyles) and epiphysis region of femur; tissue blocks were impregnated and embedded in paraffin. From each block, two 5-mm-thick sections were cut, each section was stained in Toluidine blue and pathological changes in the knee joints and bone cells in epiphysis region were observed under microscope (Hoegh-Andersen et al., 2004). Number of bone cells per focus area of epiphysis region of femur was counted by ImageJ software. 'Osteoarthritis Research Society International' (OARSI) system, described by Pritzker et al. (Pritzker et al., 2006) was used for bone surface erosion analyses. A qualitative OARSI score was reported. OARSI Grades 0 to 5 were observed as; Grade 0: surface intact, cartilage morphology intact; Grade1: surface intact, superficial fibrillation, and edema or cell death or cell proliferation; Grade 2: surface discontinuity; Grade 3: vertical fissures (clefts); Grade 4: erosion; Grade 5: denudation;

Grade 6: deformation. The scoring was performed by a single trained researcher in a blinded fashion (Takahashi et al., 2018; Waldstein et al., 2016).

4.5.7. Tensile strength test:

Biomechanical testing was performed to investigate the effects of peptide on the structure–function properties of bone and to understand behavior of whole bone based on mechanical properties (Oksztulska-Kolanek et al., 2016; Sharir et al., 2008). After careful dissection and cleaning of all adherent soft tissues, femurs of approximately similar length (~34mm) and diameter (~2.98mm) were separated from rats of all groups and stored in phosphate buffered saline solution (PBS), till mechanical analysis.

Tensile strength testing was performed using a universal testing machine (UTM) (UNITEK 94100). Both ends of the bone were fixed and tensile strength was assessed, operating the equipment at a speed of 1 mm/min, until the bone was fractured, followed by generation of load-displacement and stress-strain curve. Maximum tensile strength value (maximum load/original area of cross-section) was obtained, by a graph between load (KN) and displacement (mm) (Nazarian et al., 2011; Sanada et al., 2015). Total *e*nergy absorbed before failure (fracture) by the samples was decided by calculating area under the load-displacement *curve*. Load displacement curve represents extrinsic properties of bone. Tensile strength reflects the general integrity of the bone structure and area under curve represents the amount of energy necessary to break the bone and ultimate displacement is conversely related to the brittleness of the bone. The slope of the stress-strain curve within its elastic region is called Young's modulus, which reflects intrinsic stiffness of the bone. Young's modulus (E) was obtained by stress-strain curve (E= stress/strain) (Ritchie et al., 2008; Sharir et al., 2008; Varghese et al., 2011). Stress–

strain curve also distinguishes the elastic and plastic strain regions which are separated by the yield point i.e. the point where the stress–strain curve begins to become nonlinear (Oksztulska-Kolanek et al., 2016).

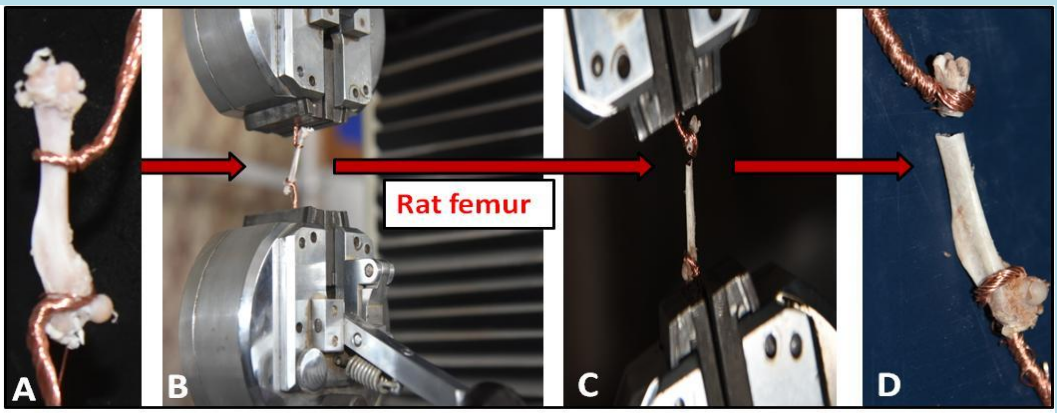


Fig. 4.6: Flow diagram for bone tensile strength test. A= Rat femur, B=Femur attached in universal testing machine (UTM) (UNITEK 94100), C= Force applied and bone fracture D= Fractured bone.

4.5.8. Microcomputed tomography (µCT) analysis:

Micro-computed tomographic (µCT) analysis of excised bones (tibia) was carried out by Sky scan 1076 micro CT scanner (SKyScan, Belgium). After harvesting, bone was fixed before storage in 70% isopropanol and scanning were done at 70 kV, 142mA using 1 mm filter at a resolution of 18 micro meter/pixel. Reconstruction was done by Nrecon software based on modified feldkamp algorithm. For analysis 100 slices region of interest (ROI) was drawn bellow the growth plate. CTAn software was used for micro architectural parameter measurement, encompassing bone volume fraction (BV/TV), thickness of trabecularized spicules (Tb.Th), trabecular separation (Tb.Sp) and trabecular number (Tb.N) were assessed, representative 3D µCT images were also obtained (Bouxsein et al., 2010; Genant et al., 2008; Wu et al., 2015).

4.5.8. Statistical analysis:

Data obtained were analyzed using one-way Analysis of Variance (ANOVA) followed by Tukey's test using GraphPad Prism, version 7.00 (San Diego, CA, USA). Data obtained were considered as statistically significant, if $p < 0.05$.

5. Results

5.1. Beta-casein gene sequence analysis:

The whole genomic DNA from cattle blood samples was isolated (**Fig. 5.1A**) using standard blood DNA isolation protocols with minor modifications. The CSN2 exon VII was amplified using the isolated whole genomic DNA and the designed primers. An amplification of ~500bp was obtained (**Fig. 5.1B**), as expected. The amplicons were purified and sequenced commercially. The obtained sequences were analyzed by BLASTn (NCBI) to verify the authenticity of the amplicons. The chromatograms (**Fig. 5.2**) were thoroughly screened to identify the nucleotide mutation in the sequence and thus characterized into three different alleles A1A1, A2A2 & A1A2. All the obtained sequences of exon VII were further analyzed by multiple alignments with M55158 (*Bos taurus* exon VII) (**Fig. 5.3**). The overall genotype of HF cattle identified had 9% -A1A1, 69.7% -A1A2 and 21.3% -A2A2 alleles. Most of the desi cattle were found to possess A2A2 genotype, while A1A2 was observed in mixed zebu breeds and A1A1 was not observed in any of the tested samples. The A2A2 genotype prevailed over the A1A2 genotype in mixed breeds. The allele frequency analysis of HF cows was 0.44 for A1 allele, 0.56 for A2 allele and 0.09, 0.70, 0.21 for genotypes A1A1, A1A2, and A2A2, respectively. The allele frequency analysis for Zebu cows was 0.1 for A1 allele, 0.9 for A2 allele and 0.00, 0.10, 0.9 for genotypes A1A1, A1A2, and A2A2, respectively. The distribution of the genotypes was within the Hardy–Weinberg equilibrium in the tested population ($P>0.05$). The value of gene homozygosity (Ho), gene heterozygosity (He) and fixation index (FIS) were found to be 0.40, 0.60, and - 0.20, respectively. Average milk yield in HF cattle was 16.5 L/day for A1A1, 15.5L/day for A1A2, 15.25L/day for A2A2.

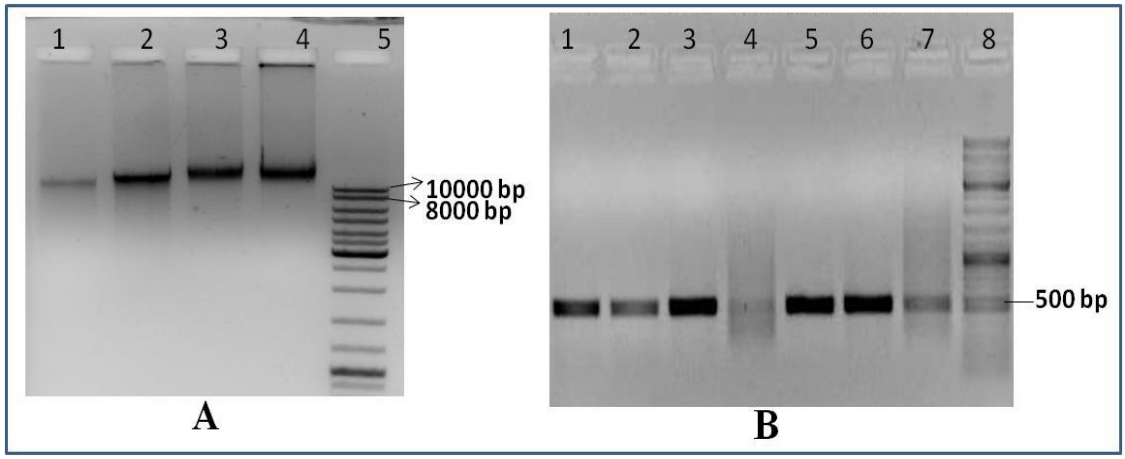


Fig. 5.1: A. Agarose gel electrophoretogram of DNA isolated from cow blood. Lane 1-4: DNA from different cow; lane 5: Gene Ruler DNA ladder mix (SM0331, Fermentas); **B**. **Beta-casein gene amplification product:** Lane 1-7: amplicons of different samples; lane 8: Gene Ruler DNA ladder mix (SM0331, Fermentas).

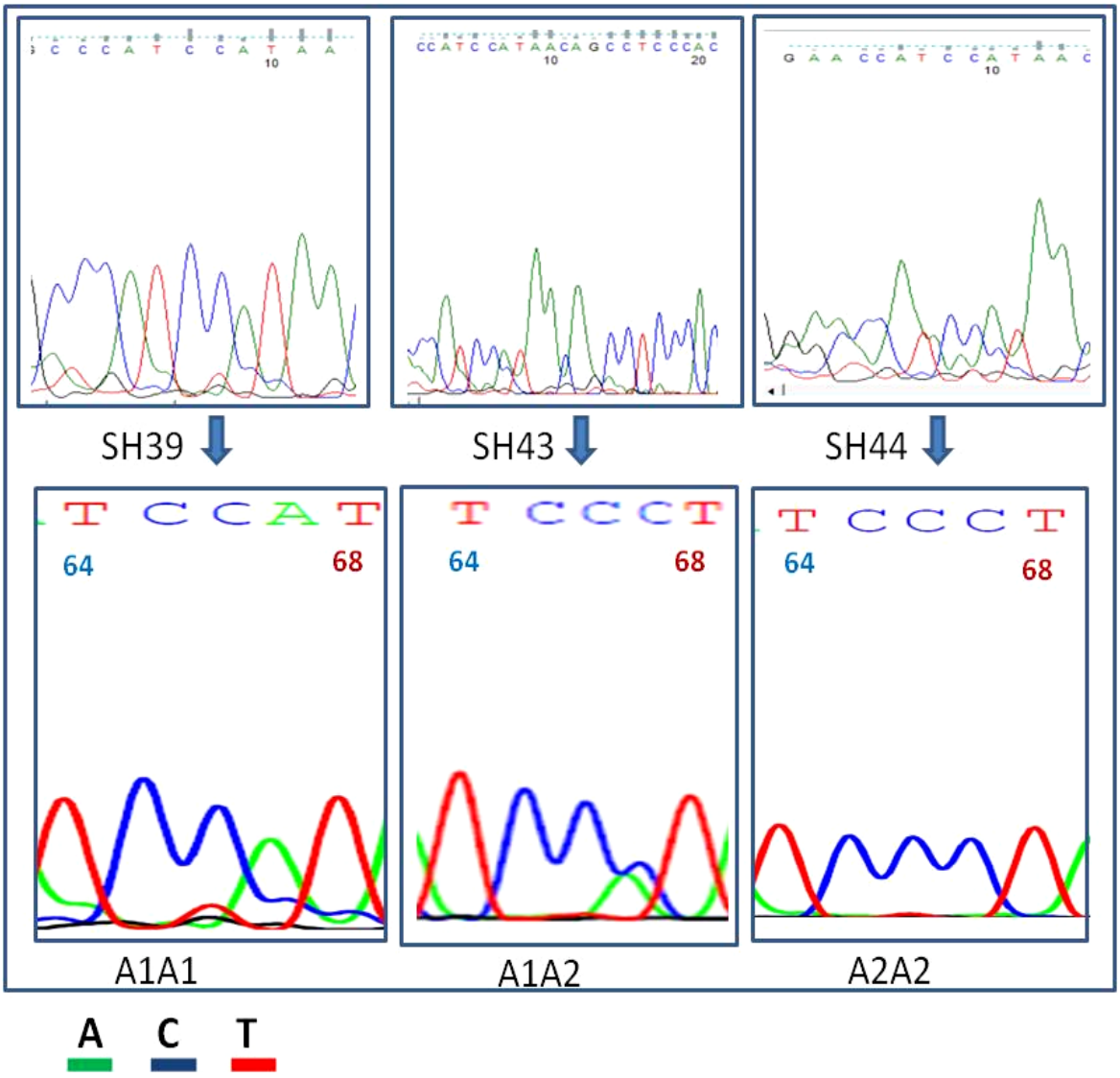


Fig. 5.2: Chromatogram regions showing nucleotide variations; SH39-HF, SH43-HF, SH44-HF

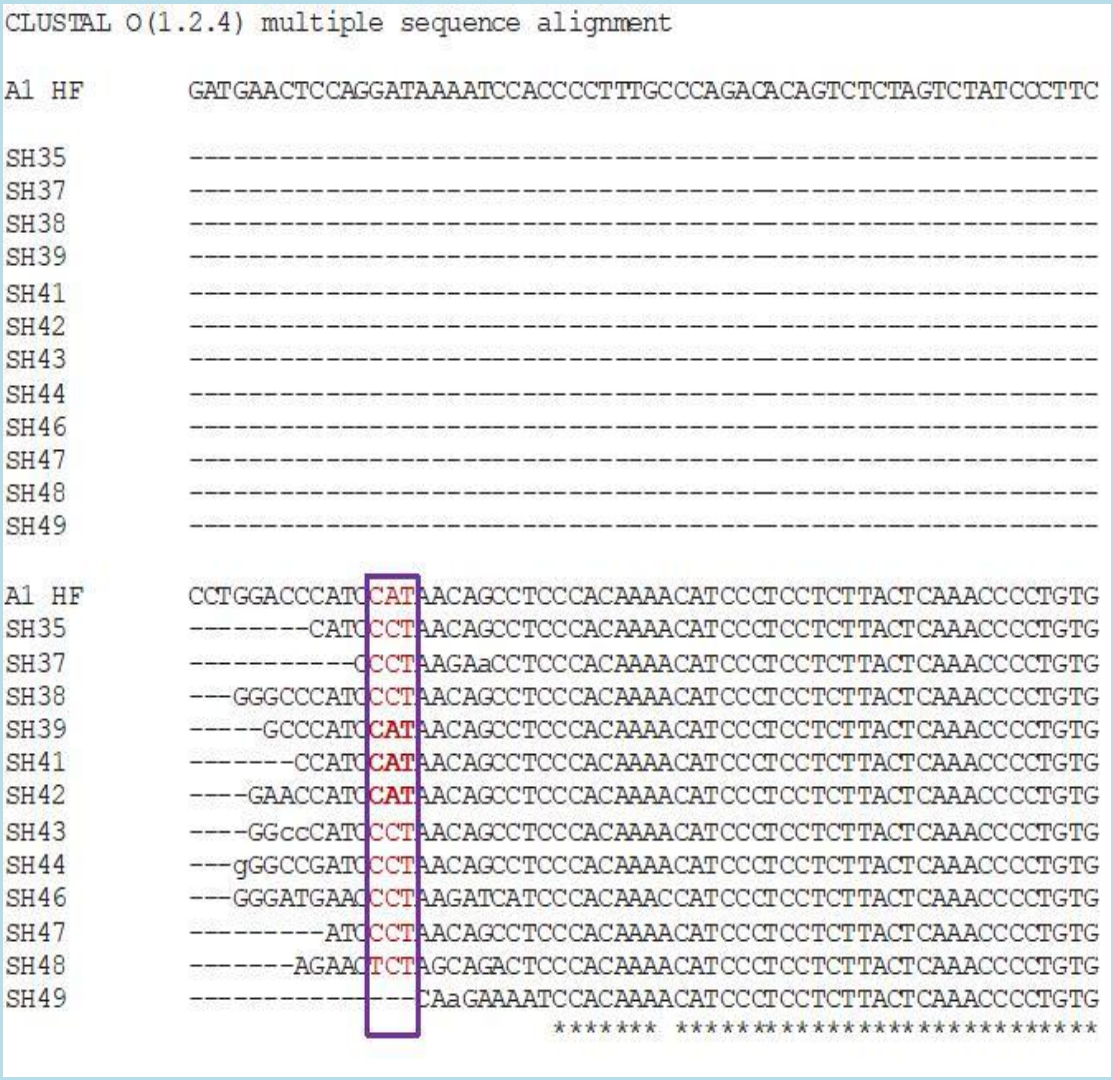


Fig 5.3: Clustal **Omega Multiple sequence alignment of Indian isolates and M55158 exon VII.** Sample Ids; A1 HF- A1 reference sequence from NCBI; SH35-HF, SH37-HF, SH38-desi breed, SH39-HF, SH41-HF, SH42-HF, SH43-HF, SH44-desi breed, SH46-desi breed, SH47-HF, SH49-HF.

5.2. Selection pressure analysis:

For the selection pressure analysis of *Bos taurus* beta-casein gene, 12 closely related mammal's beta-casein genes were selected. Out of all selected mammals, only a few have

more than one variant of beta-casein gene. Gene and site-specific selection pressure were analyzed to identify the evolution pattern of this gene.

5.2.1 Gene specific selection pressure analysis:

Five different variants of *Bos taurus* beta-casein genes, were analyzed. For A2 and I variant's, all pair wise comparisons were less than 1, indicating that these beta-casein gene variants evolved under purifying selection, whereas A1 variant selection value was near to 1 (0.9882). While A3 and B variant's selection pressure analysis values were more than 1, indicating that these variants were evolved under positive selection.

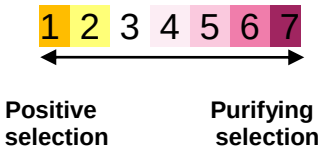
5.2.2. Site specific selection pressure analysis:

A2 is a wild type variant, which has 7 mutation prone sites (Dai et al., 2016). Among these, five sites seem to have evolved under purifying selection, including 72nd site coding for glutamine, shown in magenta color (**Table 5.1**). Proline is coded at 67th position in beta-casein A2 variant and indicated to have evolved under purifying selection as per the color scale. However, the 67th site mutation in A1 and B variants seems to have evolved by positive selection, shown in yellow color. 106th and 122nd sites in A3 and B variants seem to have evolved under positive selection in all variants, as depicted by yellow color irrespective of amino acid variation. Similarly, A3, B and I variants' mutation sites also seem to have evolved by positive selection (**Table 5.1**). The Likelihood Test Ratio (LTR) results showed statistically significant levels (0.001).

Table 5.1: Site specific natural selection result for different variants of CSN2. Indicated as per selection scale, where orange color indicates significant positive selection (Ka/Ks>=1) and magenta indicates significant purifying selection (Ka/Ks <1) for site specific selection. Adapted and modified from Dai et al., 2016.

CSN2 Variants	Amino acid positions						
	67	72	88	93	106	122	138
A2	CCT P	CAA Q	CTT L	ATG M	CAC H	AGC S	CCT P
A1	CAT H						
A3					CAA Q		
B	CAT H					AGG R	
I				CTG L			

The selection scale:



5.3. A1 Protein isolation

Beta-casein fractions isolated by acetic acid and rennin were analysed using 18% SDS - polyacrylamide gel electrophoresis (PAGE). When the rennin processed sample was analysed using 18% SDS-PAGE, multiple bands were observed, indicating sub-fractions of casein or whey proteins. Similarly when the acetic acid processed sample was analysed using 18% SDS-PAGE, three (2 intense and 1 faint) bands were observed (**Fig 5.4**). Probably principal protein was beta-casein [(β-CN), (mol. wt. ≈25KD, upper intense band)] with minor amount of α_{s1}-casein [(α_{s1}-CN), (mol. wt. ≈20KD, lower intense band)] and trace amount of alpha $_{s2}$ -casein [(α_{s2}-CN) (middle faint band)]. Further, all the bands obtained were analysed using MALDI-MS/MS.

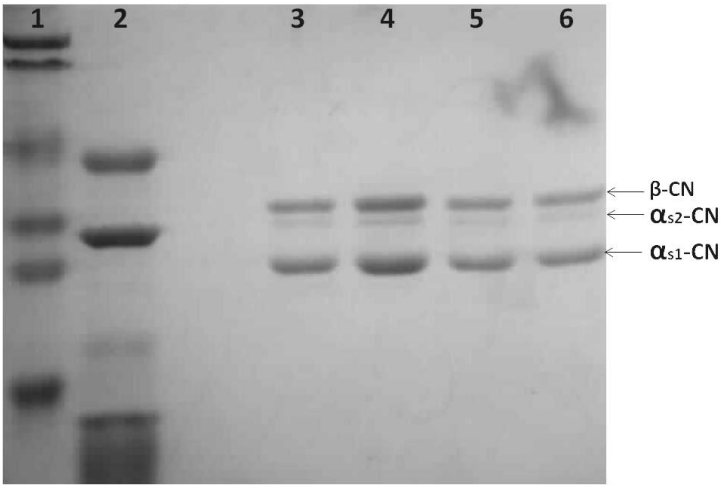


Fig. 5.4: SDS–PAGE electrophoretogram of supernatant obtained from the milk of different breeds. **Lane 1:** Holstein Friesians (HF) milk was coagulated by rennin and obtained rennet curd was heated at 80 ºC for 5 min, incubated at 4 ºC for 24h. All other samples were coagulated by acetic acid; obtained curd was washed and dissolved in AMQW, incubated at 4 ºC for 24h. **Lane 2**: low molecular weight marker (PMW-L), **Lane 3-6:** supernatant from milk samples of different cows.

5.3.1. Effect of incubation (at 4ºC) time on protein yield:

Impact of the incubation time on protein yield was also observed, maximum yield was observed at 18 hours incubation. In **Fig. 5.5**, bands of SDS–PAGE electrophoretogram of supernatant obtained at different incubation time, are shown. Same volume of supernatant was loaded in each well. All samples were coagulated by acetic acid and obtained curd was washed and dissolved in AMQW, incubated at 4ºC up to 48h. Lane 1 denotes incubation at 0 hour, Lane 2 denotes incubation at 6 hours, Lane 3 denotes incubation at 12 hour, Lane 4 denotes incubation at 18 hours, Lane 5 denotes incubation at 24 hour, Lane 6 denotes incubation at 48 hours, and Lane 7 denotes incubation at PMW-L. There was an increase in the yield of beta-casein as we increase the incubation time up to 18hours, where as maximum yield was obtained between 18-

24 hours (**Fig. 5.5 & 5.6)**. There was not any significant increase in protein yield beyond 18 hour incubation.

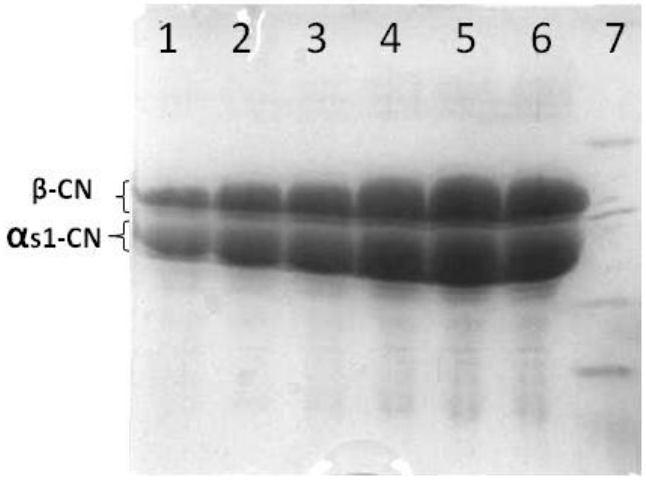


Fig. 5.5: SDS–PAGE electrophoretogram of supernatant obtained at different incubation time. Same volume of supernatant was loaded in each well. All samples were coagulated by acetic acid and obtained curd was washed and dissolved in AMQW, Incubated at 4 ºC up to 48h. **Lane 1:** 0h, **Lane 2:** 6h, **Lane3:** 12h, **Lane 4:** 18h, **Lane 5:** 24h, **Lane 6:** 48h, **Lane 7:** PMW-L.

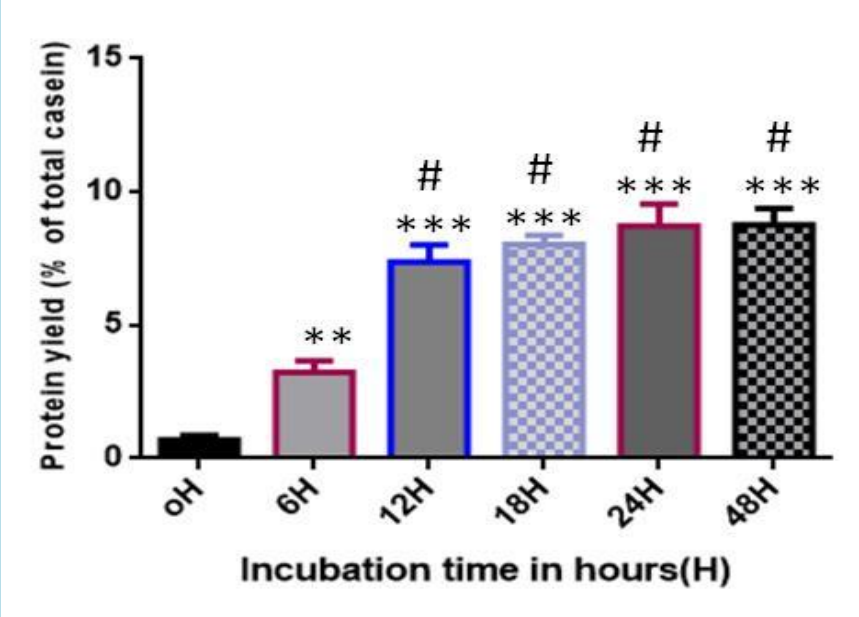


Fig. 5.6: Effect of incubation time at 4 ºC on protein yield expressed as % of total casein obtained. The columns represent mean of values. Error bars indicate ± S.D. * significant difference as compared with 0H VS 6H-48H, # significant difference as compared with 6H VS 12H-48H, *P<0.05, ** P<0.002, #P<0.05 N=3.

5.3.2. Effect of initial incubation temperature on protein yield:

The effect of initial incubation temperature, on protein yield, expressed as percentage of total casein obtained was analysed (**Fig. 5.7**). There was no significant effect of temperature on initial incubation of milk and on protein yield. Below 20°C, coagulation didn't happen in desired incubation time. Between 20-24 °C, coagulation was not proper and at room temperature (25°C) and higher temperature coagulation was proper and not affected by higher temperature (up to 80°C).

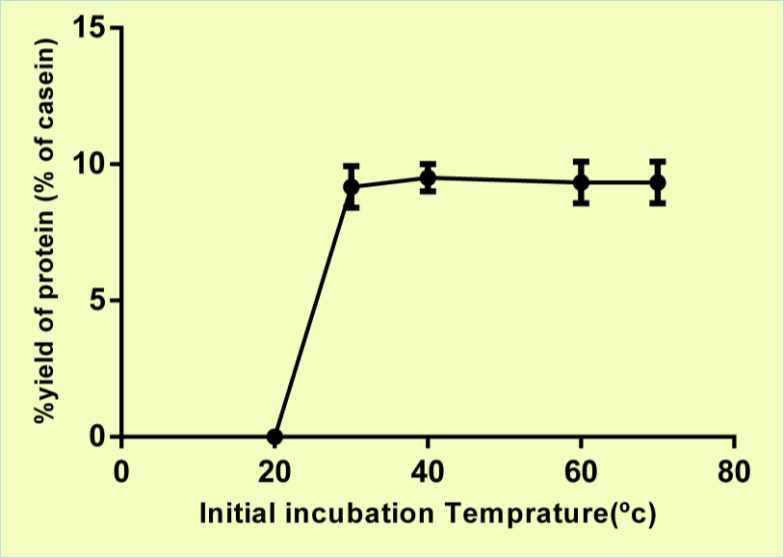


Fig. 5.7: Effect of initial incubation temperature, on protein yield, expressed as % of total casein obtained. The values are mean of data obtained from triplicate experiments on individual milk samples. Error bars indicate ± S.D., P<0.05, N=3.

5.3.3. Effect of incubation time on quantity of sediment:

Remaining sediment obtained from acetic acid method, after filtration, was also estimated at different incubation time (2-48hrs). All samples were coagulated by acetic acid, curd was washed and dissolved in AMQW, incubated at 4 º C for 24 hours curd was washed with AMQW and also dissolved in AMQW. Incubated at 4 ºC for 0 hour (lane 1), 6 hours (lane 2), 12 hours (lane 3), 18 hours (lane 4), 24h (lane 5) or 48 hours (lane 6). Some protein still remained in sediment (curd) even after 48-hour incubation. Same quantity of sediment was loaded in each well of SDS–PAGE electrophoretogram, bands were observed (**Fig. 5.8**). Quantity of beta-casein in sediment decreased as incubation time increased up to 18 hours. There was no significant decrease beyond 18 hours incubation.

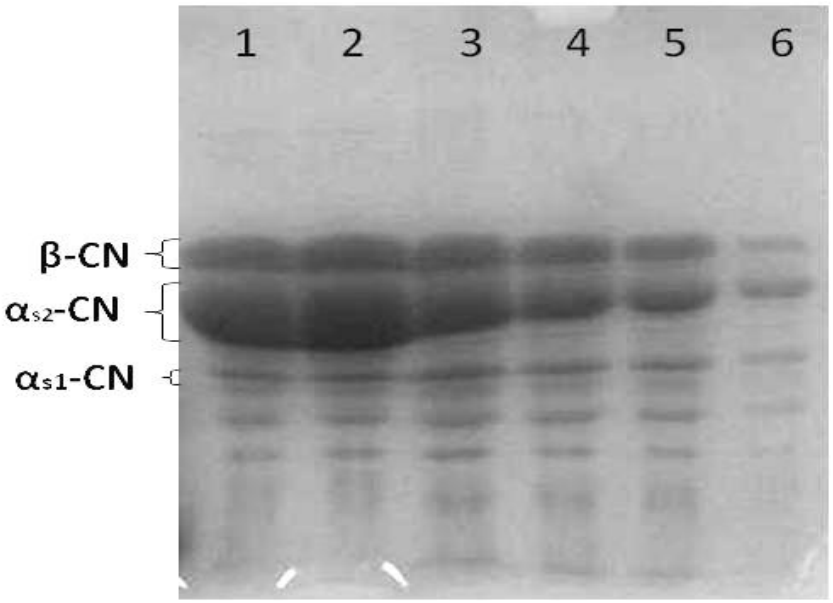


Fig. 5.8: SDS–PAGE electrophoretogram of sediment (curd) remaining after filtration at different incubation time. Same quantity of sediment was loaded in each well. All samples were coagulated by acetic acid, curd was washed and dissolved in AMQW, incubated at 4 º C for 24h curd was washed with AMQW and also dissolved in AMQW. Incubated at 4 ºC, for 0h (lane 1), 6h (lane 2), 12h (lane 3), 18h (lane 4), 24h (lane 5) or 48h (lane 6).

5.3.4. MALDI-MS/MS analysis:

Identification of protein is carried out by peptide mass fingerprinting technique (PMF). Based on accurate mass measurement of a group of peptide derived from a protein by sequence-specific proteolysis (trypsin). Spectrum of identified peptide masses is unique for a specific protein and known as mass finger printing. From the PMF selected masses are searched from the available data base of the known protein. Protein from desired bands was gel eluted and confirmed by MALDI-MS/MS and the results presented below as mascot score histogram (**Fig. 5.9)** and index charts (**table 5.2** & **5.3)**. First histogram showing upper band supports the presence of beta-casein and second histogram showing lower band supports the presence of α_{S1}-casein. Protein score was calculated as -10*Log (P), where P is the probability that the observed match is a random event. Protein scores greater than 78, are considered significant ($p<0.05$). Protein scores are derived from ions scores as a non-probabilistic basis, for ranking protein hits. Beta-

casein obtained from upper band gel elusion are indexed in **table 5.2** and αS1-casein obtained from gel elusion of lower band are indexed in **table 5.3**.

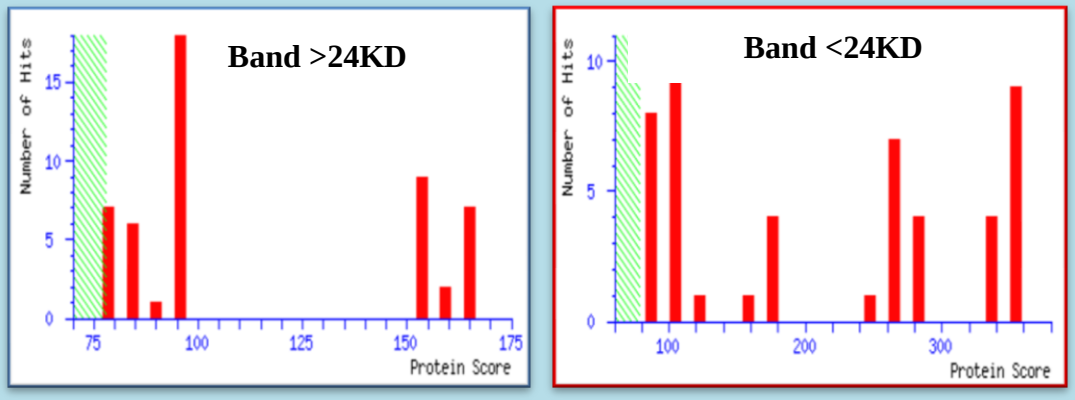


Fig. 5.9: Mascot Score Histogram of both bands. Protein score is -10*Log (P), where P is the probability that the observed match is a random event. Protein scores greater than 78 are significant (p<0.05). Protein scores are derived from ions scores as a non-probabilistic basis.

Table 5.2: Index of beta-casein obtained from gel elusion of band >24KD.

S.No.	Accession	Mass	Score	Description
1	**XP_014335715.1**	24207	165	PREDICTED: beta-casein isoform X4 [Bos mutus]
2	**AGT56763.1**	25042	165	beta-casein [Bos taurus]
3	**AAA30431.1**	25188	165	beta-casein [Bos taurus]
4	**XP_010850446.1**	25148	165	PREDICTED: beta-casein [Bison bison bison]
5	**AAI11173.1**	25257	165	CSN2 protein [Bos taurus]
6	**XP_014335714.1**	25468	164	PREDICTED: beta-casein isoform X3 [Bos mutus]
7	**XP_015327157.1**	28209	163	PREDICTED: beta-casein isoform X2 [Bos taurus]
8	**XP_010804480.1**	29150	162	PREDICTED: beta-casein isoform X1 [Bos taurus]
9	**XP_014335713.1**	30412	162	PREDICTED: beta-casein isoform X1 [Bos mutus]
10	**NP_001009373.1**	24956	154	beta-casein precursor [Ovis aries]
11	**P11839.3**	24916	154	RecName: Full=Beta-casein; Flags: Precursor
12	**XP_005985492.1**	24960	154	PREDICTED: beta-casein [Pantholops hodgsonii]
13	**CAB39200.1**	24906	154	beta-casein [Capra hircus]
14	**P33048.1**	24906	154	RecName: Full=Beta-casein; Flags: Precursor
15	**AAK97639.1**	25033	154	beta-casein precursor [Capra hircus]
16	**AAB29137.1**	25139	154	beta-casein A3 [Bos taurus]
17	**XP_005681778.2**	28899	153	PREDICTED: beta-casein isoform X1 [Capra hircus]
18	**XP_013820153.1**	28914	153	PREDICTED: beta-casein isoform X2 [Capra hircus]
19	**AGZ84117.1**	17174	97	beta-casein, partial [Bos indicus]
20	**ABY27644.1**	17302	97	beta-casein, partial [Bos indicus]

Table 5.3: Index of alpha S1-casein obtained from gel elusion of band <24KD.

S.No.	Accession	Mass	Score	Description
1	**ABW98943.1**	23473	354	alpha S1 casein, partial [Bos taurus]
2	**ABW98940.1**	23598	354	alpha S1 casein, partial [Bos taurus]
3	**XP_015327132.1**	24442	353	PREDICTED: alpha-S1-casein isoform X1 [Bos taurus]
4	**ACG63494.1**	24484	353	alpha S1 casein [Bos taurus]
5	**NP_851372.1**	24570	353	alpha-S1-casein precursor [Bos taurus]
6	**XP_010850445.1**	24498	353	PREDICTED: alpha-S1-casein [Bison bison bison]
7	**XP_015327133.1**	23558	353	PREDICTED: alpha-S1-casein isoform X2 [Bos taurus]
8	**XP_005902100.1**	24498	351	PREDICTED: alpha-S1-casein [Bos mutus]
9	**XP_015327135.1**	23703	350	PREDICTED: alpha-S1-casein isoform X4 [Bos taurus]
10	**ABW98941.1**	18728	342	alpha S1 casein, partial [Bos taurus]
11	**AAA30429.1**	24536	340	alpha-S1-casein [Bos taurus]
12	**XP_005208086.1**	20227	340	PREDICTED: alpha-S1-casein isoform X13 [Bos taurus]
13	**ABW98949.1**	22442	337	alpha S1 casein, partial [Bos taurus]
14	**ABW98954.1**	14704	278	alpha S1 casein, partial [Bos taurus]
15	**ABW98937.1**	15733	276	alpha S1 casein, partial [Bos taurus]
16	**ABW98942.1**	16321	276	alpha S1 casein, partial [Bos taurus]
17	**ABW98950.1**	17842	274	alpha S1 casein, partial [Bos taurus]
18	**ABW98945.1**	19792	268	alpha S1 casein, partial [Bos taurus]
19	**ABW98955.1**	12985	265	alpha S1 casein, partial [Bos taurus]
20	**ABW98948.1**	9519	264	alpha S1 casein, partial [Bos taurus]

5.3.5. Comparison of Glacial Acetic Acid (A) and Rennin (B) methods for beta-casein isolation: A comparative analysis was performed on the use of glacial acetic acid as described earlier with the method for isolation of beta-casein from milk using rennin, as described by Huppertz et al., 2006. The comparative analysis is shown in **Table 5.4**.

Table 5.4: Table showing comparison of glacial acetic acid (A) and rennin (B) methods for beta -casein isolation.

S.No.	Property	Our method (A)	Enzymatic method (B)
1	Use of coagulant	Glacial Acetic Acid	Rennin
2	Cost for coagulation	For 1-litre milk, 1 ml acetic acid is required which cost approximately INR. 0.5	For 1-litre milk, 0.5g rennin is required which cost approximately INR. 250.00
3	Temperature conditions	Mostly room temperature, no need of high temperature.	High temperature is required to inactivate rennin.
5	beta-casein yield	1250µg/ml i.e. 85% of total beta-casein or 30% of total casein	400µg/ml i.e. 27.4% of total beta-casein or 9.67 % of total casein
6	Purity of beta-casein	Highly pure (65-75 %)	Multiple and faint bands as shown in **Fig.5.4**
7	Common impurities	α_{s1} and α_{s2} caseins (α_{s2} = 15-20 %, α_{s1} =2-3%)	α_{s1} and $\alpha_{s2,}$ kappa caseins and some whey proteins, there were multiple and faint bands as shown in **Fig.5.4**

Note: Protein estimation was done by using standard Bradford assay of gel eluted protein from different bands obtained by using 18% SDS-PAGE.

5.4. Animal study

5.4.1. Estimation of serum osteocalcin levels:

Osteocalcin is a vitamin K-dependent calcium-binding non-collagen protein, specifically produced by osteoblast, and has been used as one of the osteoblast marker. Osteocalcin is

released from bone matrix into blood by action of various enzymes during bone metabolism. Osteocalcin on catabolism produces its characteristic amino acid, gamma-carboxyglutamic acid (Gla), that is excreted in the urine. Both serum osteocalcin and urine Gla, are currently used for the clinical assessment of bone disease. Rat osteocalcin consists of 50 amino acids with three glutamate (Glu) residues at position 17, 21 and 24, affording osteocalcin its ability to bind with bone matrix.

Further to estimate serum osteocalcin, Anti-Glu, specific monoclonal antibody (as described by Kit manufacturer) was used. There was a significant decline in serum osteocalcin level of OVX (0.5ng/ml), BCM7 PO (0.5ng/ml), BCM7 IP (0.30ng/ml), isolated protein (0.4ng/ml), and A1 natural milk fed rats (0.45ng/ml), as compared with normal control rats (1.00ng/ml), (**Fig.5.10**). Lowest level of osteocalcin was observed in BCM7 (IP) treated rats, while normal level of osteocalcin was observed in sham operated and A2 natural milk fed rats. There was comparatively less decrease of osteocalcin in BCM7 (PO) treated rats, as compared with BCM7 (IP) treated rats.

5.4.2. Estimation of serum calcium and phosphorus:

There was a significant decrease in serum calcium and phosphorus levels in ovariectomized, BCM7 treated, beta-casein protein and A1 natural milk fed rats, as compared with normal control rats, at the end of the study. A2 natural milk fed rats reported better levels of both the minerals as compared with normal control rats. A significant difference in serum calcium (**Fig. 5.11**) and phosphorus (**Fig. 5.12**) levels was observed after 3 months of dosing, not earlier.

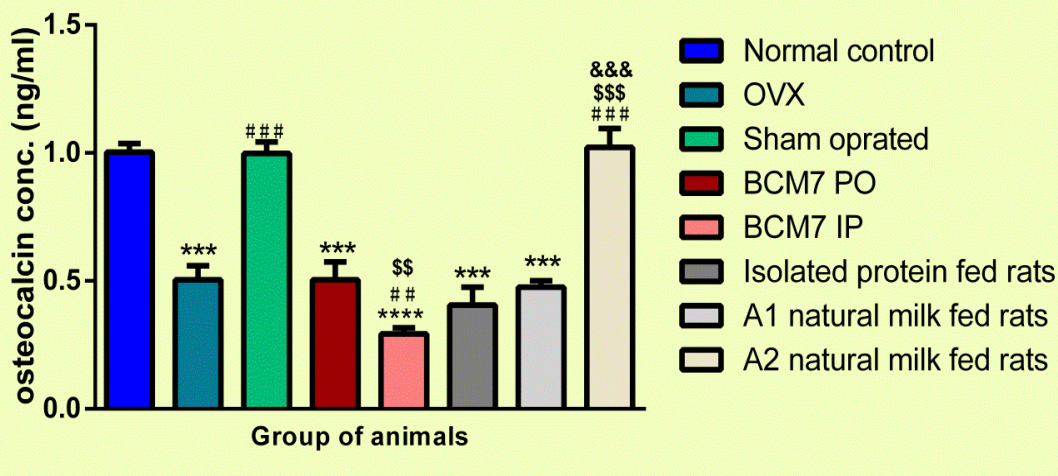


Fig. 5.10: Serum osteocalcin levels in rats. Serum osteocalcin levels were estimated by commercial available high sensitive EIA Kit. Values are represented as mean ± S.D., n=6/group. *,#,$ and & indicate significant difference as compared with normal control, OVX, BCM7 PO and A1 natural milk fed rats, respectively. *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001, P values are similar for #, $ and &.

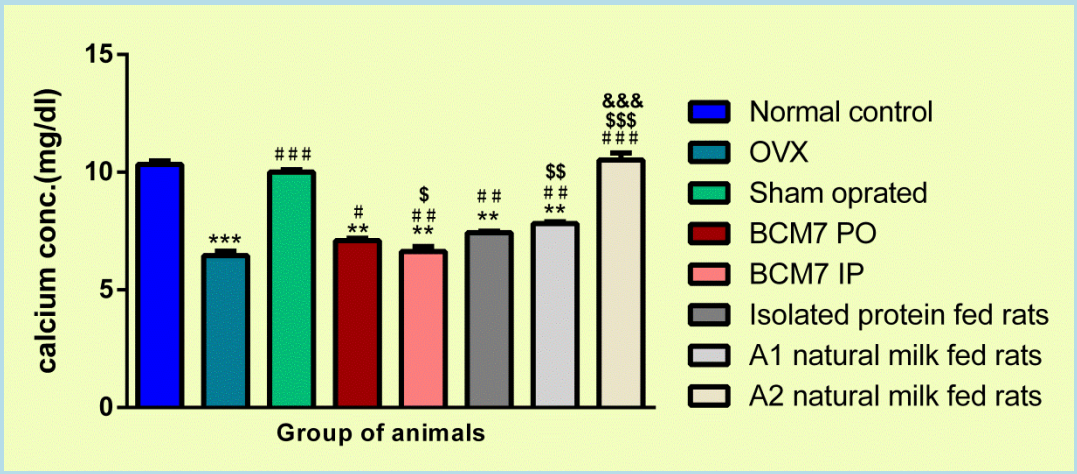


Fig. 5.11: Serum calcium levels in rats. Calcium levels were estimated by colorimetric kits. Values are represented as mean ± S.D., n=6/group. *,#,$ and & indicate significant difference as compared with normal control, OVX, BCM7 PO and A1 natural milk fed rats, respectively. *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001, P values are similar for #, $ and &.

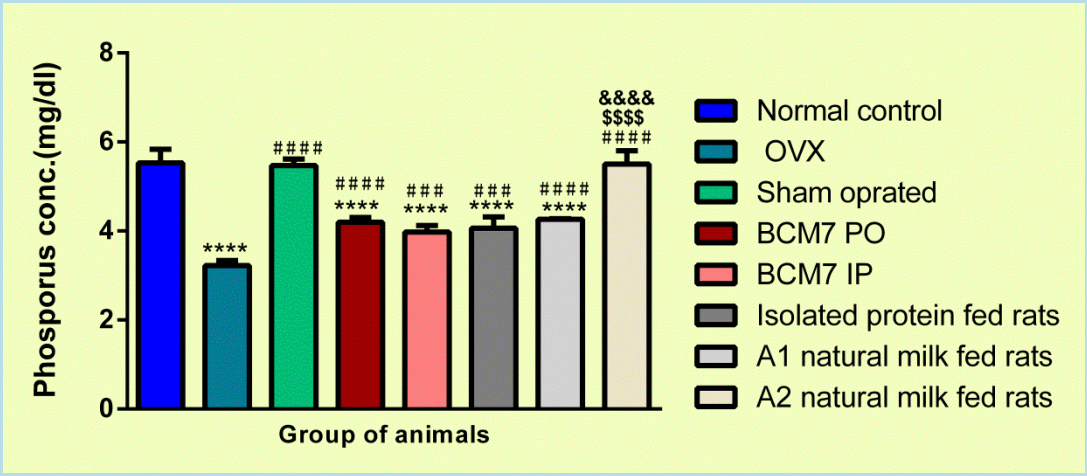


Fig. 5.12: Serum phosphorus levels in rat. Phosphorus levels were estimated by colorimetric kits. Values are represented as mean ± S.D., n=6/group. *,#,$ and & indicate significant difference as compared with normal control, OVX, BCM7 PO and A1 natural milk fed rats, respectively. *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001, P values are similar for #, $ and &.

5.4.3. Analysis of body and uterus masses:

Hypogonadism impairs the normal body metabolism, by lowering the level of estrogen and testosterone, resulting in increased body mass, in all females and males, respectively. Significant increase in body mass was observed in OVX, BCM7, beta-casein protein, A1 and A2 natural milk treated rats, as compared with control rats. Mass gain in control rats at termination of study (6.5 months) was 34% and in OVX rats, 38%. Ovariectomy induced tremendous regression of the uterus in OVX rats, there was a significant decrease in uterus mass in BCM7 (IP), beta-casein protein and A1natural milk treated rats (Table 2).

Table 5.5: Comparison of body mass of rats under study and uterine masses at termination of study (after 15 weeks dosing).

Group of rats	Body mass (g)		Uterine mass at end of study(g)
	At start of study (3 months)	At end of study (6.5 months)	
Normal control	191.00 ± 1.15	288.67 ± 1.53	1.144 ± 0.012
OVX	200.00 ± 1.73	322.00 ± 1.52****	0.34 ± 0.011****
Sham operated	198.34 ± 0.88	291.67 ± 1.21 ns	1.21 ± 0.038 ns
BCM7 PO	185.67 ± 1.2	292.00 ± 1.52 ns	1.1 ± 0.052 ns
BCM7 IP	193.00 ± 1.73	301.33 ± 1.85**	0.87 ± 0.021**
A1 beta-casein protein	192.67 ± 1.45	301.00 ± 3.21**	0.99 ± 0.047*
A1 natural milk	198.00 ± 0.5	301.33 ± 3.28**	0.94 ± 0.008**
A2 natural milk	200.00 ± 1.15	301.00 ± 2.08**	1.2 ± 0.058 ns

All Values are represented as mean ± S.D., n=6/group. Difference body mass and terminal uterine mass between normal control and other groups rats were assessed using One- way ANOVA. *P≤0.05, **P < 0.01, ****P < 0.0001.

5.4.4. Histological analyses:

5.4.4.1. Observation of bone cells:

Observations on cells involved in bone remodeling, revealed a significant increase of osteoclast cells and decrease of osteoblast cells in OVX rats, BCM7 treated rats, beta-casein protein and A1 natural milk fed rats, as compared with control group rats. Significant increase of osteoblast cells were observed in BCM7IP rats and decrease in beta-casein protein and A1 natural milk fed rats, as compared with BCM7PO rats. Significantly low numbers of osteoclasts were observed in A2 natural milk fed rats as compared with A1 natural milk fed rats (**Fig.5.13 & 5.14**).

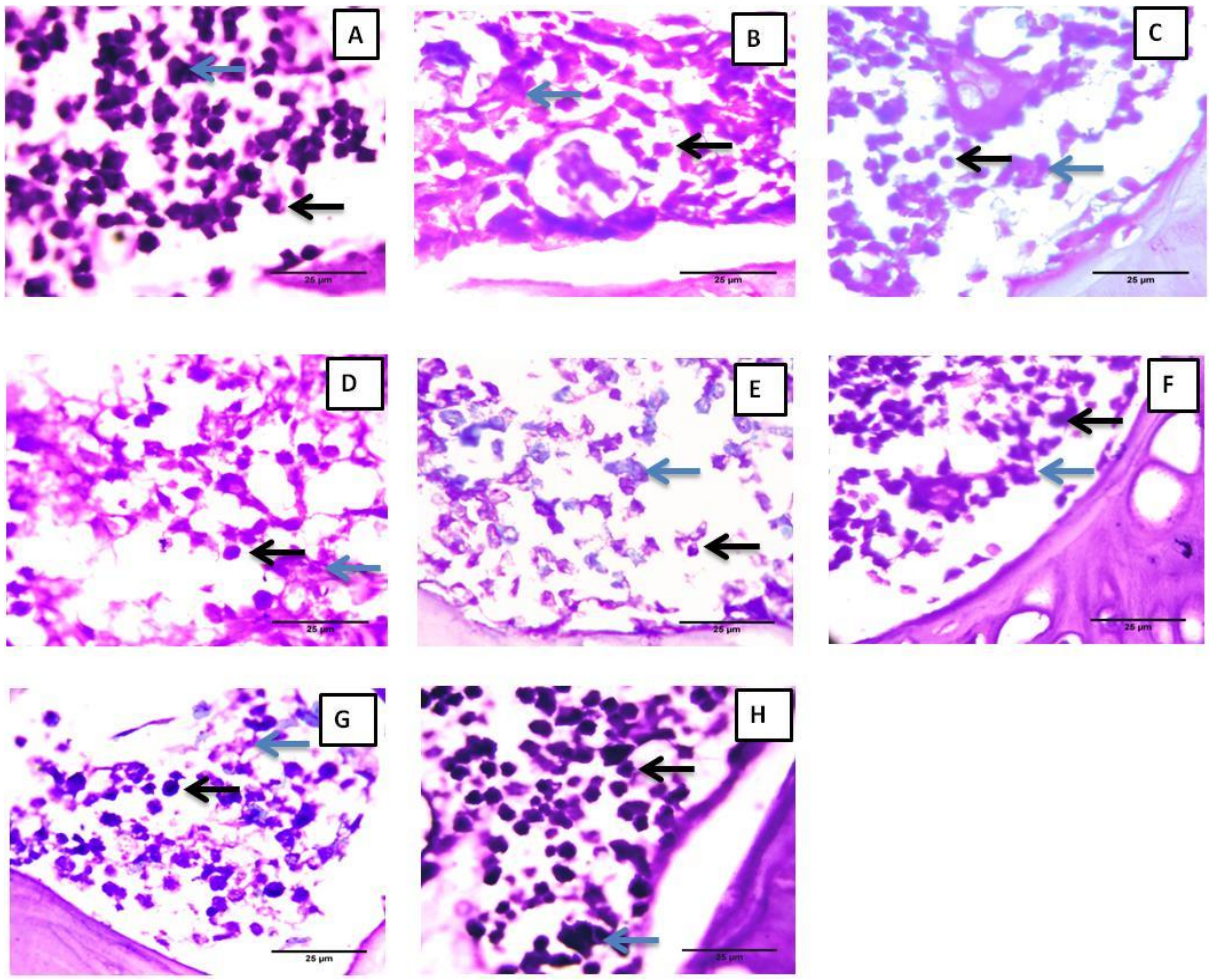


Fig. 5.13: Bone section from epiphysis region of the femur in 6.5 month old rats, stained with Toluidine blue. Black arrow indicates osteoblast and blue arrow indicates osteoclast cells. All histological images were observed with a magnification X100 (B-193, Optika, Italy) and captured by a digital camera D750 (Nikon, Japan), Scale bars: 25µm. Experimental animal groups are **A:** control, **B:** OVX, **C**: sham operated, **D**: BCM7 PO, **E**: BCM7 IP, **F**: isolated protein fed, **G**: A1 natural milk fed, and **H**: A2 natural milk fed rats.

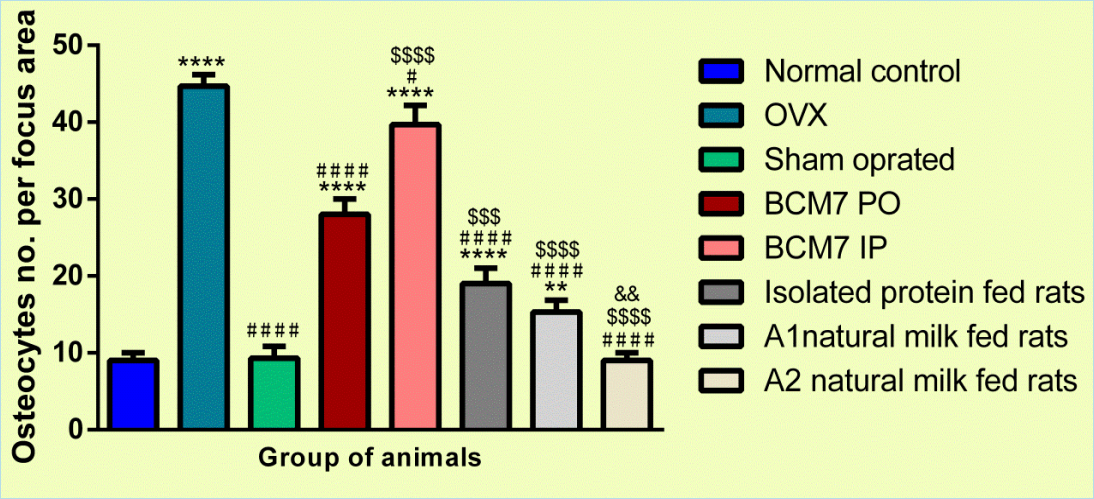


Fig. 5.14: Quantification of osteoclast cells in different rat groups. Cells were counted by ImageJ software and the number of osteoclast cells per focus area was represented. Values are represented as mean ± S.D., n=6/group. *,#,$ and & indicate significant difference as compared with normal control, OVX, BCM7 PO and A1 natural milk fed rats, respectively. *P≤0.05, ** P≤0.01, ***P≤0.001, ****P≤0.0001, P values are similar for #, $ and &.

5.4.4.2. Articular surface erosion:

Bone surface erosion was analyzed histologically by looking at cross section of the femoral and tibial condyles stained by Toluidine-blue. Surface erosion indicates alterations in the structure of the collagen fibers in comparison with intact cartilage surface of normal control rats (**Fig.5.15**). To clarify the histopathological changes in cartilage surface, we quantitatively evaluated the changes using the OARSI scoring system. Surface erosion was more pronounced in OVX rats (Grade 4, **Fig.5.15B**), BCM7 IP treated rats (Grade 4, **Fig.5.15E**) and clearly observed in BCM7 PO treated rats (Grade 3, **Fig.5.15D**), isolated protein fed rats (Grade 2, **Fig.5.15F**) and A1 natural milk fed rats (Grade 1, **Fig.5.15G**). No significant changes were observed in control rats (Grade 0, **Fig.5.15A**), sham operated rats (Grade 0, **Fig.5.15C**) and A2 natural milk fed rats (Grade 0, **Fig.5.15H**).

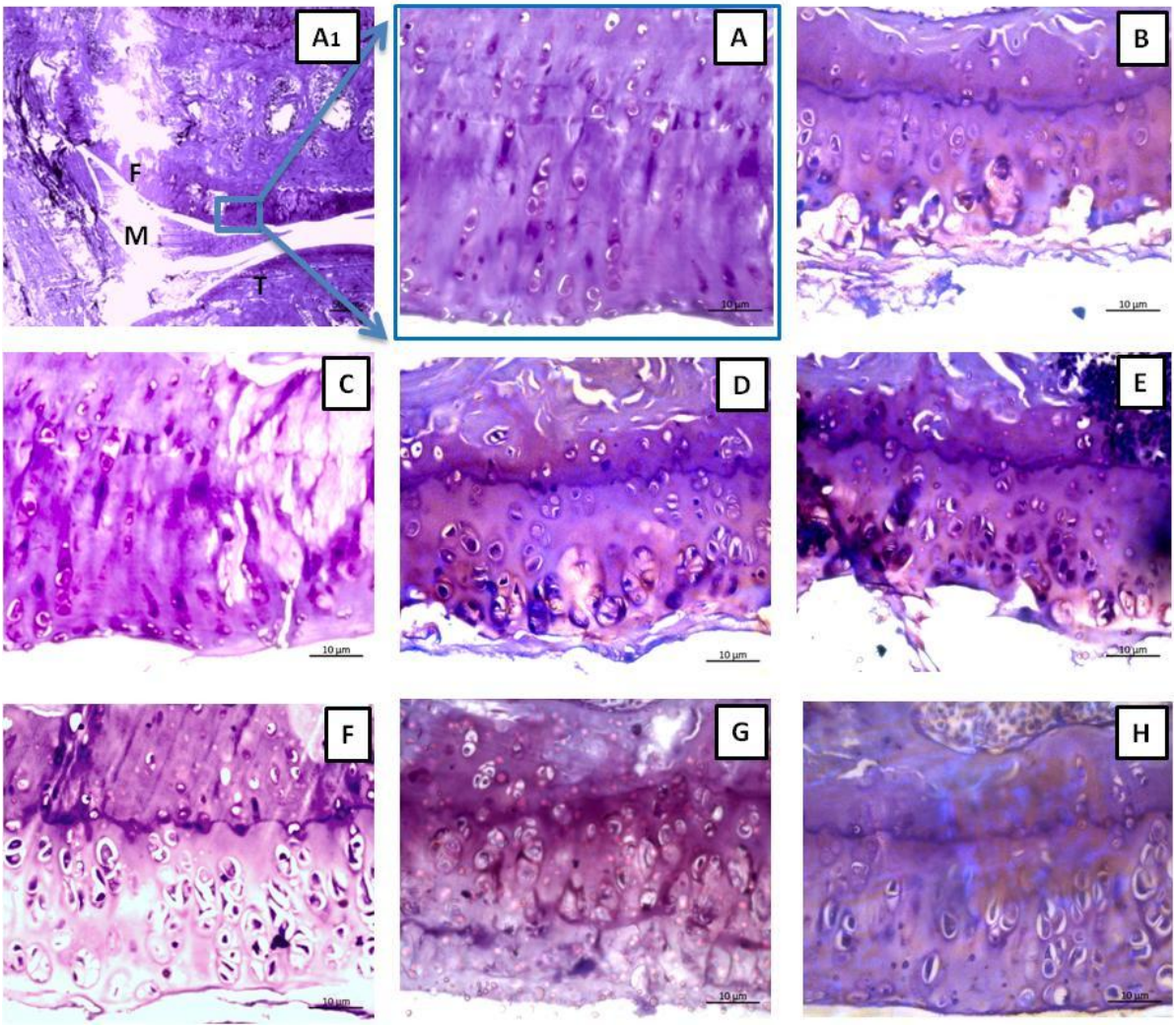


Fig. 5.15: Knee and femur sections of 6.5-month-old rats, stained with Toluidine blue.

A1: Showing the distal part of femur (F), proximal part of tibia (T) with the meniscus (M) and is a representative knee image of control group. **A**: control, **B**: OVX, **C**: sham operated, **D**: BCM7 PO, **E**: BCM7 IP, **F**: isolated protein fed, **G**: A1 natural milk fed and **H**: A2 natural milk fed rats. Toluidine blue stained microscopical sections of knee and femur were observed under X5 magnification and final images were captured with a magnification X40 using Zen 2.3 microscope (Zeiss, Germany). Scale bars: 50 µm (A1), 10 µm (A-H).

5.4.5. Biomechanical analysis:

The data extrapolated from the graphs (load-displacement and stress-strain) as obtained from UTM, showed a clear difference in strength of bones for different groups. Maximum tensile strength (**Fig.5.16**), yielding point (**Fig.5.17**), energy (**Fig.5.18**) and Young's modulus (**Fig.5.19**) were recorded for A2 natural milk fed rats, while minimum for OVX rats. Significant difference in all the mechanical parameters was observed in all treatment groups, as compared with control group rats. Significant decrease in tensile strength, yielding point, total energy absorbed and Young's modulus was observed in BCM7 IP, isolated protein and A1 natural milk fed rats, as compared with BCM7 PO treated rats. Significant increase in mechanical parameters was observed in A2 natural milk fed rats, as compared to A1 natural milk fed rats, indicating good effect of A2 natural milk on bone health. There was significant decrease in elongation capacity of bone in all OVX and treated rats (**Fig.5.20**).

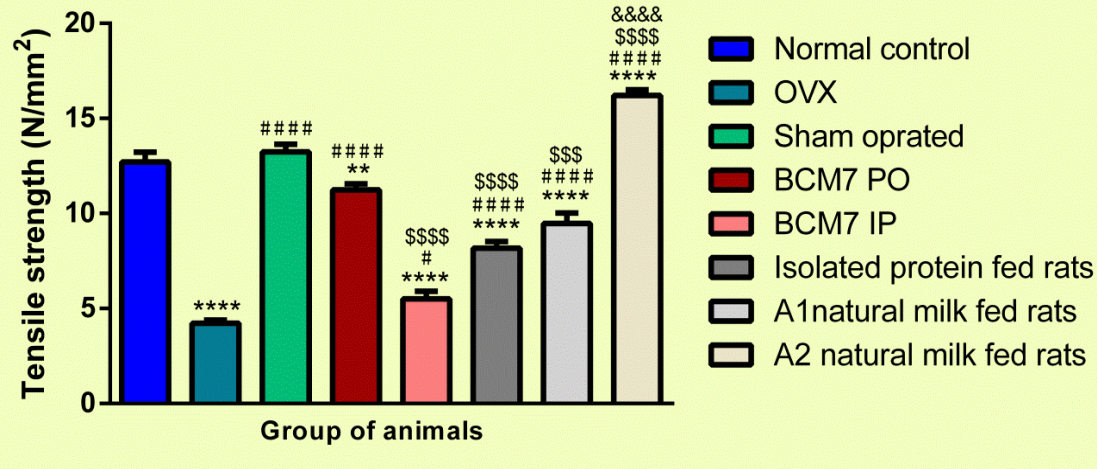


Fig. 5.16: Tensile strength (KN/mm^2) analysis of femur, from rats of different groups at the end of study.

Tensile strength was obtained by universal testing machine (UTM). Values are represented as mean ± S.D., n=6/group. *, #, $ and & indicate significant difference as compared with normal control, OVX, BCM7 PO and A1 natural milk fed rats, respectively. *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001, P values are similar for #, $ and &.

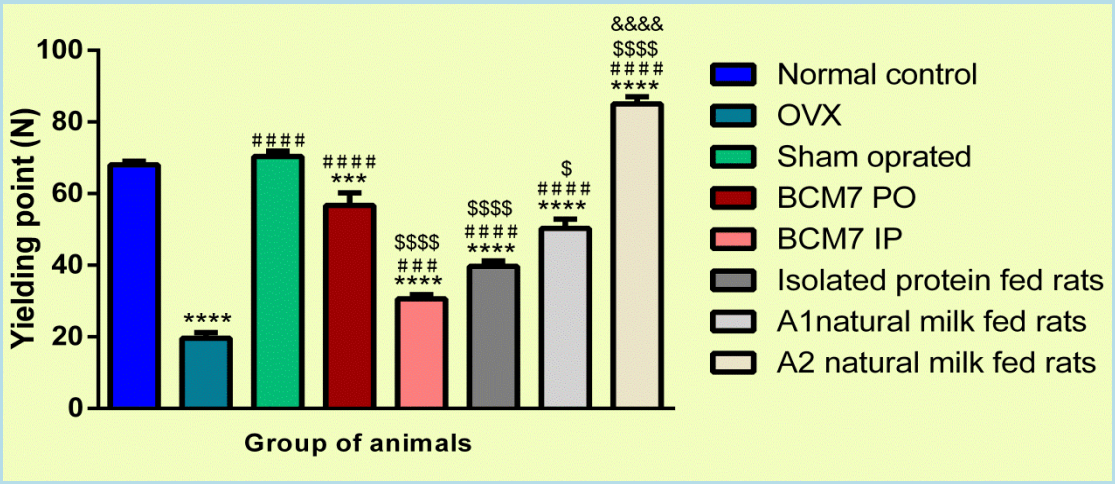


Fig. 5.17: Yielding point (N) analysis of femur, from rats of different groups at the end of study. Yielding point was obtained by universal testing machine (UTM). Values are represented as mean ± S.D., n=6/group. *,#,$ and & indicate significant difference as compared with normal control, OVX, BCM7 PO and A1 natural milk fed rats, respectively. *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001, P values are similar for #, $ and &.

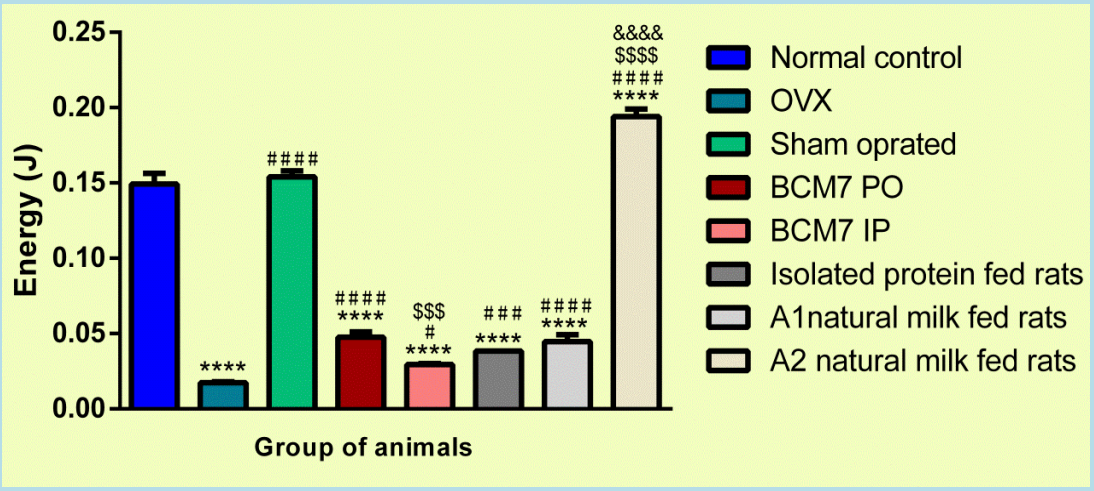


Fig. 5.18: Total energy absorbed (J) analysis of femur, from rats of different groups at the end of study. Total energy absorbed was obtained by universal testing machine (UTM). Values are represented as mean ± S.D., n=6/group. *, #, $ and & indicate significant difference as compared with normal control, OVX, BCM7 PO and A1 natural milk fed rats, respectively. *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001, P values are similar for #, $ and &.

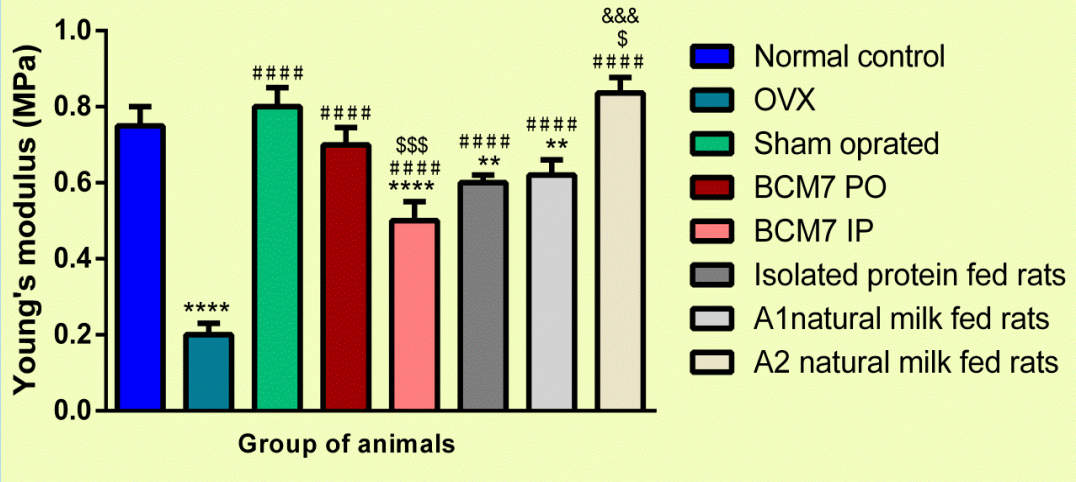


Fig. 5.19: Young's modulus (MPa) analysis of femur, from rats of different groups at the end of study.

Young's modulus (MPa) was obtained by universal testing machine (UTM). Values are represented as mean ± S.D., n=6/group. *,#,$ and & indicate significant difference as compared with normal control, OVX, BCM7 PO and A1 natural milk fed rats, respectively. *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001, P values are similar for #, $ and &.

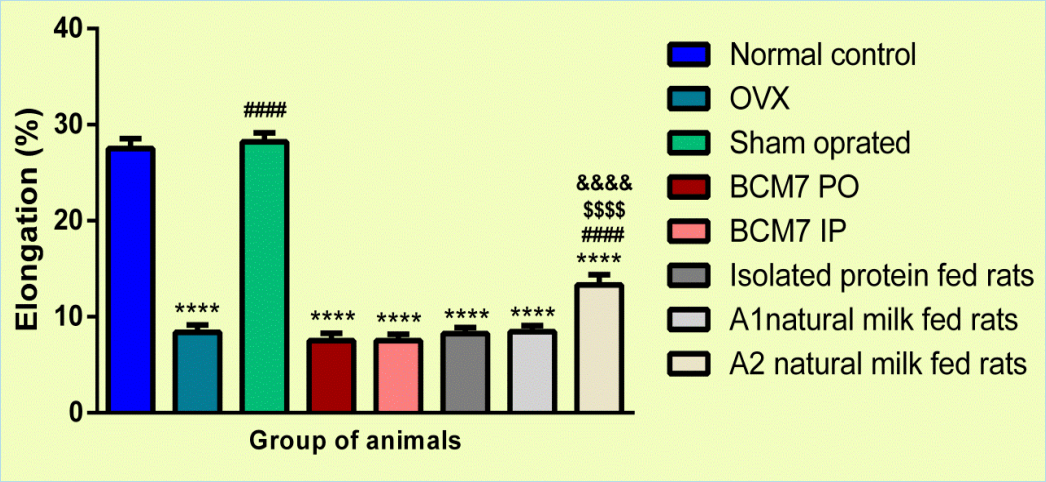


Fig. 5.20: Elongation (%) analysis of femur, from rats of different groups at the end of study.

Elongation was obtained by universal testing machine (UTM). Values are represented as mean ± S.D., n=6/group. *,#,$ and & indicate significant difference as compared with normal control, OVX, BCM7 PO and A1 natural milk fed rats, respectively. *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001, P values are similar for #, $ and &.

5.4.6. Microcomputed tomography (µCT) analysis:

µCT images (**Fig.5.21**) showed the highest bone loss in OVX rats, followed by BCM7 I/P rats. Less bone loss was observed in BCM7 oral rats, as compared to BCM7 I/P, which indicated that BCM7 is not absorbed completely, it either degraded/digested in GIT or pass through it. In BCM7 IP rats, peptide directly reaches in the circulation, bypassed the GIT, which results, potentially the lack of degradation/digestion. Isolated protein fed rats had less bone loss, as compared with BCM7 treatment rats, but higher than A1 natural milk fed rats.

Quantification of the trabecular bone BV/TV (**Fig.5.22**), Tb.N (**Fig.5.23**), Tb.Th. (**Fig.5.24**) and Tb.Sp. (**Fig.5.25**) was conducted using µCT scans. BV/TV values (%) of OVX, BCM7 PO, BCM7 IP, isolated protein and A1 natural milk fed rats are, 16,26,23,26 and 28 % respectively, indicated significant decrease, as compared with control rats (34%). Minimum Tb.N and maximum Tb.Sp. was observed in A1 natural milk fed and BCM7 IP rats, respectively, in aforementioned treatment groups. Tb.Th. (mm) for OVX (0.090), BCM7 PO (0.085), and BCM7 IP (0.096) are almost similar, but significant difference was reported, as compared with control rats (0.125). Significant increase in BV/TV, Tb.N, and decrease in Tb.Sp. was observed in A2 natural milk fed rats, as compared with A1 natural milk fed rats.

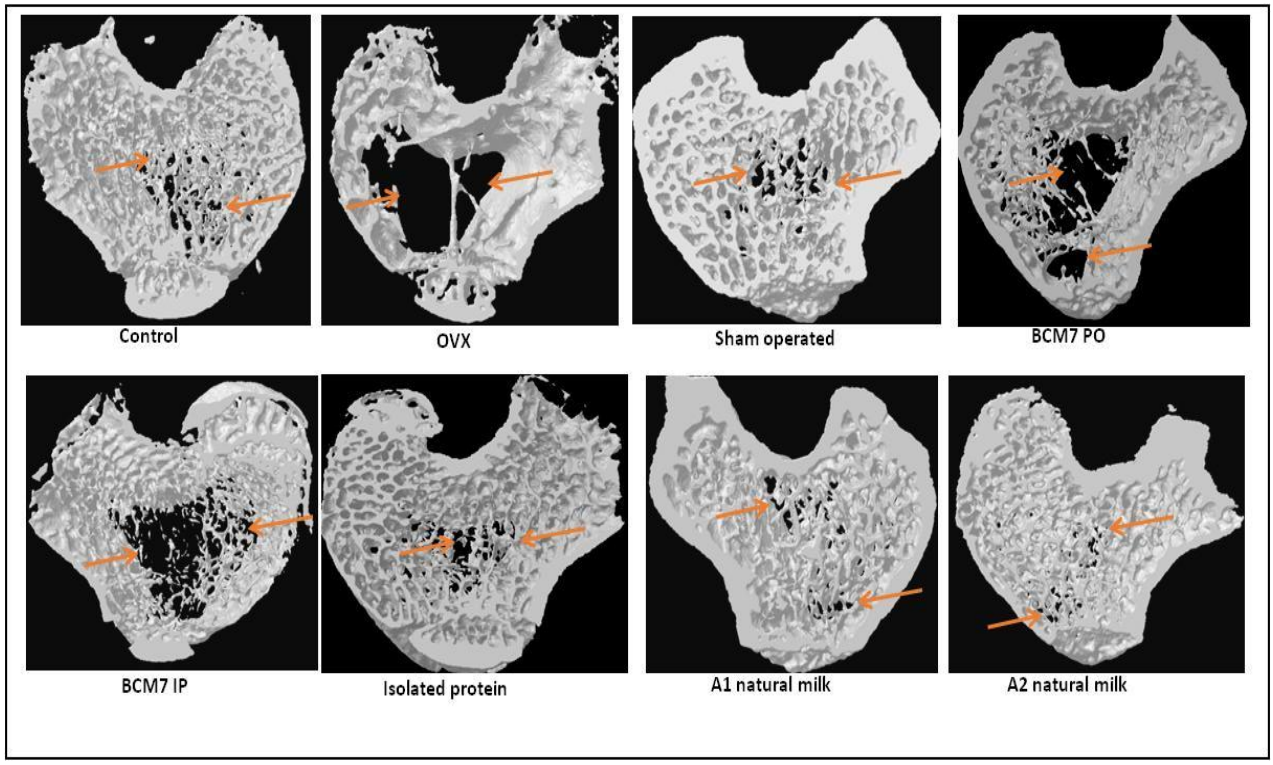


Fig. 5.21: Representative 3D images generated by µCT in different groups of rats at age of 6.5 months. ROI was drawn bellow the growth plate of tibia. Images acquired at 70 kV, 142mA using 1 mm filter at a resolution of 18 micro meter/ pixel. Groups of rats are: I-control, II-OVX, III-sham operated, IV-BCM7 oral, V-BCM7 I/P, VI-isolated beta-casein, VII- A1 milk, and VIII-A2 milk.

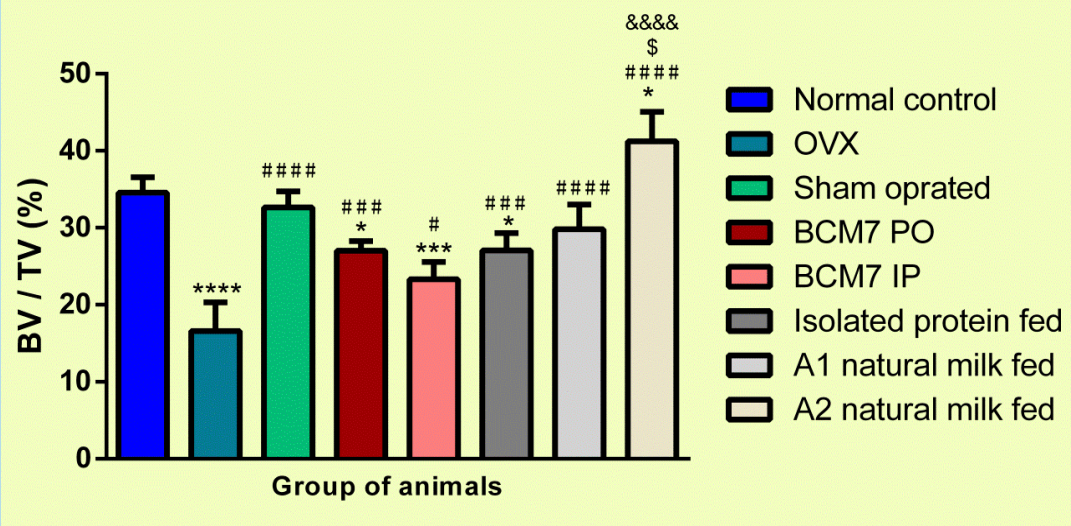


Fig. 5.22: Bone volume/tissue volume ratio (BV/TV), describing trebecular bone morphology in different group of rats. Values are represented as mean ± S.D., n=6/group. *,#,$ and & indicate significant difference as compared with normal control, OVX, BCM7 PO and A1 natural milk fed rats, respectively. *P≤0.05, ** P≤0.01, ***P≤0.001, ****P≤0.0001, P values are similar for #, $ and &.

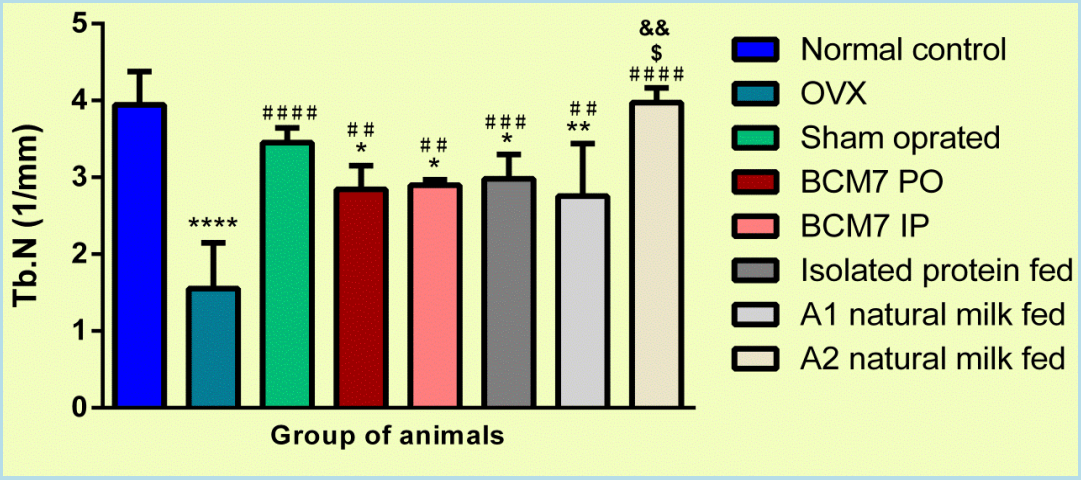


Fig. 5.23: Trebecular number (Tb.N.), describing trebecular bone morphology in different group of rats. Values are represented as mean ± S.D., n=6/group. *,#,$ and & indicate significant difference as compared with normal control, OVX, BCM7 PO and A1 natural milk fed rats, respectively. *P≤0.05, ** P≤0.01, ***P≤0.001, ****P≤0.0001, P values are similar for #, $ and &.

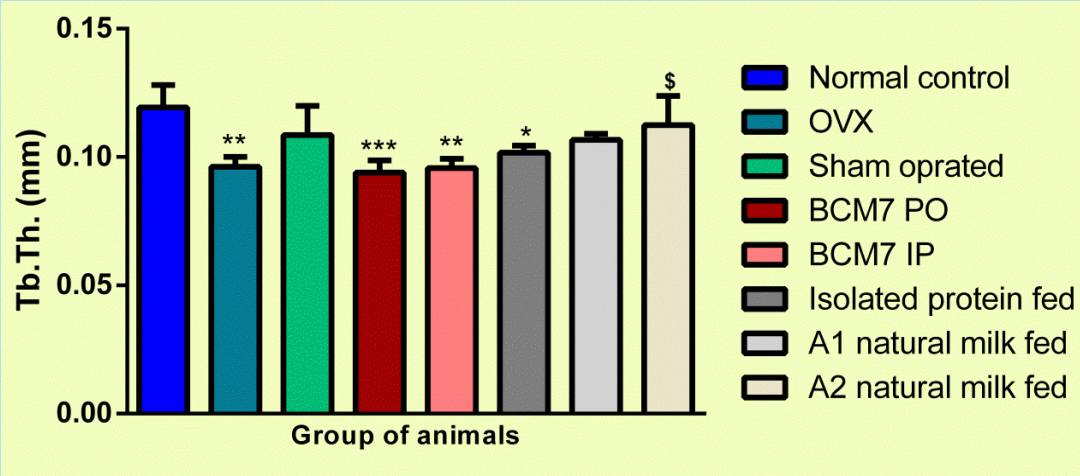


Fig. 5.24: Trebecular thickness (Tb.Th.), describing trebecular bone morphology in different group of rats. Values are represented as mean ± S.D., n=6/group. *,#,$ and & indicate significant difference as compared with normal control, OVX, BCM7 PO and A1 natural milk fed rats, respectively. *P≤0.05, ** P≤0.01, ***P≤0.001, ****P≤0.0001, P values are similar for #, $ and &.

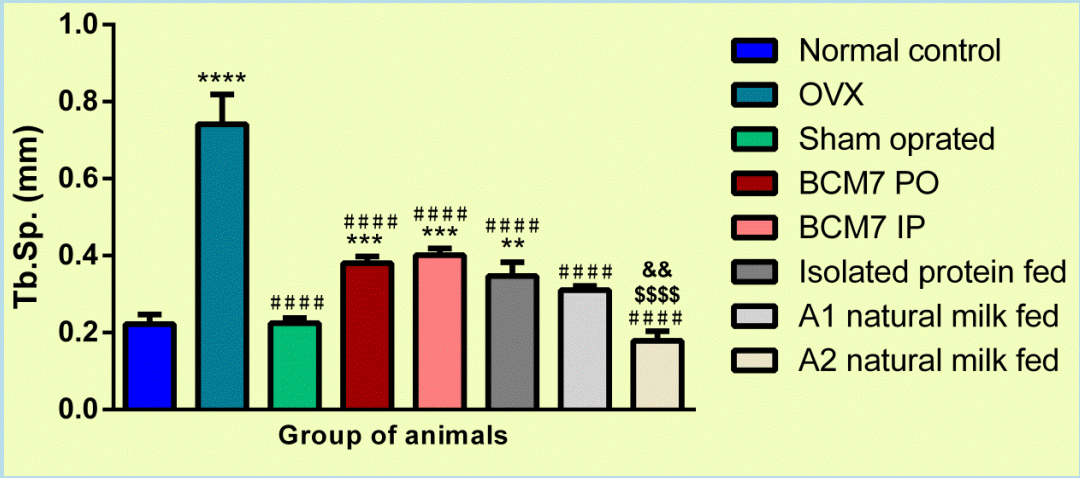


Fig. 5.25: Trebecular space (Tb.Sp.), describing trebecular bone morphology in different group of rats. Values are represented as mean ± S.D., n=6/group. *,#,$ and & indicate significant difference as compared with normal control, OVX, BCM7 PO and A1 natural milk fed rats, respectively. *P≤0.05, ** P≤0.01, ***P≤0.001, ****P≤0.0001, P values are similar for #, $ and &.

6. Discussion

6.1. Genotypic global status of cattle:

Last few decades, India has become the highest milk producer in the world and exotic cattle played a major role in this achievement. The exotic/crossbred milch cattle increased up to 34.78%, from 14.4 (in 2007) million to 19.42 (in 2012) million, whereas the indigenous milch cattle increased marginally by 0.17%, which was from 48.04 million (in 2007) to 48.12 million (in 2012), (DADF, 2012). This increase may be attenuated to replacement or mixing of indigenous zebu cattle germplasm with the exotic cattle due to unorganized breeding practices and inclination of farmer's for high milk production. Most of the cattle farms in developing countries like India are unorganized, involved in unplanned breeding, due to lack of resources, awareness, genetic and paternal breed data of cattle. These circumstances are acting as a major problem in the implementation of proper breeding policies.

India has only one marketing channel for both A1&A2 milk, whereby milk from all cattle, is marketed together, in common packages. While some countries like New Zealand have separate marketing channels for A1 and A2 milk and milk products. In 2000, a New Zealand company, A2 Corporation Limited, was founded to identify A2 type of cows based on genetic testing and market A2 milk. In 2003, it even petitioned Food Standards Australia-New Zealand, a bi-national government agency, to print health warnings on the packages of A1 milk. In 2006, Keith Woodford, author of the book 'Devil in the Milk', linked A1 beta-casein intake to T1DM, gave a boost to A2 milk sales in New Zealand and Australia.

6.2 Genotypic global status of cattle as identified in Indian breeds:

During the current study, a large proportion (69.7%) of HF cows having heterogeneous (A1A2) genotype was identified, which indicated to an abrupt increase of A1 allele as compared with desi cows (90%) homologous (A2A2) genotype. It is observed that continuous breeding of such HF cattle (A1A2 or A1A1), may replace the A2 allele from the population. Under Indian circumstances, genotypic estimation may play a major role in promoting breeding for A2 allele. New breeding policies may be required to increase insemination of A2A2 semen only. Awareness among farmers about these alleles can play an important role to achieve a higher proportion of A2A2 cows.

We observed that A1A1 genotype cattle are higher milk producers in Pilani region and a similar observation was also reported by Muhammed and Stephan (2012) from Kerala, India (Muhammed et al., 2012). Natural selection analysis also supports that A1 variant appeared to have evolved through positive selection, indicating that the mutation is favored and evolutionarily advantageous. The wild type variant was evident to have evolved through purifying selection. The other variants included in this study, seemed to have evolved through positive selection but currently, their frequency was not identified in cattle population studied. This may support to the fact stated in previous reports that the A3, B and I variants are rare.

6.2. Nonenzymatic method for isolation of beta-casein from natural milk:

The methods followed till date for isolation of beta-casein, were observed to have major bottlenecks at small and large scale. Keeping in mind these gaps, it is proposed in our work, a nonenzymatic method for isolation of beta-casein from natural milk. The developed method suits both small and large scale isolation of beta-casein having substantially fewer fractions of impurities with a substantially good yield. Selective

solubilization of beta-casein at low temperature is the key principle for isolation of pure beta-casein. There is a large increase in the level of non-sedimentable beta-casein, when milk coagulum is incubated at low temperature, whereas non-sedimentable levels of other caseins, increase only slightly. The weakening of hydrophobic bonds at low temperature is the most probable reason for high solubilization of beta-casein. Plasmin produces γ-caseins by hydrolysis of beta-casein, which is optimally active at 37ºC. At low temperature, there are negligible chances of plasmin-induced hydrolysis of beta-casein. Therefore, the present method provides a method for isolation of beta-casein from milk, at a temperature, lower than optimal activity of the plasmin. It was observed that there was no significant effect of initial incubation temperature on total yield of beta-casein up to 80ºc, However initial room temperature (25 ºc) is required for proper coagulation of milk, in desired incubation time. These factors favoured with low plasmin activity.

Glacial acetic acid was used in an optimum range, in the range of 1µl-1.25µl/ml of milk. At high concentration of glacial acetic acid, contamination of α_{s1}-casein increased, due to increased dissociation of micellar α_{s1}-casein at lower pH (Dalgleish et al., 1988), and at lower concentration of glacial acetic acid, milk does not coagulate. However, excess glacial acetic acid in coagulum, was washed away during curd washing, with AMQ.

This method was faster, efficient and economical, as compared to the use of enzymatic method for isolation of beta-casein from milk. The cost for coagulation of milk using glacial acetic acid is approximately 500 times lower than use of enzymes such as rennin. Also, the overall cost of the present method was approximately 5-7 times lower than the enzymatic method. Further, high temperature was required to inactivate rennin. In comparison, present method was carried out at room temperature, thus scaling up of the

method; will not add extra cost of high temperature. Thus, the method comprises use of inexpensive extraction agent and processing at room temperature, which reduces time as well as the use of additional heating aids.

The total casein comprises of 25 to 35 % of beta-casein, 5-8% of α_S2 casein, 2-3 % α_S1 casein and 1-2 % kappa casein. The yield of total casein normally ranges between 14 to 16% of total casein. The yield of beta-casein would be about 85 % of total beta-casein or 25-30% of total casein. The yield of beta-casein from present method was approximately two times higher than the enzymatic method. Beta-casein obtained by the present method had a purity of approximately 65% to 75%, as determined by MALDI-MS/MS and by Bradford method, before the purification step. Thus, the present method with with much lesser cost yielded, more pure and double quantity of beta-casein, as compared with rennin method. Hence it is suggested that the method can be scaled up for higher quantities of milk, with minor setup changes.

Using the proposed method, pure beta-casein can be obtained from milk of all dairy animals such as cow, buffalo or goat. Further, the method provides other milk fractions such as cream, whey protein or curd. Said milk fractions are normally useful in the manufacture of other dairy products. An example, the cream removed, can used for the manufacture of ghee and whey for the manufacture of whey protein concentrate. Curd obtained with partial concentration, contains beta-casein content, which may be used in the manufacture of processed cheese or similar products.

6.3. OVX rats as a model of osteoporosis:

Ovariectomy (female) and immobilization (both male & female), induced osteoporosis in

rat, bears a strong resemblance to human osteopenia, both in its anatomical features, as well as, in bone dynamics, and hence considered significant. The pathological changes observed in the OVX rats, are of a similar nature to the very early changes observed in human osteoarthritis (Walter et al., 1998). OVX rats provide a useful experimental model for the clinical assessment of chondroprotective effects of novel therapeutic compounds and the model may be an in vivo representation of osteoarthritis in postmenopausal women. In OVX rats, hypogonadism is induced, by removal of primary source of estrogen production (ovaries). Subsequent to ovariectomy, low levels of estrogen, affect the normal physiological functions of estrogen responsive tissues, such as endometrium, bone, and breasts. As a result, the amount of bone resorption becomes greater than the amount of bone formation, leading to a net bone loss (Cavailles, 2002).

6.4. Osteoporosis:

A large population of different age group of individuals, consume milk for different nutritional requirements. The main issue related to consumption of A1 milk is the generation of opioid peptide BCM7, that has been linked to various diseases like T1DM, schizophrenia etc. In the past, many researchers have established direct correlation between opioids and osteoporosis by developing hypogonadism (McLachlan, 2001; Keiler et al., 2012; McLachlan, 2001; Shapses et al., 2017; Tegeder et al., 2004). Low levels of osteocalcin in pregnant women addicted to heroin and cocaine was reported by Rico et al., (1990) and Pérez-Castrillón et al., (2000) also observed low levels of osteocalcin in osteoblast like cells (MG-63) and suggested, a direct toxic effect of these drugs on osteoblasts.

The probable mechanism involved in indirect effect of BCM7, that suggests precipitation of osteoporosis is the suppression of HPG axis, by action of BCM7 on μ-opioid receptors

(**Fig.6.1**). Normally, GnRH, released by hypothalamus, activates the anterior pituitary gland to release LH and FSH. These hormones, via systemic circulation, exhibit their effect on testes and ovaries, to produce testosterone or estrogen, respectively. The structural similarity of BCM7 with opioids, indicate their possibility to either act on μ-opioid receptors, suppressing the HPG axis (**Fig. 6.1**) or on the ovaries and testes, leading to the reduction of estrogen and testicular testosterone production respectively, resulting in the precipitation of osteoporosis, as similar to that of opioids (Brennan, 2013; Colameco et al., 2009; Delitala et al., 1983; Petraglia et al., 1986) (**Fig. 1.6 &1.7**). The HPA axis is another endocrine system that is affected by the peptide (Allolio et al., 1987; Palm et al., 1997).

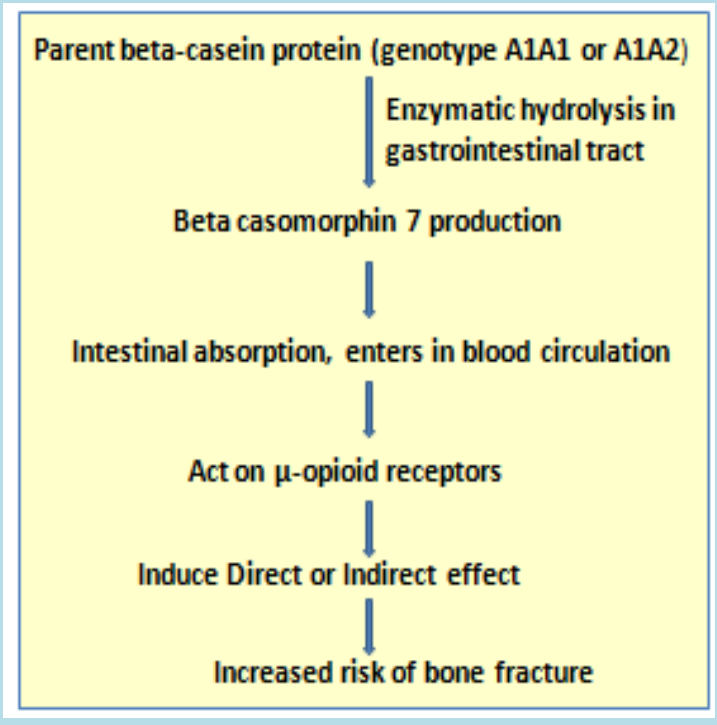


Fig. 6.1: Flow chart depicting suggestive pathway of BCM7, in increasing risk of bone fracture.

Serum osteocalcin measurements have been well established as a non-invasive specific marker for bone synthesis, and is exclusively synthesized by osteoblast and odontoblasts. Thus, a decrease in serum osteocalcin level indicates, low osteoblast and high osteoclast activity or increased bone resorption. Low levels of osteocalcin in OVX rats, BCM7 treated rats, beta-casein protein and A1 natural milk fed, rats support the same. There was comparatively less decrease of osteocalcin in BCM7 (PO) treated rats, possibly due to the degradation of the peptide in the stomach or other parts of the gastro-intestinal tract (GIT).

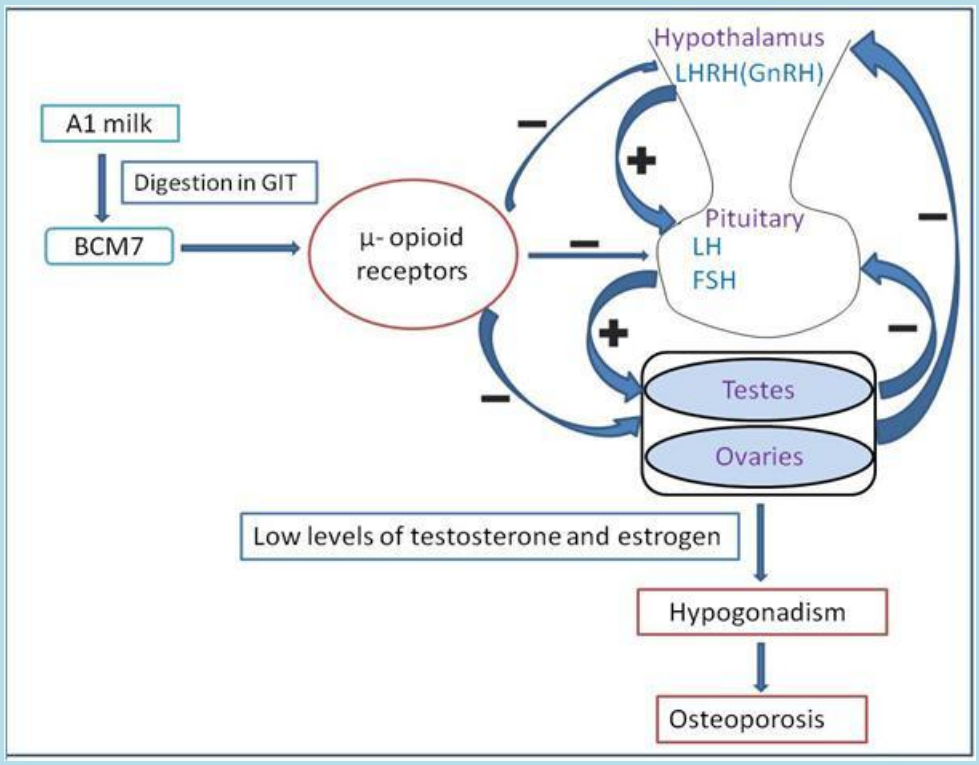


Fig .6.2: Flow chart suggesting interactions between BCM7 and the endocrine system, induction of osteoporosis. LHRH-luteinizing hormone releasing hormone, GnRH- gonadotropin releasing hormone, LH-luteinizing hormone, FSH- follicle stimulating hormone, + = positive effect, - = negative effect (Katz et al., 2009).

As calcium and phosphorus in milk, play a pivotal role in maintaining healthy bone, there was a significant decrease of both these minerals in BCM7 treated, beta-casein protein and A1 natural milk fed rats. Low calcium levels stimulates the secretion of PTH, which in turn increases the precipitation of osteoporosis (Ostergaard et al., 1997) by triggering bone metabolism, causing bone resorption and bone loss, besides releasing calcium ions in the blood (Boonen, Aerssens, et al., 1996; Boonen, Broos, et al., 1997; Boonen, Lesaffre, et al., 1996; Boonen, Vanderschueren, et al., 1997; Lips et al., 2001; McKenna et al., 1998; Reginster et al., 1999). PTH also increases renal clearance of phosphorus, resulting in hypophosphatemia. Low levels of both these minerals and osteocalcin, in BCM7 treated, beta-casein protein, and A1 natural milk fed rats, indicated unhealthy bone. However, better levels of these minerals in A2 milk treated rats, supported the fact that natural milk is a good source of calcium and phosphorus and its pivotal role in maintaining healthy bones.

Decrease in uterine mass was also observed, indicating a low uterine activity or lower hormonal activity, in uterus. Increase in body mass and regression of uterus, are not healthy indicators for bone health and are an alarm for development of osteoporosis. High proportion of osteoclast cells without corresponding increase in osteoblast cells along with articular surface erosion in the treatment (BCM7/protein/A1 milk) animals, strongly indicate the early development of osteoporosis (Ostergaard et al., 1997).

In addition to biochemical analysis, biomechanical and Micro-CT scanning could be most accurate methods for measuring bone properties and plays an important role in preclinical experimental models concerning osteoporosis. Micro-CT data revealed that there was high loss of bone in BCM7, A1 milk protein and A1 natural milk fed rats and decrease in the biomechanical parameters like tensile strength, Young's modulus,

energy, etc. in these animals also indicated the weakening of rat bones and supported the hypothesis. Data also indicated that OVX rats bone are weakest and A2 natural milk fed rats strongest bone , maximum impact was observed on BCM7 IP, followed as: BCM7 PO> isolated protein> A1 natural milk fed rats.

Most of the dairy farms in developing countries like India are involved in unorganized breeding due to lack of genetic and paternal breed data of cattle, acting as a major problem in implementation of policies. Under such circumstances, genotypic estimation may play major role in promoting breeding for A2 allele. Further specific studies, in a larger population and human trials, may be of importance to strengthen the findings and the hypothesis. However in-depth systemic in vivo study is required to understand the actual mechanism involved in these diseases.

7.1. Summary & Conclusions

Osteoporosis is the disease of masses and there is a growing prevalence rate of osteoporosis in India. Along with usual reasons like deficiency of calcium and vitamin D, opioids are the new risk factors for osteoporosis. Opioid like peptides derived from milk proteins, act on μ opioid receptors and induce endocrine and metabolic changes. BCM7, one such peptide, directly impairs osteoblast activity and reduces BMD and synthesis of osteocalcin. Epidemiological evidences suggest that BCM7 is the emerging risk factor for the development of osteoporosis.

A1 and A2 are two important variants of cattle beta-casein expressing gene, based on a point mutation at position 67 of exon VII, on 6th chromosome. As a result of this mutation, a conversion from cytosine to adenine base, leads to the replacement of proline (A2 allele, codon; CCT) by histidine (A1 allele, codon; CAT) amino acid, at position 67. These variants are reported, as the presence of both homogenous (A1A1 or A2A2) alleles and heterozygous (A1A2) allele of CSN2 in a given population and are also associated with co-dominance of allele. Presence of other alleles in population i.e. A3 E, D, H and I, are also obtained, but their presence is rare, compared to A1 & A2. The A1 beta-casein protein, upon protease degradation, generates BCM7. Peptides other than BCM7, are also produced in very less amount, which are less stable and further degraded in the intestine, as well as in the blood.

The A2 allele is recognized as the progenitor of CSN2, in the genus Bos. A1 is most frequent in Holstein-Friesian, Ayrshire and Red cattle. In contrast, a high concentration of A2 is observed in Guernsey, Jersey, Indian Zebu cattle and Indian buffalo. The overall genotype of HF cattle identified had 9% A1A1, 69.7% A1A2 and 21.3% A2A2 alleles. Most of the desi cows were observed to possess A2A2 genotype, A1A2 was observed only in mixed zebu breeds and A1A1 was not observed in any of the desi

cows. The A2A2 genotype, prevailed over the A1A2 genotype in mixed breeds. The allele frequency analysis of HF cows was 0.44 for A1 allele, 0.56 for A2 allele and 0.09, 0.70, 0.21 for genotypes A1A1, A1A2, and A2A2, respectively. The allele frequency analysis for Zebu cows was 0.1 for A1 allele, 0.9 for A2 allele and 0.00, 0.10, 0.9 for genotypes A1A1, A1A2, and A2A2, respectively. The distribution of the genotypes was within the Hardy–Weinberg equilibrium, in the tested population (P>0.05). The value of gene homozygosity (Ho), gene heterozygosity (He) and fixation index (FIS) were found to be 0.40, 0.60, and - 0.20, respectively. Average milk yield in HF cattle was A1A1-16.5 L/day, A1A2-15.5L/day, and A2A2-15.25 L/day. It is high time to generate awareness amongst the farmers, breeding policy planners, field functionaries and all stake holders to avoid mixing Indian zebu cattle with exotic cattle.

Various methods have been developed at laboratory level as well as industry level for economic yield of pure beta-casein. The method developed in present research work is faster, efficient and economical as compared to use of the enzymatic method for isolation of beta-casein from milk. The cost for coagulation of milk using glacial acetic acid is approximately 500 times lower than use of enzymes such as rennin. Besides, the overall cost of the developed method is approximately 5-7 times lower than the enzymatic method. The yield of total casein can be from 14 to 16% of total casein. The yield of β-casein can be 85% of total beta-casein or 25-30% of total casein. The yield of beta-casein from present method is approximately two times higher than the enzymatic method. Beta-casein obtained by the present method has a purity of approximately 65% to 75% before the purification step, and 99.9% pure beta-casein can be obtained after the purification step. Thus, by this method, with almost 50 times less cost, more pure and double quantity of beta-casein can be obtained in comparison to rennin method. The

method can be adopted for large-scale isolation/purification method with minor setup changes.

The main issue related to consumption of A1 milk is the generation of opioid peptide BCM7, that has been linked to various diseases like T1DM, schizophrenia etc. In the past, many researchers have established direct correlation between opioids and osteoporosis by developing hypogonadism. Reports have also suggested reduction in synthesis of osteocalcin, in cells incubated with morphine, where the effect was completely reversed by Naloxone (μ-opioid receptor competitive antagonist), suggesting a direct toxic effect of these drugs, on osteoblasts. The structural similarity of BCM7 with opioids, indicate their possibility to either act on μ-opioid receptors, suppressing the HPG axis (**Fig. 6**) or on the ovaries and testes, leading to the reduction of estrogen and testicular testosterone production respectively, resulting in the precipitation of osteoporosis.

In the present study, following biochemical/mechanical/histomorphological parameters were observed in OVX, BCM7 treated, A1 beta-casein protein and A1 natural milk fed rats as compared to normal control, sham operated and A2 natural milk fed rats.

- Low level of serum osteocalcin
- Low level of serum calcium
- Low level of serum phosphorus
- Decrease in bone tensile strength, yield point, energy and Young's modulus.
- Decrease in uterus mass
- Increase in body mass
- Decreased trabecular bone volume **(**BV/TV), trabecular number (Tb.N), and trabecular thickness (Tb.Th)
- Increased trabecular space (Tb.S)

- Increase in number of osteoclast cells
- Articular surface erosion

Aforementioned parameters indicated low osteoblast and high osteoclast activity or increased bone resorption supporting the hypothesis of the study. Decrease in uterus weight, indicating a low uterine activity or lower hormonal activity, in uterus. Increase in body weight and regression of uterus, are not healthy indicators for bone health and are an alarm for development of osteoporosis. Micro-CT measurements and 3D images showed decreased BV/TV, Tb.Th, Tb.N and increased Tb.S, becides articular surface erosion in treated rats, strongly indicating weakening of rat bones, further strengthening the hypothesis of the study.

Based on these aforementioned animal studies, it may be inferred that people consuming milk of A1 variant cattle on a regular basis, may be at high risk of osteoporosis, or its early precipitation. Further specific studies, in a human population, may be of importance to strengthen the findings and the hypothesis. In developing countries like India, there is a requirement of a structured programme to enhance the awareness and set standards of care for health professionals, which have had a significant impact on osteoporosis care in India. Coding (milk packet) the milk obtained from various allelic polymorphs as in developed countries such as US, New Zealand, can be practiced, for information as well as for awareness of consumers.

7.2. CONCLUSIONS:

Observations and conclusions derived from the present study are as follows:

- There is an abrupt increase in the A1 allele (0.44) frequency, large no. of A1A2 cattle, indicating that our original germplasm is being mixed with A1 (exotic) cattle, which needs to be conserved.
- Natural selection analysis also supports that A1 variant appeared to have evolved through positive selection indicating that the mutation is favored and evolutionarily advantageous.
- Nonenzymatic method developed for isolation of beta-casein from natural milk is faster, efficient and economical as compared to use of enzymatic method for isolation of beta-casein from milk.
- BCM7 that acts on μ-opioid receptor of osteoblast cells, induced hypogonadism, by suppressing HPG and HPA axes, resulting in bone resorption, and decreased bone formation.
- Low levels of serum osteocalcin, calcium and phosphorus, decreased bone tensile strength, high count of osteoclast cells, increase in body weight and decreased uterine weight in BCM7/A1 protein/A1 natural milk treated rats, clearly indicated the adverse effect of BCM7, on bone formation and strength.
- μCT measurements and 3D images analysis showed a significant decrease of trabecular bone volume, trabecular number, trabecular thickness and increased trabecular space, which strongly indicated degradation of bone, in treated rats.
- The studies conducted leads to an inference that people consuming milk of A1 variant cattle on a regular basis, may be at high risk of osteoporosis, or its early precipitation.

8.1. Limitations of the current study

Following are some of the important limitations of the present research work

- Samples were collected from small area with similar husbandry and climatic conditions.
- Method for genotype identification is a long process.
- In beta-casein isolation method, yield of protein was 85% and purity approximately 65% to 75%, before the purification step. For purification, separate setup is required, to enhance percentage of purity.
- Animal model (Ovareictomy) is only for mature female rats, which mimics the postmenopause condition of a human female. Another model that emulates early/general osteoporosis is required.
- Sex hormones were not estimated.

8.3. Plan for future research

The present research work comprehensively investigated the genotype of cattle (exotic and desi) in the local area of Pilani and adjacent area (100Km radius), isolation of beta-casein from natural milk of cows and the effect of BCM7, on precipitation of osteoporosis, in Wistar rats. Based on current research work, further research as mentioned below can be conducted to add more value to the work;

- Development of a kit for identification of A1 or A2 cattle at the spot.
- Assessment of Genotypes, A1 and A2 milk producing cattle will help play a major role in promoting breeding of A2 allele type and lower exposure to A1 allele type, with regulated/proper breeding plan. Awareness among farmers about these alleles would help achieve higher proportion of A2A2 cows.

- Reconfirming the findings for precipitation of osteoporosis in rats, through a clinical study on the human population.

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LIST OF PUBLICATIONS

Research publications from thesis work

Patent:

1. **Sushil Kumar Yadav**, Mahesh Radhakrishnan, Vishal Saxena, Non-enzymatic method for large scale isolation of ß-casein from milk. Patent application no. 201811018301, dated: 16/05/ 2018
2. **Sushil Kumar Yadav**, Mahesh Radhakrishnan, Vishal Saxena A method for determining A1/A2 genotype of milch animals. Patent application no. 201911018953, dated: 13/05/ 2019

Paper:

1. Sushil Kumar Yadav, Radhakrishnan Mahesh, Vishal Saxena, Impact of beta-casomorphin 7 (BCM7) on precipitation of osteoporosis, Journal of Animal Physiology and Animal Nutrition. (under review)
2. Gagandeep S. Saggu, Shilpi Garg, Zarna R. Pala, **Sushil Kumar Yadav**, Sanjay K. Kochar, Dhanpat K. Kochar, Vishal Saxena Characterization of 4-hydroxy-3-methylbut-2-en-1-yl diphosphatesynthase (IspG) from *Plasmodium vivax* and it's potential as an antimalarial drug target. International Journal of Biological Macromolecules, 2017; 96, 466–473.
3. Zarna R Pala, Vishal Saxena, Gagandeep S Saggu, **Sushil Kumar Yadav**, Rajendra P Pareek, Sanjay K Kochar, Dhanpat K Kochar, Shilpi Garg. Structural and Functional Characterization of an Iron-Sulfur Cluster Assembly Scaffold ProteinSufA from Plasmodium vivax. Gene, 2016; 585, 159-165.
4. Ashwin Kumar R., **Sushil Kumar Yadav**, Sanjay Kumar Verma. Antidepressive-like effect of microcystin-fr in swiss albino mice tested by a battery of behavioural depression models. International Journal of Pharmacy and Pharmaceutical Sciences, 2012; 4, 293-299.
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6. Dilip Kumar Pandey, **Sushil Kumar Yadav**, Radhakrishnan Mahesh. Depression-like and anxiety-like behavioural aftermaths of impact accelerated traumatic brain injury in rats: A model of comorbid depression and anxiety. Behavioural Brain Research, 2009; 205, 436-442.
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9. Radhakrishnan Mahesh, Thangaraj Devadoss, Dilip Kumar Pandey, **Sushil Kumar Yadav**. Quinoxalin-2-carboxamides: synthesis and pharmacological evaluation as serotonin type-3 (5-HT3) receptor antagonists. Journal of Enzyme Inhibition and Medicinal Chemistry, 2010, 1–6

Research Posters Presented in Conference

1. **Sushil Kumar Yadav**, R Mahesh, Vishal Saxena, Gautam Singhvi “Separation and Analysis of Beta Casein Variants from Cow Milk and their Application in Solubility Enhancement of Poorly Water Soluble Drug”. 8th Annual International Conference on Dissolution Science and Applications - Disso India 2019, organized at NIPER-SAS Nagar, Mohali, India, from September 11 - 13, 2019.
2. **Sushil Kumar Yadav**, R Mahesh, Vishal Saxena Milk derived peptide induced histopathological changes in rat bone. International Conference on Life Science Research & its Interface with Engineering and Allied Sciences (LSRIEAS 2018) organized at BITS, Pilani, Pilani Campus, Nov 1-3, 2018. (**Best Poster Award**)
3. **Sushil Kumar Yadav**, R Mahesh, Vishal Saxena Epidemiological study of beta-casein alleles. Golden Jubilee Concluding Celebrations & Annual Confrence of Indian Pharmacological Society (IPSCON-2017), organized by Shobhaben Pratapbhai Patel School of Pharmacy & Technology Management, SVKM’S NMIMS, Mumbai, India, Feb 15-17, 2018.
4. **Sushil Kumar Yadav**, R Mahesh, Vishal Saxena Development of new analytical method for the analysis of β- casein variants in cattle from Pilani region. **International**

Conference on Challenges in Drug Discovery and Delivery (ICCD3-2017), organized at BITS, Pilani, Pilani Campus, March2-4, 2017

5. Zarna R. Pala, Vishal Saxena, Gagandeep S. Saggu, **Sushil K. Yadav**, R. P. Pareek, Sanjay K. Kochar, Dhanpat K. Kochar, Shilpi Garg. "Structural and Functional Characterization of an Iron-Sulfur Cluster Assembly Scaffold Protein-SufA from Plasmodium vivax." BITS Conference on Gene and Genome Regulation, at BITS Pilani, India, Feb 18–20, **2016** (**Best Poster Award**).
6. Gagandeep S. Saggu, Shilpi Garg, Zarna Pala, **Sushil K. Yadav**, Sanjay K. Kochar, Dhanpat K. Kochar, Vishal Saxena. "Characterization of 4-Hydroxy-3-Methylbut-2- en-1-yl diphosphate Synthase (GcpE) enzyme from Plasmodium vivax" in BITS conference on Gene and Genome Regulation (BCGGR 2016) at Department of Biological Sciences, Birla Institute of Technology and Sciences, Pilani, India from Feb 18-20, 2016.

Workshop

1. National workshop on Improving Synergy Between Teaching and Research in Indian Academia. Organized by TLC, BITS, Pilani, Pilani campus**,** March, 26-27, 2018.
2. One week Training programme of CPCSEA Nominees, organized by Ministry of Environment, Forests and Climate Change, Government of India, at National Institute of Animal Welfare (NIAW), Ballabgarh, Faridabad, Sep 7-11, 2015.
3. National workshop on Latex and Matlab for beginners (NWLMB-2014). Organized by Department of Mathematics, BITS, Pilani, Pilani campus**, Dec**, 24-28, 2014

Biography of Prof. R. Mahesh

Prof. Mahesh is currently working as Professor, Department of Pharmacy BITS, Pilani, Pilani campus. He was awarded PhD (Medicinal Chemistry) in 1997 from BITS, Pilani. He has been involved in teaching and research for past more than two and a half decades. He has vast experience in the field of Molecular Modeling and Drug Design, Medicinal Chemistry, Neuropharmacology and Clinical Pharmacy and Therapeutics. He has successfully completed several funded projects and some ongoing projects as Principal Investigator by UGC, DBT, DST and ICMR. He has guided eleven PhD students. He has guided several postgraduate and undergraduate students on various projects. Several of his projects have won several awards in Academic Exhibitions. He has published more than 100 papers in peer reviewed international/national journals and in conferences of international/national repute. He is life time member of Association of Pharmaceutical Teachers of India (APTI), Indian Pharmacological Society (IPS) and Society of Neurochemistry, India.

Biography of Prof. Vishal Saxena, Ph. D.

Prof. Vishal Saxena is working as an Associate Professor in Molecular Parasitology & Systems Biology Lab, Department of Biological Sciences, Birla Institute of Technology and Science, Pilani, Rajasthan, India since 2015. Previous to this he joined as Assistant Professor in the current department in 2006. Prof. Saxena did B. Pharmacy (University of Rajasthan, Jaipur, August 1999) followed by M. E. Biotechnology (BITS, Pilani, June 2001) and Ph. D. (Biological Sciences Group, BITS, Pilani, October 2006). During his Ph.D., he had worked as CSIR-Senior Research Fellow (2003-2006). He has over 19 years (7 year's Pre-doctoral experiences, about 12 year's job tenure) of teaching and research experience. His major thrust area of research is Molecular Biology and Immunology with special emphasis to Genomics and Proteomics of Malaria parasites, *Plasmodium vivax* and *P. falciparum*. His group is focusing on various aspects related to *P. vivax* infections in humans, apicoplast and its genome, metabolic pathways functional in the apicoplast, hypothetical proteins encoded by *P. vivax* nuclear genome. He worked twice as a Visiting Scholar at Department of Global Health, College of Public Health, University of South Florida, Tampa, Florida, USA; initially from June- July 2015 under the University Immersion Faculty Exchange Award from BITS Pilani and later from August – December 2017 under the "2017 ASM-IUSSTF Indo-US Research Professorship award"

bestowed by American Society of Microbiology, USA & Indo-US Science & Technology Forum, India. He was also a recipient of the INSA Bilateral Exchange 2017 Fellowship to visit Institute of Parasitology, Ceske Budejovice, Czech Republic during June 2017. He was recipient of DST Fast Track Project (2010 – 2013). He is actively involved in teaching and research, has handled projects from various funding agencies, has supervised 4 Ph.D. thesis, many graduate and post-graduate student thesis and is currently supervising 4 PhD students. He was a recipient of Young Scientist Award for Best Poster Presentation at International Conference on Molecular Epidemiology & Immunology of Malaria and other vector Borne Diseases at RMRCT, Jabalpur, M. P., INDIA, 2007. He has published research papers in Scopus indexed journals of high repute and good impact with total h-index of 8 and citation index of 766. He has authored a book on Genetic Engineering. Prof. Saxena has participated and presented his work in many national and international conferences within India and abroad.

Biography of Dr. Sushil Kumar Yadav

Dr. Sushil Ku mar Yadav has completed his Bachelor degree, Bachler of Veterinary Sciences & Animal Husbandry (BVSc & AH) in 2005 from Karnataka Veterinary, Animal & Fisheries Sciences University Bidar-585401. Post graduation: Master of Engineering (ME) in Biotechnology in 2012, from BITS, Pilani. His work experience and appreciations/awards are as below mentioned

Professional career:

S. No.	Designation	Institute	Duration
1	Field veterinarian	Private	Sep. 2005 to Feb, 2007
2	Marketing and Recovery Officer	State Bank of India	24thMarch 2007 to 24thSep. 2007
3	Veterinary In-charge	BITS, Pilani	25thSep.2007 to 31st march 2012
4	Senior Veterinary In-charge	BITS, Pilani	1stApril 2012 to till date

Award/Prize/Certificate etc.

S. No.	Award/Prize/Certificate	Institution	Year
1	Best Poster Award	International Conference on Life Science Research & its Interface with Engineering and Allied Sciences (LSRIEAS), BITS, Pilani	2018
2	Best Paper in Pharmacology	Association of pharmaceutical	2011

		teachers of India.	
3	Best Performance Award	BITS, Pilani.	2011
3	4th All India Inter-Agriculture University Youth festival (Debate, Elocation, quiz) Certificate of participation	Indian Council of Agricultural Research, New Delhi	2003
4	Inter Collegiate Youth Festival (Certificate of Merit)	University of Agricultural sciences Dharwad, Karnataka	2003
5	Certificate of Earn While You Learn Scheme	Karnataka Veterinary, Animal & Fisheries Sciences University Bidar	2003
6	All India Inter-University Seminar	Shiksha Mandal, Wardha.	2002
7	Inter-University National Debate Competition on cooperation (Certificate of participation)	National Council for Cooperative Training of National Cooperative Union of India, New Delhi	2001
8	Certificate of Appreciation (for Literary Excellence)	University of Agricultural sciences Dharwad, Karnataka	2001-2002 and 2002-2003
	Second Prize	VET-FEST 2002, University of Agriculture Sciences Dharwad, Karnataka	2002
	First Prize	Inter college Elocution competition, University of Agriculture Sciences Dharwad, Karnataka	2001
	First Prize	Inter college debate competition, University of Agriculture Sciences Dharwad, Karnataka	2001

He is registered as a practicenar in Veterinary Council of India (VCI) and Rajasthan Veterinary Council RVC). He joined BITS Pilani for his doctoral research studies in Jan 2012. His areas of interest are Veterinary Sciences, Genetics, Biotechnology and pharmacology. During his PhD studies he was also involved in conducting experimental laboratories for post graduates and undergraduates.