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Role of Cholesterol and Its Fractions in Buffalo Ovarian Follicular Atresia

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Abstract The present study was undertaken to ascertain the transfer process of total cholesterol and its fractions (HDL-C, LDL-C, Free-C and esterified-C) from blood into various compartments (Follicular Wall-FW; Granulosa Cells – GC; Follicular Fluid – FF) of the buffalo ovarian follicles across the "blood-follicle barrier" and also to correlate the potential role of the cholesterol and its fractions as a substrate for steroidogenesis in non-atretic and early atretic buffalo ovarian follicles. The FW, GC and FF from non-atretic and early-atretic follicles of pre-ovulatory and ovulatory stages of buffalo ovaries were collected and pooled category-wise. From these samples lipids were extracted as per the standard protocol. The total cholesterol, HDL-C and LDL-C present in these samples was estimated spectrophotometrically, while the free-C and esterified-C were initially separated by partition chromatography and then estimated spectrophotometrically. The concentration of total cholesterol and its fractions decreased progressively from FW to GC, followed by FF, which indicate that all these fractions originate from serum only. Within the health (non-atretic and atretic) status of the follicles, their concentration increased from pre-ovulatory to ovulatory stage, indicating a higher steroidogenic potential and an increase in the number of steroidogenic cells within the follicles, as they grow. Other observations include: higher concentration of total cholesterol and HDL-C in the GC and FF than the FW of early-atretic follicles; higher free-C in all the compartments of early atretic follicles. From these results it is evident that there is sufficient supply of the substrate (total-C and HDL-C) to the thecal cells (FW) for the testosterone synthesis even at the onset of atresia, but the limitation is the availability of the free-C in the GC of the atretic follicles. This may be the cause for the switch over in steroidogenic pattern i.e. from estradiol to progesterone synthesis in atretic follicles.

Keywords *Cholesterol, HDL-C, LDL-C, Free-C, Esterified-C, Buffalo, Follicular Atresia*

1. Introduction

Ovary is the dynamic female reproductive organ and follicle is the ovarian compartment that enables the ovary to fulfill its dual function of gametogenesis and steroidogenesis. Folliculogenesis is a dynamic event culminating in ovulation or atresia. Follicular growth and maturation represent a series of sequential sub-cellular and molecular transformations of various components of the follicle such as oocyte, granulosa and theca. These are governed by several paracrine, autocrine and endocrine signals that regulate the recruitment, selection, differentiation, maturation and dominance of follicles (Hafez, 1987). The major challenge met by the mammalian ovary is to maintain the continuous development of small follicles and at the same time to allow species-specific number of follicles to ovulate and become corpora lutea. They also inhibit other co-existing follicles from development deviating them towards a degenerative change called atresia (Fortune, 1994). In mammals it has been established that a vast majority (more than 99 percent) of follicles present at birth eventually become atretic, whereas significantly less number (less than 1 percent) actually attain ovulation during the active reproductive life of an animal (Erickson,1966). Therefore, to augment reproductive performance, the number of ovulations needs to be increased (superovulation) or rate of atresia decreased.

A) Follicular Atresia

Manny morphological and biochemical characteristics of atretic follicles have been identified (Richards, 1980; Ryan, 1981; Farookhi, 1981), e.g. lower estradiol / progesterone, lack of carotene cleavage activity, non-activation of plasminogen, accumulation of chondroitin sulphate in follicular fluid have all been claimed as biochemical markers of atresia; but, neither the mechanism nor the factors initiating atresia have been established or identified. Moreover species-wise difference in many of the chemical constituents of follicular fluid indicates the need to study different farm animals separately. Buffalo is an important farm animal in India and South East Asian countries. It is well known for higher milk fat percentage, better feed conversion ratio, disease resistance etc., but suffers from many reproductive problems like delayed age of maturity, longer calving interval, weak/silent oestrus, seasonality of breeding etc. and the available literature on buffalo folliculogenesis and atresia are scanty.

B) Cholesterol and Follicular Atresia

Cholesterol is the precursor for all the steroid hormones synthesised in all the tissues including ovaries. Sangha *et al.* (1989) reported that the cholesterol content increased with growing follicles and free fatty acids are present in minor quantities in the growing follicles of rat ovary. Granulosa cells *in vitro* were able to utilise low density lipoproteins (LDL-C) for the production of cholesterol and subsequently for the progesterone synthesis (Savion *et al.,* 1981). But studies on follicular fluid show lower content of LDL and higher content of high density lipoproteins (HDL-C) and hence, HDL-C may have important role in steroidogenesis *in vivo.* This difference in LDL-C and HDL-C concentration would have risen due to the presence of “blood-follicle barrier” (*Enk et al.,* 1986). Zerbinatti and Dyer (1999) have shown that the thecal cell androgen synthesis in rats is stimulated by apolipoprotein E and the whole process is mediated by the members of LDL receptor superfamily. Though no direct relationship has been established so far between cholesterol and atresia, it can be speculated that cholesterol may serve as a marker for follicle atresia, as they are the substrate for steroidogenesis.

Hence, the present study was undertaken to ascertain the transfer process of total cholesterol and its fractions (HDL-C, LDL-C, Free-C and esterified-C) from blood into various compartments (Follicular wall-FW; Granulosa Cells – GC; Follicular Fluid – FF) of the buffalo ovarian follicles across the “blood-follicle barrier” and also to correlate the potential role of the cholesterol and its fractions as a substrate for steroidogenesis in non-atretic and early atretic buffalo ovarian follicles.

2. Materials and Methods

A) Chemicals

The total cholesterol and HDL-C kits were procured from M/s. Qualigens fine chemicals Ltd. (India) and all other routine chemicals used were of AR grade.

B) Collection of Ovaries, Pre-Ovulatory and Ovulatory Follicles

Buffalo ovaries were collected from local abattoir immediately after slaughter and transported to the laboratory in chilled saline. They were kept cool throughout the collection of experimental materials. After thorough washing in chilled normal saline, the diameters of each follicle protruding on the surface of the ovaries were measured at two perpendicular points (short and long axes). Based on the average of the two measurements follicles were either categorised as pre-ovulatory (5-8 mm) or ovulatory (>10 mm) follicles. These follicles were dissected free of stromal tissue using scissors and micro-dissection forceps under stereo-zoom microscope.

C) Microscopic Evaluation of Follicles

All the isolated follicles of both the above categories were examined under stereo-zoom microscope (Trycon 100z) for the following characters:

a. Degree of translucency
b. Degree of thecal vascularisation
c. Continuity of membrana granulosa
d. Presence or absence of free floating particles inside follicular cavities

Follicles with a uniformly bright, translucent appearance, extensive vascularisation, a regular continuous granulosa layer and absence of free floating particles in the follicular cavity were graded non-atretic, while the follicles characterized by loss of translucency, slight greyish appearance, observable discontinuities of granulosa layer with acceptable vascularisation were graded as early-atretic as described by Moor *et.al.* (1978) and Kruip and Dieleman (1982).
Thus the following four classes of follicles were taken for study:

1. Preovulatory non-atretic (PN)
2. Preovulatory early-atretic (PA)
3. Ovulatory non-atretic (ON)
4. Ovulatory atretic (OA)

D) Collection of Experimental Materials

The experimental materials *viz.* follicular fluid (FF), granulosa cells (GC) and follicular walls / sacs (FW) were collected from each class of the above follicles and pooled separately as follows:

Follicular fluid was aspirated from each follicle using a 5 ml syringe with 22G needle. Then the follicular wall was slit open and the inner surface was scraped thoroughly to collect the remnant GC. These cell scrapings were added to the centrifuge tubes containing FF of respective classes and were centrifuged at 3000 rpm for 10 min. The cell-free supernatant, which is FF sample was collected and stored at -20°C till further analysis.

The pellet containing GC was suspended in known volume of 0.25 M sucrose solution and stored at -20°C till further analysis. Likewise the empty follicular walls representing the thecal cells were weighed, suspended in 0.25 M sucrose solution and stored at -20°C till further analysis.

E) Preparation of Lipid Extracts

Lipids were extracted from the FW, GC and FF samples by the Folch method (Folch *et al.,* 1951) with little modification. The samples were hot extracted in about 20 vol. of chloroform: methanol (2:1), the extracting solvent (after homogenisation in case of FW and GC, followed by filtration and drying of the lipid residues). Finally, the dried lipid residues were dissolved in chloroform: methanol (2:1) and stored at 4° C in microfuge tubes sealed with parafilm until used.

F) Estimation of Total Cholesterol

The cholesterol present in the lipid extracts of the samples were estimated by using a diagnostic kit (Qualigens diagnostics, India) as per the standard protocol prescribed by the manufacturer. The increase in the absorbance was measured at 560 nm and the results were expressed as mg or µg total cholesterol per mg protein in all the samples.

G) Estimation of HDL- C:

The HDL-C present in the lipid extracts of the samples were estimated by using a diagnostic kit (Qualigens diagnostics, India) as per the standard protocol prescribed by the manufacturer i.e. LDL-C and HDL-C were precipitated using the precipitating reagent and then the HDL-C was determined in the supernatants recovered by centrifugation. The procedure from this step is same as that for total cholesterol and the increase in the absorbance was measured at 560 nm and the results were expressed as µg HDL-C per mg protein in all the samples.

H) Estimation of LDL- C

The LDL-C content was calculated using the Friedewald's formula (Friedewald, 1972).
LDL-C = (Total Cholesterol) – (Total triglyceride / 5) – (HDL-C)
The results were expressed as mg or µg LDL-C per mg protein in all the samples.

I) Estimation of Esterified and Free Cholesterol in FW, GC and FF Samples

A known amount of total cholesterol was resolved on alumina column (adsorption chromatography) as per the method of Kerr and Bauld (1953) to yield two fractions *viz.* esterified cholesterol and free cholesterol. The cholesterol content in each of these fractions was determined by the method of Henly (1957) using ferric chloride reagent.

25 ml glass chromatographic column having a length of 10-12 cm and an internal diameter of 0.4 cm was used. The alumina was packed as slurry in light petroleum to a height of 3 cm over a small plug of glass wool on the tip of the pipette. The rate of flow was regulated at 20 ml/h. About 0.15 mg of total cholesterol of each sample (lipid extracts in chloroform: methanol – 2:1) taken in eppendorf tubes was evaporated to dryness in boiling water bath. Then they were placed in a desiccator for an hour and dissolved in 0.5 ml of light petroleum by warming the tubes in hot water. These samples were applied to the column. The eppendorf tubes were washed three more times, each with 0.5 ml light petroleum and were applied to the column. First the esterified cholesterol was eluted with 10 ml 25% benzene in light petroleum and then the free cholesterol with 10 ml of 1% ethanol in benzene. These two fractions (eluates) of 10 ml each were collected for each class of the sample and taken for cholesterol estimation.

All the eluates collected were evaporated to dryness and so also 10 ml of one of the solvents to serve as solvent blank. After drying the samples, 5.0 ml of ferric chloride reagent (10g $FeCl_3.6H_2O$ in 100ml of glacial acetic acid) was added to all the sample tubes, standard tube containing 0.15 mg total cholesterol and the blank tube. Then 3.0 ml of Conc. H_2SO_4 was added to each tube and mixed well. After cooling the tubes, the red colour developed was read at 560 nm. Thus the content of esterified and free cholesterol present in this 0.15 mg of total cholesterol were calculated and accordingly converted to percentage composition in total cholesterol content (that has been estimated earlier as mentioned above in all the samples). Thus the content of free and esterified cholesterol were determined and are expressed as mg or µg free-C per mg protein and mg or µg esterified-C per mg protein in all the samples.

J) Estimation of Protein

Protein content in the samples was estimated according to the method of Lowry *et.al.* (1951) using bovine serum albumin as standard.

K) Statistical Analysis

Data were analysed statistically using One-Way Analysis of Variance (Snedcor and Cochram, 1994).

3. Results

A) Total Cholesterol

The results of concentration of total cholesterol are presented in Table 1 and 1A and are expressed as Mean ± SE. The concentration of total cholesterol was highest in FW samples of ovulatory follicles (ON and OA) and differed significantly ($P<0.01$) from the pre-ovulatory follicles (PN and PA). Though the difference was not significant within the ovulatory follicles (ON and OA) it differed significantly ($P<0.01$) between the pre-ovulatory follicles, showing a higher concentration in PA. In case of GC all the four categories showed significant differences ($P<0.01$) among each other, with the highest value present in OA, followed by ON, PA and PN. The FF picture was totally different as the OA class had the highest concentration which significantly differed ($P<0.01$) from the other three classes and no significant difference was observed among these three classes (i.e. ON, PA and PN).

Table 1: *Concentration of Total Cholesterol in Different Components of Buffalo Ovarian Follicles*

Sl. No.	Class of Follicles	Follicular Wall mg/mg prot	Granulosa Cells µg/mg prot	Follicular Fluid µg/mg prot
1.	PN	2.19 ± 0.017^{c}	31.76 ± 0.863^{d}	20.16 ± 0.799^{b}
2.	PA	2.30 ± 0.018^{b}	38.23 ± 0.927^{c}	21.29 ± 0.796^{b}
3.	ON	4.02 ± 0.022^{a}	57.13 ± 0.023^{b}	22.87 ± 0.954^{b}
4.	OA	4.04 ± 0.026^{a}	76.24 ± 0.391^{a}	28.05 ± 0.342^{a}

Different superscripts in each column indicate significant difference

Table 1A: *Comparative Level of Significance between Different Classes of Follicles with Respect to Total Cholesterol Concentration*

Sl. No.	Class of Follicles	Level of Significance		
		Follicular Wall	Granulosa Cells	Follicular Fluid
1.	Between PN and PA	P = <0.01	P = <0.01	NS
2.	Between ON and OA	NS	P = <0.01	P = <0.01
3.	Between PN and ON	P = <0.01	P = <0.01	NS
4.	Between PA and OA	P = <0.01	P = <0.01	P = <0.01

NS = Not significant

B) HDL-C

The results of concentration of HDL- cholesterol are presented in Table 2 and 2A and are expressed as Mean ± SE. The pattern of HDL-C in FW sample was also similar to that of total cholesterol but for a minor change in pre-ovulatory follicles. The PN follicles showed a significantly higher concentration (P<0.01) than PA follicles and not PA over PN. In case of GC the pattern of HDL-C was exactly the same as the total cholesterol concentration. But, in FF there was some variation in the pattern of HDL-C when compared to the total cholesterol. Though the OA class of FF had the highest concentration, which differed significantly (P<0.01) from all the other three classes (ON, PN and PA) as in total cholesterol, the lowest concentration was observed in PN class and it significantly differed (P<0.01) from PA and OA classes. But there was no significant difference between PA and OA classes of FF with respect to HDL-C content.

Table 2: *Concentration of HDL- Cholesterol in Different Components of Buffalo Ovarian Follicles*

Sl. No.	Class of Follicles	Follicular Wall µg/mg prot	Granulosa Cells µg/mg prot	Follicular Fluid µg/mg prot
1.	PN	193.13±0.228^{b}	14.12±0.440^{d}	11.74±0.044^{c}
2.	PA	187.73±0.046^{c}	16.47±0.237^{c}	14.45±0.188^{b}
3.	ON	466.87±0.227^{a}	24.40±0.414^{b}	14.69±0.228^{b}
4.	OA	464.24±0.990^{a}	31.27±0.668^{a}	15.74±0.590^{a}

Different Superscripts in Each Column Indicate Significant Difference

Table 2A: *Comparative Level of Significance between Different Classes of Follicles with Respect to HDL-C Concentration*

Sl. No.	Class of Follicles	Level of Significance		
		Follicular Wall	Granulosa Cells	Follicular Fluid
1.	Between PN and PA	P = <0.01	P = <0.01	P = <0.01
2.	Between ON and OA	NS	P = <0.01	P = <0.01
3.	Between PN and ON	P = <0.01	P = <0.01	P = <0.01
4.	Between PA and OA	P = <0.01	P = <0.01	P = <0.01

C) LDL-C

The results of concentration of LDL- cholesterol are interesting and are presented in Table 3 and 3A; they are expressed as Mean ± SE. The LDL-C was not detected in any of the GC samples, in PA class of FW sample as well as in ON class of FF sample. All other classes in FW and FF samples had LDL-C. In case of FW, the ON, OA and PN classes showed a significant (P<0.01) variation among each other and the highest concentration was present in ON, followed by OA and PN. In FF, the OA, PN and PA classes also differed significantly (P<0.01) from each other, with the highest concentration of LDL-C being found in OA, followed by PN and PA.

Table 3: *Concentration of LDL- Cholesterol in Different Components of Buffalo Ovarian Follicles*

Sl. No.	Class of Follicles	Follicular Wall mg/mg prot	Granulosa Cells µg/mg prot	Follicular Fluid µg/mg prot
1.	PN	0.38±0.082^{c}	ND	1.45±0.012^{b}
2.	PA	ND	ND	0.99±0.026^{c}
3.	ON	1.78±0.262^{a}	ND	ND
4.	OA	1.30±0.278^{b}	ND	7.97±0.027^{a}

Different superscripts in each column indicate significant difference
ND= Not detected

Table 3A: *Comparative Level of Significance between Different Classes of Follicles with Respect To LDL-C Concentration*

Sl. No.	Class of Follicles	Level of Significance		
		Follicular Wall	Granulosa Cells	Follicular Fluid
1.	Between PN and PA	-	-	P = <0.01
2.	Between ON and OA	P = <0.01	-	-
3.	Between PN and ON	P = <0.01	-	-
4.	Between PA and OA	-	-	P = <0.01

(-) indicate statistical analysis not done as LDL-C concentration was ND

D) Esterified Cholesterol

The results of concentration of esterified cholesterol are presented in Table 4 and 4A and are expressed as Mean ± SE. There was a significant difference (P<0.01) among all the four categories of FW and GC samples with the highest concentration in OA, followed by ON, PA and PN. In case of FF, a significant difference (P<0.01), existed among OA, PA and non-atretic classes (PN and ON) and there was no significant difference within the non-atretic classes i.e. between PN and ON. In FF, the highest concentration of esterified cholesterol was found in the OA, followed by PA and non-atretic classes (PN and ON).

Table 4: *Concentration of Esterified- Cholesterol in Different Components of Buffalo Ovarian Follicles*

Sl. No.	Class of Follicles	Follicular Wall mg/mg prot	Granulosa Cells µg/mg prot	Follicular Fluid µg/mg prot
1.	PN	1.24±0.009^{d}	22.21±0.596^{d}	14.53±0.212^{c}
2.	PA	1.39±0.009^{c}	29.28±0.389^{c}	16.73±0.228^{b}
3.	ON	2.41±0.013^{b}	42.99±0.654^{b}	14.57±0.079^{c}
4.	OA	2.70±0.018^{a}	69.40±0.535^{a}	22.25±0.272^{a}

Different superscripts in each column indicate significant difference

Table 4A: *Comparative Level Of Significance between Different Classes of Follicles with Respect to Esterified-C Concentration*

Sl. No.	Class of Follicles	Level of Significance		
		Follicular Wall	Granulosa Cells	Follicular Fluid
1.	Between PN and PA	P = <0.01	P = <0.01	P = <0.01
2.	Between ON and OA	P = <0.01	P = <0.01	P = <0.01
3.	Between PN and ON	P = <0.01	P = <0.01	NS
4.	Between PA and OA	P = <0.01	P = <0.01	P = <0.01

NS = Not significant

E) Free Cholesterol

The results of concentration of free cholesterol are presented in Table 5 and 5A and are expressed as Mean ± SE. The FW sample showed a significant variation (P<0.01) among all the four classes and the highest concentration was present in ON, followed by OA, PN and PA. In case of GC a significant difference (P<0.01) was noted among ON, PN and early atretic (PA and OA) classes, but the difference was not significant within early-atretic follicles i.e. between PA and OA. The ON class of GC sample had the highest concentration followed by PN and early atretic follicles (PA and OA). In case of FF, the lowest value was seen in PA which differed significantly from (P<0.01) the other three classes (i.e. PN, ON and OA). Moreover there was no significant difference among these three classes i.e. PN, ON and OA).

Table 5: *Concentration of Free - Cholesterol in Different Components of Buffalo Ovarian Follicles*

Sl. No.	Class of Follicles	Follicular Wall mg/mg prot	Granulosa Cells µg/mg prot	Follicular Fluid µg/mg prot
1.	PN	0.95±0.005^{c}	9.52±0.255^{b}	6.18±0.245^{a}
2.	PA	0.91±0.008^{d}	7.89±0.189^{c}	5.11±0.192^{b}
3.	ON	1.61±0.010^{a}	14.07±0.214^{a}	5.95±0.505^{a}
4.	OA	1.34±0.010^{b}	8.13±0.148^{c}	5.80±0.070^{a}

Different superscripts in each column indicate significant difference

Table 5A: *Comparative Level of Significance between Different Classes of Follicles with Respect to Free-C Concentration*

Sl. No.	Class of Follicles	Level of Significance		
		Follicular Wall	Granulosa Cells	Follicular Fluid
1.	Between PN and PA	P = <0.01	P = <0.01	P = <0.01
2.	Between ON and OA	P = <0.01	P = <0.01	NS
3.	Between PN and ON	P = <0.01	P = <0.01	NS
4.	Between PA and OA	P = <0.01	NS	P = <0.01

NS = Not significant

4. Discussion

A) Total Cholesterol

The present study in buffalo ovarian follicles indicates a considerable lower level of total cholesterol than the serum (Mondola *et al.,* 1987) similar to those reported in the FF of rats (Sangha and Guraya, 1989), pigs (Chang *et al.,* 1976; Yao *et al.,* 1980) and humans (Menezo *et al.,* 1984; Enk *et al.,* 1986). Further it showed highest concentration in FW followed by GC and finally FF. These findings suggest that the follicular total cholesterol is mainly transudated from blood across the "blood –follicle barrier" into the follicles. The *de novo synthesis* by granulosa cells can also contribute but only to a minor extent (Gwynne and Strauss, 1982). The presence of low level of cholesterol in the follicles (the substrate for steroidogenesis) may also be an important factor leading to the low reproductive performance of buffaloes. The increase in their concentration from pre-ovulatory to ovulatory stage of development is attributable to both the increased steroidogenic potential and the increase in the number of cells that a follicle acquires as it grows. The concentration of total cholesterol was higher in the non-atretic follicles from only FW samples, whereas in the GC and FF samples early-atretic follicles (GCOA and FFOA) had the higher concentration of total cholesterol than the non-atretic. These findings are supported by the observations made by Guraya (1979) in his histochemical study on buffalo anestrous ovaries where he reported accumulation of abundant sudonophilic lipid droplets consisting mainly of triglycerides and cholesterol and /or its esters in the degenerating granulosa cells of atretic follicles. He suggested that GC may undergo abortive luteinisation during atresia. These findings suggest that:

1. It is the granulosa cells where the atresia sets in and not the thecal cells (FW) of the follicle.
2. The accumulation of cholesterol in early atretic GC and FF may arise either due to an increase in its synthesis or a decrease in its utilisation.

B) LDL vs. HDL Cholesterol

It is known that cholesterol and /or its esters are the principal substrate for steroid synthesis by the granulosa cells and the circulating lipoproteins which get internalised in the cells by receptor mediated endocytosis supply this substrate (Enk *et al.,* 1986). However, the controversy regarding the main carrier LDL or HDL or both still exists! The result of LDL-C in the present study appears a bit difficult to explain as it was detected in some categories of the component while it went undetected in others. This ambiguity in the results may be attributed to the indirect methodology (Friedewald's formula) used in this study. However, striking observation is the absence of LDL in all the categories of granulosa cells. This is an important finding as a similar observation has been made in the FF of pigs (Chang *et al.,* 1976) and humans (Enk *et al.,* 1986) due to the presence of "blood follicle barrier" (the membrana limitans) which do not allow the passage of particles above 850 kD (Shalgi *et al.,* 1973) like IgM, LDL, VLDL, etc. from serum into the follicles.

HDL was present in all the components of the follicle and its concentration decreased progressively from FW to GC and FF. This indicates that the "blood follicle barrier" allows the HDL to pass through into the follicle via FW, GC and FF. Buffalo serum has higher concentration of HDL than LDL (Mondola *et al.* 1987). So this may be another reason for the presence of more HDL inside the follicle. With respect to size, the HDL concentration increases as the follicle develops and it can be attributed to higher steroidogenic potential and /or more number of follicular cells. The HDL picture in relation to atresia indicates a high concentration of HDL in non-atretic FW than early atretic FW and the pattern is reverse in GC and FF i.e. more in early-atretic than non-atretic. This observation signifies that:

1. In atresia, there is sufficient supply of the substrate to thecal cells, which do utilise them and synthesise testosterone.
2. The accumulation of HDL in GC and FF during atresia indicates that the GCs are not able to utilise them and this may be due to the lack of the Cyt.P450 side chain cleavage enzyme, which needs to be confirmed.

In general, the production of steroids in the steroidogenic cells depends upon three mechanisms (Enk *et al.,* 1986):

a. intracellular *de novo* synthesis
b. de-esterification of intracellular stored cholesterol and /or ester
c. uptake from lipoprotein bound cholesterol

Gwynne and Strauss (1982) suggested that the *de novo* synthesis of cholesterol from acetate plays a minor role in ovaries. The cholesterol ester hydrolase and the lecithin cholesterol acyl transferase (LCAT) activities have been identified in the pig follicular fluid (Chang *et al.,* 1976) and this will contribute to the second mechanism proposed. However it is the third mechanism of uptake from lipoprotein which is expected to contribute significant steroidogenesis.

LDL stimulates steroid production in *in vitro* GC (Savion *et al.,* 1981). However, studies on follicles indicate a low level of LDL in FF (Enk *et al.,* 1986). On the other hand reports by Savion *et al.,* (1981) and Schreiber *et al.,* (1982) showed that GC can utilise cholesterol provided by HDL *in vitro.*

An overview of the literature available and the present findings suggest that the gonadotropins *in vivo* can facilitate the delivery of HDL bound cholesterol to granulosa cells for steroidogenesis. However, confirmation of this concept needs further investigation.

C) Free vs. Esterified Cholesterol

The present study revealed nearly about 40: 60 ratio of free: esterified cholesterol in FW and 30 : 70 ratio in GC and FF. The pig and human FF were also reported to have a similar free: esterified cholesterol ratio (Chang *et al.,* 1976; Menezo et al., 1984). In general an increase in this ratio implies a higher steroidogenic activity. There was a progressive decrease in concentration of both the forms of cholesterol from FW to GC and FF. Their ratio also increases with the stage of development in all the components. These observations also indicate that the cholesterol and its esters are derived from serum mainly and it increases with steroidogenic capacity.

As far as the atresia is concerned the presence of more free cholesterol in non-atretic follicles and esterified cholesterol in early-atretic follicles reflect their capability of steroidogenesis. Thus, there is a switch over in the steroidogenesis pattern i.e. from estradiol to progesterone in early stage of atresia itself. Mondola *et al.,* (1987) also reported that the free cholesterol is only 15% to that of total cholesterol.

5. Conclusion

It is very clear from the results of this study that cholesterol and its fractions get transudated mainly from the serum into the follicular compartment through the "blood-follicle barrier "and the contribution by thecal and granulosa cells through *de novo* synthesis are minimal. There is accumulation of total cholesterol and HDL-C in granulosa cells of early atretic follicles which indicates that there is supply of substrate for ovarian steroidogenesis but it is not being utilized, which may be due to lack of the Cyt.P450 side chain cleavage enzyme or any other similar factor. Moreover, there is very low concentration of free cholesterol and absolute absence of LDL-C in the granulosa cells of early-atretic follicles. Hence it is concluded that cholesterol (free form) and / or LDL-C are the major limiting factors in buffalo steroidogenesis, leading to follicular atresia and perhaps it can also result in the lower reproductive efficiency of buffaloes.

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Research Article **Open Access**

Blood Biochemical, Enzymatic and Haematological Status of Dogs Affected with Dilated Cardiomyopathy

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Abstract Dilated Cardiomyopathy (DCM) occurs more frequently in large breed dogs and the common form is Idiopathic DCM. Blood biochemical and enzymatic parameters pertaining to cardiology were analysed in both healthy and DCM affected dogs. The activity of creatine kinase increased significantly while the concentration of sodium and calcium decreased significantly in DCM cases. Significant differences in the concentration of potassium, creatinine, urea and total cholesterol were observed, but the values were well within the normal range. The concentration of other blood biochemical parameters like glucose, total proteins, HDL-cholesterol, LDL-cholesterol, triglycerides and the activity of the enzymes AST and LDH were all within the normal range and showed no significant variation between the healthy and DCM dogs. Similarly, haematological parameters were also analysed in both healthy and DCM affected dogs. There was a significant decrease in the concentration of Haemoglobin, PCV (%), RBC count and Platelet count in DCM affected dogs. However, the total WBC counts were significantly higher; with DLC showing neutrophilia in DCM affected dogs. Thus the overall picture indicated Ischemia with acute infection.

Keywords *DCM, Dogs, Cardiology, Biochemical Parameters, Cardiac Enzyme Markers, Haematological Parameters*

1. Introduction

Dilated Cardiomyopathy (DCM) characterized by chamber dilatation and myocardial systolic and diastolic dysfunction is one of the most common heart diseases in dogs (Tidholm, *et.al.,* 2001). DCM occurs more frequently in large and giant breed dogs (Doberman Pinschers, Boxers, etc) and rarely in small breeds (cocker spaniels) or mixed breeds (Mc Evan and Joanna Dukes, 2000). The most common form is Idiopathic DCM, as many processes could lead to myocardial injury in dogs – *Viz.*

genetic, nutritional, metabolic, inflammatory, and infectious or drug – or toxin induced (Borgarelli, *et.al*., 2001, Aleksandra Domanjko Petric and Katerina Tomsic, 2008).

The usefulness of biochemical markers in the diagnosis and prognosis of heart disease is well documented in humans and similar documentation is coming up in companion animals. The veterinary biochemical markers of cardiac dysfunction on date are of two types: The first group comprises of biochemical markers of myocardial injury and necrosis like cardiac enzymes - creatine kinase (CK-MB), aspartate transaminase (AST), lactate dehydrogenase (LDH), and the recently evaluated structural myocardial proteins – troponins. The second group of biochemical markers is used to assess the degree of cardiac dysfunction and is represented by the plasma neurohormones. The most commonly used indicators of neuroendocrine activation are plasma norepinephrine (NE), the atrial natriuretic peptide (ANP), the B-type peptide (BNP), peptides of the renin - angiotensin system (RAS) – vasopressin and aldosterone, and plasma big endothelin –I (Aleksandra Domanjko Petric and Katerina Tomsic, 2008).

Hence the present study was undertaken to identify some of the routine blood biochemical, enzymatic and haematological parameters that will help in the diagnosis and prognosis of DCM.

2. Materials and Methods

The blood samples were collected from the dogs attending the OP ward of Madras Veterinary College Teaching Hospital, Chennai. Fifty blood samples were collected from Healthy dogs that served as control (Group I) after routine clinical examination and fifty blood samples were collected from DCM diagnosed dogs (Group-II) after screening for cardiac dysfunction tests by ECG, Chest Radiography and Doppler echocardiography.

A) Biochemical and Enzyme Analysis

From each dog about 5 mL of blood was collected from the cephalic vein and serum was separated and stored at 4˚C till analysis. All the biochemical and enzymatic parameters were analyzed on the same day, using diagnostic kits (M/S. Agappe Diagnostics Ltd., Ernakulum, and Kerala, India) as per the manufacturer's protocol except LDH-1 isoenzyme which was standardised in the lab and measured as per the method of Welshman *et al.* (1967). The concentrations were estimated spectrophotometrically, using UV-Vis Spectrophotometer (CE-2021, Cecil, UK) available in the department.

B) Haematological Analysis

Another 2.5 mL of blood was collected separately from the same dogs from the cephalic vein in vacutainers with anticoagulants for haematology. Thus the whole blood collected from both the DCM and Healthy canine subjects were analyzed for routine haematological parameters (like Hb, PCV, RBC, WBC, DLC and platelet count) using Auto-analyzer (Biosystems, A15).

C) Statistical Analysis

The data obtained was statistical analysed as per Snedecor and Cochran (1994), by unpaired 't'-test. A value of $p < 0.01$ was considered statistically significant.

3. Results and Discussion

A) Blood Biochemical Parameters

The results of the routine blood biochemical parameters for both the healthy (control) and DCM groups along with the reference values are presented in Table 1. It is evident from the table that there was no significant difference with respect to the following parameters between both the groups: glucose, proteins, potassium, HDL- cholesterol, LDL-cholesterol and triglycerides and all the values were within the normal reference range. It indicates that the DCM cases encountered are of per acute nature and hence the metabolic derangements upon carbohydrate, protein and lipid metabolism are yet to be observed.

Table 1: *Routine Biochemical Parameters in Control and DCM of Canines*

Sl. No.	Parameters	Control (n=50)	DCM (n=50)	Reference Range	Result
1.	GLUCOSE (mg/dl)	92.04 ± 3.0156	92.19 ± 6.4021	65.0 - 118.0	NS
2.	PROTEIN (g/dl)	5.01 ± 0.0738	4.80 ± 0.1907	5.4 - 7.1	NS
3.	TRIGLYCERIDES (mg/dl)	90.44 ± 11.5616	64.68 ± 7.2640	38.1	NS
4.	TOTAL CHOLESTEROL (mg/dl)	198.90 ± 8.3308	157.44 ± 11. 4258	135 - 270	**
5.	HDL CHOLESTEROL (mg/dl)	88.70 ± 0.8706	87.75 ± 6.4889	37.8 - 75.6	NS
6.	LDL CHOLESTEROL (mg/dl)	53.08 ± 3.0147	58.10 ± 9.2349	40.5 - 81.0	NS
7.	UREA (mg/dl)	22.96 ± 0.9163	50.71 ± 6.1571	16 - 58	***
8.	CREATININE (mg/dl)	1.11 ± 0.0495	1.58 ± 0.1415	0.5 - 1.5	***
9.	SODIUM (mEq/l)	147.76 ± 0.6410	94.13 ± 7.3025	141.0 - 152.0	***
10.	POTASSIUM (mEq/l)	5.06 ± 0.0658	4.04 ± 0.5279	4.5 - 5.5	NS
11.	CALCIUM (mg/dl)	10.33 ± 0.1535	8.23 ± 0.3608	9.0 - 11.3	***

NS = Not Significant; ** = P<0.05; *** = P<0.01

There was a significant difference between the two groups with respect to the concentration of sodium and calcium, showing lower values in DCM cases. The sodium concentration in control was found to be 147.76 ± 0.6410 mEq/L and in DCM it was 94.13 ± 7.3025 mEq/L. Similarly the calcium concentration in control was found to be 10.33 ± 0.1535 mg/dl and in DCM it was 8.23 ± 0.3608 mg/dl. This may be attributed to the fact that there is drainage of sodium and calcium from the blood into cardiac tissue for depolarization and excitation of cardiac muscle respectively, which is one of the physiological responses at the time of cardiac arrest (Guyton, 2011).

Though statistically a significant difference in the concentration of total cholesterol and urea have been observed between the two groups (with higher values for DCM cases), they are clinically not significant as they fall well within the normal reference range.

B) Cardiac Enzyme Profile

The results of the cardiac enzyme markers like creatine kinase (CK), aspartate transaminase (AST), total lactate dehydrogenase (LDH) and the isoenzyme LDH-1 along with the reference range for both the groups are presented in Table 2. Again a significant difference was observed only for the total creatine kinase activity between the two groups, which confirms the per-acute form of DCM. It indicates that the patients have been brought to the hospital within 6 hours of cardiac damage, due to which only the CK activity had increased. The rise in the LDH and LDH-1 activity happens generally within 12-24 hrs and that of AST after 2-3 days of cardiac arrest (Kaneko, 2008).

Table 2: *Cardiac Marker Enzymes in Control and DCM of Canines*

Sl. No.	Parameters	Control (n=50)	DCM (n=50)	Reference Range	Result
1.	TOTAL - CK (U/L)	20.23 ± 1.0387	107.89 ± 23.3291	1.15 - 28.40	***
2.	LDH (WU/ml)	352.98 ± 17.51	388.12 ± 40.9138	93.15 - 482.31	NS
3.	LDH - 1(WU /ml)	31.31 ± 3.1691	28 ± 6.7178	3.5 - 51.34	NS
4.	AST (U/L)	45.91 ± 1.9208	59.26 ± 7.4831	23.0 - 66.0	NS

NS = Not Significant; *** = P<0.01

C) Haematological Parameters

The results of routine haematological parameters for both the groups along with the reference range are presented in Table 3.

Table 3: *Haematological Parameters in Control and DCM of Canines*

Sl. No.	Parameters	Control (n=50)	DCM (n=50)	Reference Range	Result
1.	HAEMOGLOBIN (g/dl)	14.72 ± 0.4690	10.94 ± 0.4637	12 - 18	**
2.	PCV (%)	41.74 ± 0.8231	30.98 ± 1.4565	37 - 55	***
3.	RBC (millions/cu.mm)	6.48 ± 0.2454	4.81 ± 0.1755	5.5 - 8.5	***
4.	WBC (cells/cu.mm)	8721 ± 387	13673 ± 1081	6,000 - 7,000	***
4.	LYMPHOCYTES (%)	23.89 ± 1.417	15.16 ± 1.0686	8 - 35	***
5.	NEUTROPHILS (%)	66.74 ± 1.4924	80.58 ± 0.9500	51 - 72	***
6.	MONOCYTES (%)	5.89 ± 0.5614	2.58 ± 0.3771	1 - 9	***
7.	BASOPHILS (%)	0.16 ± 0.1150	0.00 ± 0.00	0 - 2	NS
8.	EOSINOPHILS (%)	3.42 ± 0.5988	2.47 ± 0.4483	0 - 9	NS
9.	PLATELETS (lakhs/cu.mm)	470700 ± 64493. 7637	56500 ± 10062.3059	1, 50, 000 - 4, 00, 000	***

NS = Not Significant; ** = P<0.05; *** = P<0.01

A significant difference in the values for Hb, PCV, RBC and platelet count have been observed with lower values in DCM cases. The Hb was found to be 14.72 ± 0.4690 g/dl in control group and 10.94 ± 0.4637 g/dl in DCM. Similarly the PCV values (in %) in control group were 41.74 ± 0.8231, whereas it was 30.98 ± 1.4565 in DCM. The RBC count was also lower in DCM (6.48 ± 0.2454 millions/cu.mm) when compared to the control (4.81 ± 0.1755 millions/cu.mm). The platelet count was also very low (56500 ± 10062.3059 lakhs/cu.mm) in DCM when compared to the control group (470700 ± 64493 lakhs/cu.mm). The results suggest ischaemia indicating CVS disorder (Reece William O., Richardson *et al.*, 1996).

As far as the WBC count and DLC measurements are concerned a significant difference (with higher values in DCM group) was observed for all the parameters except basophils and eosinophils. Though the lymphocyte and monocyte counts were higher in DCM, they were well within the normal range. The WBC count was significantly higher in DCM (13673 ± 1081 cells/cu.mm) than the control (8721 ± 387 cells/cu.mm) group. Similarly a significantly higher value for neutrophils in DCM (80.58 ± 0.95 %) group than the control (66.74 ± 1.4924 %) group was observed. Thus a clinically as well as statistically significant difference were observed for the total WBC count and neutrophils only. This neutrophilia indicates an acute stage of infection, which may also lead to cardiac complication (Khomeriki and, Morozov, 1998).

4. Conclusion

The haematological observations give an overall picture indicating ischemia with acute infection. Among the biochemical and enzyme parameters, estimation of CK activity, sodium and calcium concentration in serum can be used for diagnosis and prognosis of DCM cases in dogs. All these results in *toto,* confirm that the samples were collected from dogs suffering with per-acute form of DCM.

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Short Communication **Open Access**

Incidence of Brucellosis in Camels in a Small Holder Farm at Al Ain, United Arab Emirates

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Abstract The incidence of brucellosis was evaluated in a small camel farm at Al-Ain, UAE by using chromatographic immunoassay. The incidence was found to be 0.2%. The test was found to be of high accuracy and positive camels were confirmed to be having brucellosis by rose Bengal and other tests. Thus the study indicated that chromatographic kits available for diagnosis of brucellosis in cattle can be used for camels with high precision.
Keywords *Camel, Brucellosis, Chromatographic Tests*

1. Introduction

Brucellosis has been described for camels in many Arabian and African countries (Kudi et al., 1997; Abbas et al., 2002). Extensive surveys in United Arab Emirates (Afzal et al., 1994; Moustafa et al., 1998) depicted the incidence of brucellosis in camels to vary between 0.1 to 1.8%. The usual testing procedure for brucellosis involves the Rose Bengal test as a herd screening test and confirmation by the tube agglutination test and the complement fixation test (Morgan et al., 1978; Afzal et al., 1994). The use of commercially available chromatographic tests (Rapid Bovine Brucella test kit) has been depicted for diagnosis of brucellosis in camels (Kudi et al. 1997). This manuscript describes the diagnosis of brucellosis at a commercial farm at UAE using commercially available chromatographic kits.

2. Materials and Methods

Blood was collected from camels (n=1864) in vacuette tubes (Griener-Bione, 2-step serum clot activator) and evaluated for the presence of Brucella abortus. Samples were processed immediately and the presence of *Brucella abortus* was evaluated using Antigen Rapid Bovine Brucella test kit (Bio Note, Inc. Korea). This test is a chromatographic immunoassay for the qualitative detection of *Brucella abortus* antibody. As mentioned by the manufacturer, the specifically selected *Brucella abortus*

antigens are used in the test which enables the Antigen Rapid B Brucella Ab kit to identify *Brucella abortus* antibodies in specimens with a high degree of accuracy. Briefly, 20 µL of whole blood was added to the cuvette of the test plate by the capillary tube supplied with the kit and 4 drops of the assay diluents were added and the results were read at 20 min. The presence of two color bands within the results window indicated a positive result whereas the appearance of only one purple color band indicated a negative result. In positive cases the presence of Brucella was confirmed by collection of another blood sample and using Rose Bengal plate test at a commercial laboratory.

3. Results

During a six year period (2006-2011) a total of 1864 racing and breeding camels were tested for the presence of *Brucella abortus* using chromatographic immunoassay. A total of 1860 camels (99.79%) were found negative for brucellosis and 4 samples were found to be doubtful. Out of these doubtful cases all the 4 camels were found positive by the Rose Bengal and further tests. Two of these camels were euthanized and the other two were taken by the Food Control Authority Abu Dhabi. Thus the total incidence of brucellosis amongst camels was 0.2%.

4. Discussion

The incidence of brucellosis during the present study was 0.21%. Previous studies in United Arab Emirates have shown that the incidence of brucellosis in camels was between 1.0 to 1.5% (Afzal et al., 1994). In another survey in Al-Ain the incidence of brucellosis had declined from 5.8% in 1991 to 0.1% in 1996 (Moustafa et al., 1998). A decline in the incidence has been possible because of strict identification and eradication measures adopted by the Food Control Authorities (Moustafa et al., 1998). In the present study a commercially available immunochromatographic kits using B abortus antigen for cattle was utilized as also mentioned previously (Kudi et al. 1997) and was effective in detection of brucellosis in camels. Such assays are increasingly being adopted by many breeders in this region. Studies on the comparative efficacy of different tests used for identification of Brucella species in camel have shown marginal differences (Akhtar et al., 2010; Gwida et al., 2011). Thus the chromatographic tests used in the present study can serve as a useful adjunct for testing of brucellosis in camels.

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Research Article | Open Access

A Comparative Study of Some Hematological and Serum Biochemical Parameters of Clinically Healthy Labrador and Spitz

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Abstract The effect of breed and sex on some hematological and serum biochemical parameters were studied in twenty two clinically healthy Labrador (eleven numbers each from male and female) and equivalent number of Spitz. Blood samples were estimated for hematological [hemoglobin (Hb), white blood cell count (WBC), packed cell volume (PCV)] and serum biochemical [total protein, albumin, urea, creatinine, alkaline phosphatase (ALKP), aspartate amino transferase (AST), alanine amino transferase (ALT)] parameters. Serum urea was significantly ($p<0.05$) higher in the male Labrador than in the female where as serum alkaline phosphatase was noted to be significantly ($p<0.05$) higher in both male and female Labrador as compared to Spitz breeds of corresponding sexes. However other parameters showed non-significant ($p>0.05$) variation between male and female group of both the breeds.
Keywords *Hematology, Serum Biochemistry, Clinically Healthy, Labrador, Spitz*

1. Introduction

Blood is one of the important body fluid to access the health status of animals. The evaluation of blood parameters is the first step in diagnosis of many diseases [2, 4]. Hematological as well as serum biochemical tests are routinely done to determine the general level of health in animals distinguishing them from diseased ones [5, 14, 18]. Blood tests are also used to determine the disease state, mineral content, drug effectiveness and organ function in a variety of animals [5, 6]. Age, sex, breed and some environmental factors affect the biochemical and hematological parameters of clinically healthy dogs [9, 12]. Alterations in these parameters have been observed between tropical and temperate animals [2, 7]. These variations have been thought due to the effect of climate, nutrition and sub clinical state of animals [7, 13].

Number of studies have been performed by different workers to find out the normal values of biochemical and hematological indices of dogs [2, 10, 11, 16] but most of these have been carried out

in American and European countries [2] in cold climates making it difficult to determine the effect of breed and sex on these parameters particularly in hot and humid climates like India.

Thus, the present study was planned to determine the alterations in some hematological and serum biochemical parameters between Labrador and Spitz in tropical climate. The comparison in baseline values between breeds and also sexes will provide a physiological reference range of these parameters in local Indian conditions which will be helpful in differentiating the diseased animals from healthy ones.

2. Materials and Methods

2.1. Animals

Twenty two clinically healthy dogs (male=11nos., female=11nos.) were selected from each breed; Labrador and Spitz presented for routine check up and vaccination at the Small Animal Clinic, Teaching Veterinary Clinical Complex (TVCC), GADVASU, Ludhiana. The age of dogs ranged between four to five years. All the dogs had been dewormed and vaccinated against rabies, canine distemper and hepatitis virus. They were also free from external parasites at the time of sample collection.

The weekly records of meteorological data viz. temperature (^{o}C) and relative humidity (%) were collected from the Department of Agricultural Meteorology, Punjab Agricultural University, Ludhiana, for pre summer (March-April) and summer seasons (May-July). The temperature humidity index (THI) was calculated using the following formula,

$$THI = (0.81 \times Ta) + (RH \div 100) \times (Ta \times 14.4) + 46.6$$

Where,

Ta = Average ambient temperature in ^{o}C

RH = Average relative humidity

2.2. Collection of Blood Samples

Five milliliters of blood was collected from cephalic vein separately in glass stoppered centrifuge tubes without any anticoagulant and those containing heparin to obtain serum and uncoagulated blood respectively. All the samples were collected in pre summer and summer season (March-July). The blood samples collected for separation of serum were kept at room temperature in slanting position for thirty minutes followed by centrifugation at 2000 rpm for 10 minutes. The serum and whole blood were immediately used for estimation of biochemical and hematological parameters respectively.

2.3. Procedure

The hemoglobin concentration, white blood cell count and packed cell volume were determined by using the methods given by Weiss and associates [17]. Total proteins, albumin , alkaline phosphatase (ALKP), aspartate amino transferase (AST), alanine amino transferase (ALT), urea and creatinine concentration in serum were estimated using Siemens Autopak kits (Siemens Healthcare Diagnostics Ltd., Gurgaon, Haryana) as per the manufacturer's protocol by semi-automatic biochemical analyzer (RA 50).

2.4. Statistical Analysis

Mean and standard deviation values were calculated by using Microsoft excel computer programme. Student's t-test analysis was used to determine the significant differences by using statistics package SPSS, version 16.0. A value of $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Hematological and Biochemical Indices

The mean hematological and serum biochemical values obtained for healthy male and female of Labrador and Spitz are compared and depicted in Table 3.

The mean serum total protein values (g/dL) determined for Labrador (Male=6.3±0.7, Female= 7.08±0.8) was non-significantly (P>0.05) higher than in the Spitz (Male=6.1±0.91, Female=6.6±1.1). Similarly the mean albumin values (g/dL) estimated for Labrador (Male=3.1±0.45, Female=3.8±0.74) and Spitz (Male=2.8±0.32, Female=3.3±0.51), were higher in the female than in the male of both the breeds.

The serum urea level (mg/dL) was significantly higher (P<0.05) in the male Labrador (6.2±0.7) than in the female (4.1±1.2) where as the serum ALKP (U/L) level was recorded to be higher (p<0.05) in both male and female Labrador (Male=56.6±10.2, Female=58.4±10.1) compared to Spitz (Male= 50.3±7.86, Female=51.6±9.1).

3.2. Meteorological data

Table 1 and 2 represents the meteorological data collected during pre-summer and summer seasons from 2012-13. In recent years, except for some seasons hot and humidity is the main feature of Indian climate. Ludhiana climate exhibits hot summer in May to July while December to March is the coolest period of the year. The maximum temperature recorded in 2012 during summer season was 40.2°C, 41.6°C and 42.7°C in the month of May, June and July respectively where as in 2013 the maximum temperature noted was 41.7°C and 43.5°C in the month of may and June. The maximum relative humidity in the month of May, June and July 2012 was 78%, 71.4% and 72.5% where as in 2013 it was recorded to be 79.8% and 78.4% in the month of May and June respectively.

Table 1: *Meteorological Records of Ludhiana during Pre-Summer and Summer Seasons in 2012*

Season	Month	Average ambient temperature (°C)	Average Relative humidity (%)	Average THI
Pre-summer	March	27.0	45.6	72.54
	April	28.0	38.0	74.22
Summer	May	31.0	76.5	85.89
	June	32.0	70.5	82.11
	July	31.5	78.0	84.66

Table 2: *Meteorological Records of Ludhiana during March to June 2013*

Season	Month	Average ambient temperature (°C)	Average Relative humidity (%)	Average THI
Pre-summer	March	27.8	46.4	71.14
	April	29.0	34.5	73.42
Summer	May	33.4	79.5	83.30
	June	34.0	77.3	87.46

The serum ALT (U/L) [Male Labrador=29.1±2.3, Female Labrador=30.4±6.5, Male Spitz=28.8±3.2, Female Spitz=29.5±2.3], AST (U/L) [Male Labrador=19.0±2.45, Female Labrador=20.6±5.10, Male Spitz=19.2±2.5, Female Spitz=20.0±5.84], and creatinine (mg/dL) [Male Labrador=1.7±0.31, Female Labrador=2.0±1.0, Male Spitz=1.8±0.41, Female Spitz=2.1±0.91], values differ non significantly (P>0.05) between the two breeds and also within each breed.

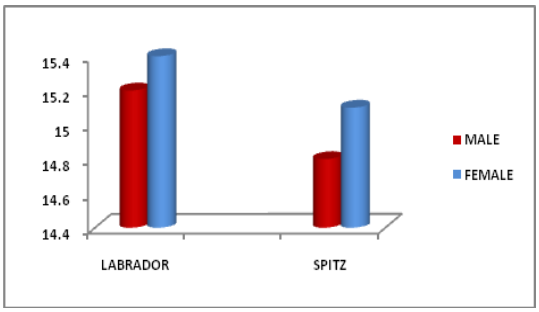
Hemoglobin (g %) [Male Labrador=15.2±0.85, Female Labrador=15.4±0.89, Male Spitz=14.8±0.56, Female Spitz=15.1±0.65], W.B.C ($\times 10^3/\mu l$) [Male Labrador=9.8±0.8, Female Labrador=10.2±1.4, Male Spitz=9.6±0.7, Female Spitz=10.0±1.2] and PCV (%) [Male Labrador=40.2±2.56, Female Labrador= 40.9±2.6, Male Spitz=39.6±2.86, Female Spitz=39.8±2.67] values also differed non-significantly (P>0.05) between both the breeds of dogs and also within each breed of dogs.

Table 3: *Hematological and Serum Biochemical Findings in Clinically Healthy Labrador and Spitz Breeds. (Data are presented as Mean ± SD of 22 Independent Experiments Performed in Duplicate)*

Parameters			Labrador		Spitz	
			Female	Male	Female	Male
Hematological		Hemoglobin (g %)	15.4 ±0.89	15.2 ± 0.85	15.1 ± 0.65	14.8 ± 0.56
		W.B.C ($\times 10^3/\mu l$)	10.2±1.4	9.8±0.8	10.0±1.2	9.6±0.7
		Packed cell volume (%)	40.9±2.6	40.2±2.56	39.8±2.67	39.6±2.86
Biochemical	**Serum Proteins**	Total serum proteins (g/dL)	7.08±0.8	6.3±0.7	6.6±1.1	6.1±0.91
		Albumin (g/dL)	3.8±0.74	3.1±0.45	3.3±0.51	2.8±0.32
	Liver Function Parameters	Alkaline phosphatase (U/L)	58.4^b ±10.1	57.6^b ±10.2	51.6^c ±9.1	50.3^c ±7.86
		Aspartate amino transferase (U/L)	20.6±5.10	19.0±2.45	20.0±5.84	19.2±2.5
		Alanine amino transferase (U/L)	30.4±6.5	29.1±2.3	29.5±2.3	28.8±3.2
	Kidney Function Parameters	Urea (mg/dL)	4.1^a ±1.2	6.2^a ±0.7	5.8±1.2	5.5±1.6
		Creatinine (mg/dL)	2.0±1.0	1.7±0.31	2.1±0.91	1.8±0.41

[a] Means with same superscript vary significantly (p<0.05) between each other
[b, c] Differences are statistically significant (p<0.05) in groups marked with different letters in the same row

(A)



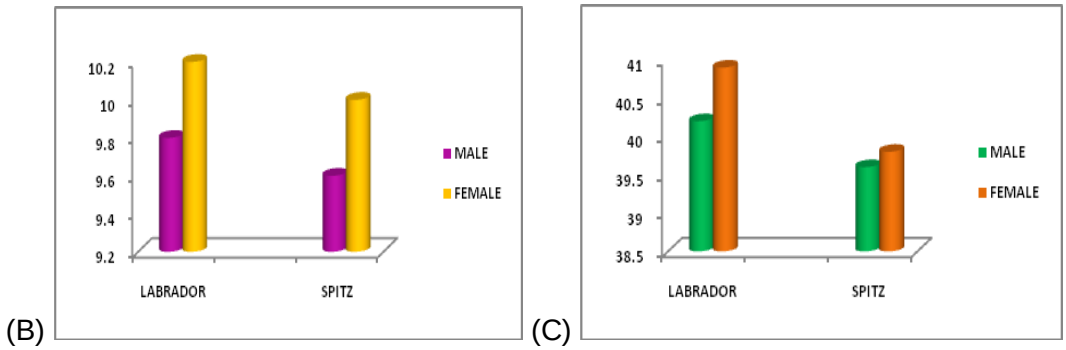


Figure 1: *Baseline Values of Hematological Parameters in Clinically Healthy Labrador and Spitz. (A) Hemoglobin (g %). (B) W.B.C (×10^3/µl). (C) Packed Cell Volume (%)*

4. Discussion

Figure 1 represents the hematological and Figure 2 the biochemical values obtained in the present study. The level of hemoglobin [Figure 1 (A)], W.B.C [Figure 1 (B)] and packed cell volume [Figure 1 (C)] obtained in this study were in line with earlier reports for tropical dogs [3, 6, 8, 13], but slightly lower than the values reported for temperate dogs [2]. The results for serum total proteins [Figure 2 (A)], albumin [Figure 2 (B)], aspartate amino transferase [Figure 2 (D)], alanine amino transferase [Figure 2 (E)] and creatinine [Figure 2 (G)] recorded in current study were similar to earlier observations by different workers [1, 2, 3, 15, 16]. Similarly the increased level of serum alkaline phosphatase [Figure 2 (C)] and urea [Figure 2 (F)] observed in the Labrador breed were in agreement with earlier reports for tropical dogs [5, 8]. Though the exact reason behind these alterations are unclear but, these differences might be due to various factors viz. individual physiological variations, age, food, handling and environmental factors [9, 11].

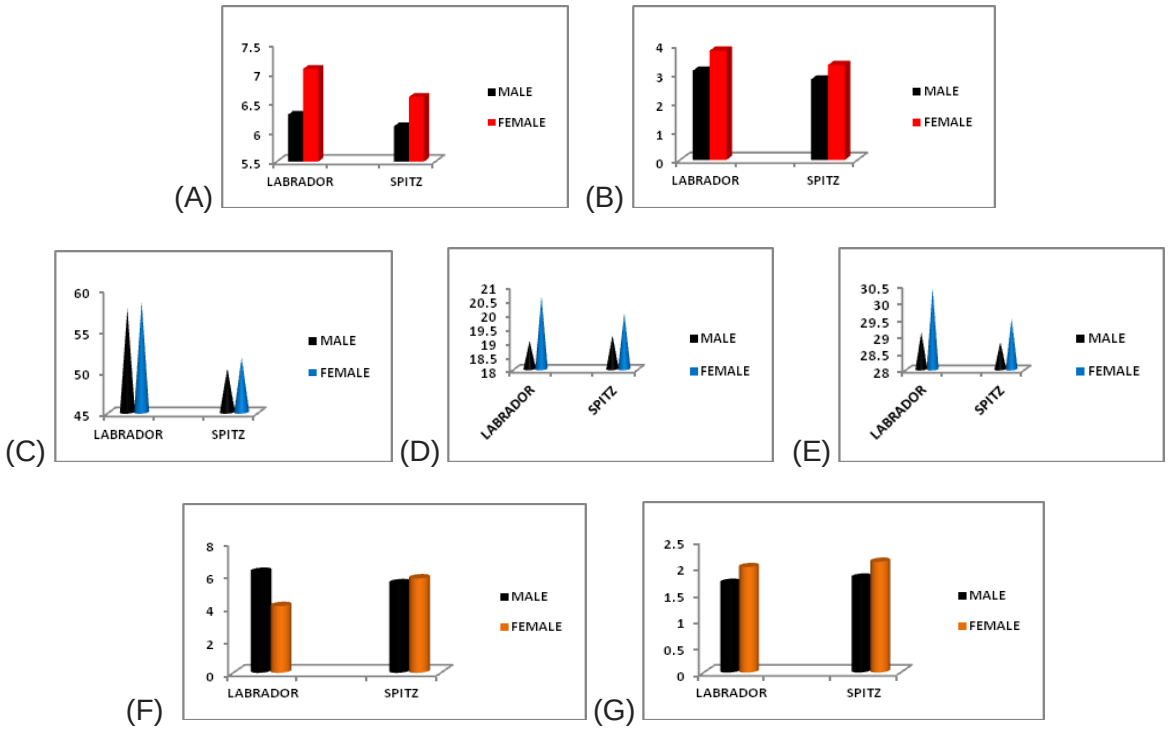


Figure 2: *Baseline Values of Serum Biochemical Parameters in Clinically Healthy Labrador and Spitz. (A) Total Serum Proteins (g/dL). (B) Albumin (g/dL). (C) Alkaline Phosphatase (U/L). (D) Aspartate Amino Transferase (U/L). (E) Alanine Amino Transferase (U/L). (F) Urea (mg/dL). (G) Creatinine (mg/dL)*

5. Conclusion

In this study, both serum urea and alkaline phosphatase values differed significantly ($p<0.05$) between male and female Labrador and Labrador and Spitz respectively. Thus, it can be concluded that breeds of dog and sex significantly affect some biochemical indices which can serve as a reference value and will be of immense importance in differentiating the healthy and diseased animals in local climatic conditions.

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Research Article | Open Access

Seroprevalence Study of Bovine Brucellosis in Slaughter House

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Abstract Brucellosis is a zoonotic bacterial diseases affecting wide range on animal species. The present study was conducted at sera samples collected from slaughter house in Chennai. The samples were subjected to three serological tests viz, RBPT, STAT and i-ELISA of these of 11 (5.23%), 7 (3.3%), 24 (11.4%) were positive by RBPT, STAT and i-ELISA respectively. Comparison of diagnostic tests to assess the sensitivity and specificity of i-ELISA with RBPT and STAT respectively and it was found to be 91.6% and 93.4% when compared to with RBPT whereas it become 100% and 91.6% when compared with STAT.
Keywords *Bovine Brucellosis, RBPT, STAT and i-ELISA*

1. Introduction

Brucellosis is a worldwide zoonotic bacterial disease which causes significant reproductive loss in livestock (OIE, 2009). In animals, bovine brucellosis is characterized by reproductive failure which can include abortion, birth of weak, unthrifty calves, orchitis and/or epididymitis in male. The organism causes abortion in cattle after the fifth month of pregnancy with retention of placenta, metritis and subsequent period of infertility. It is a highly contagious disease transmitted by both vertical and horizontally. The present study was conducted at samples collected from slaughter house in Chennai and to compare three diagnostic tests viz., RBPT, STAT and i-ELISA in terms of sensitivity and specificity for detection of Brucella antibodies in cattle. The present study was conducted at samples collected from slaughter house in Chennai and to compare three diagnostic tests viz., RBPT, STAT and i-ELISA in terms of sensitivity and specificity for detection of *Brucella* antibodies in cattle.

2. Materials and Methods

A total of two hundred and ten sera samples collected from unvaccinated bulls slaughtered at slaughter house in Chennai over a period of one year in 2010 and stored at -20^{o}C till further use. Sera samples were subjected to Rose Bengal Plate Test (RBPT) and Standard Tube Agglutination Test (STAT) as per Alton et al., (1975) using Rose Bengal Plate antigen (IVRI, Izat Nagar) and plain

Brucella abortus antigen respectively (IVPM, Ranipet). The indirect-ELISA test was carried out using kits supplied by Defence Research Development Establishment (DRDE), Gwalior.

3. Results and Discussion

The sera samples were collected out from bulls slaughtered in slaughter house. Seroprevalence of Brucellosis in Chevon goats from Bareily slaughter house was carried out by Mudit et al., (2005). Two hundred and ten sera samples collected from bulls slaughtered at slaughter house were tested by RBPT, STAT and i-ELISA for detection of Brucellosis antibodies and of these 11 (5.23%), 7 (3.3%), 24 (11.4%) were positive by RBPT, STAT and i-ELISA respectively. Agarwal et al., (2007) reported 4.9%, 3.5% and 8.4% samples from cattle to be seropositive in RBPT, STAT and i-ELISA respectively.

Comparison of diagnostic tests to assess the sensitivity and specificity of i-ELISA with RBPT and STAT respectively and it was found to be 91.6% and 93.4% when compared to with RBPT whereas it becomes 100% and 91.6% when compared with STAT. The results were shown in Table 1 & 2. The sensitivity to i-ELISA was 100% and specificity was 95.54% and 94.04% when compared to RBPT and STAT was reported by Priyadarshini et al., (2012).

Sarumathi et al., (2003) found the sensitivity of AB-ELISA to be 100%. The specificity was 88.22% and 90.59% when compared with RBPT and STAT respectively. The samples that were positive by STAT were also positive by both RBPT and i-ELISA. i-ELISA kit was based on the OMP gene31 which was specific for Brucella organism detected even low concentration of antibody and poor quality serum can also be utilized when compared to RBPT and STAT Thakur and Thapliyal (2004).

Avidin-biotin based ELISA had high sensitivity and specificity which can be used for sero-epidemiological investigation was reported by Renukaradhya et al., (2001). Our study finding were coherence with Chakraborthy et al., (2000) who observed that comparison between RBPT and STAT show that RBPT had higher sensitivity as it detected more number of samples to be positive.

4. Conclusion

Seroprevalence study of bovine brucellosis in slaughter bulls carried out. Two hundred and ten sera samples collected from bulls slaughtered at slaughter house were tested by RBPT, STAT and i-ELISA for detection of Brucellosis antibodies and of these 11 (5.23%), 7 (3.3%), 24 (11.4%) were positive by RBPT, STAT and i-ELISA respectively. It is found that i-ELISA test was more sensitive and specific when compared to other two tests viz RBPT and STAT.

Table 1: *Sensitivity and Specificity Analysis between RBPT and I-ELISA*

Test	Result	RBPT		Total
		Positive	Negative	
i-ELISA	Positive	11	13	24
	Negative	1	185	186
	Total	12	198	210

Table 2: *Sensitivity and Specificity Analysis between STAT and I-ELISA*

Test	Result	STAT		Total
		Positive	Negative	
i-ELISA	Positive	7	17	24
	Negative	0	186	186
	Total	7	203	210

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Review Article | Open Access

Economic Analysis of Metabolic Diseases in Bovines: A Review

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Abstract In a dairy farming, among more diseases, metabolic/calving diseases are of great concern to dairy producers worldwide. Dairy cattle metabolic disorders, which are disease related to disturbance of one or more metabolic processes in the organism. This paper presents an overview the evaluation of the economic impact of metabolic diseases in bovines. References will be limited to economics rather than general focus, with emphasis on the most current reviews. The search was performed with the key words being part of the title, descriptors and / or appearing anywhere in the reference in order to find as many papers dealing with metabolic diseases as possible. The important metabolic diseases such as ketosis, milk fever and downer cow complex are discussed under two headings viz., prevalence/incidence rate and quantification of economic loss.
Keywords *Downer Cow Complex, Incidence, Ketosis, Milk Fever, Prevalence*

1. Introduction

The prevalence of animal diseases in the world has been reduced in the last four decades due to its economic importance; there are still some of the livestock diseases that cause reduction in production efficiency leads severe economic losses (Johnchristy and Thirunavukkarasu, 2006; Nagategize and Kaneene, 1985). An assessment of a disease based on economics is not only due to lack of available parameters or data e.g. quantifiable data on weight loss, milk loss, extra labour cost, medicine and treatment charges, calving and fertility problems due to a disease, but it is also due to the setback of validation for such models (Singh and Shiv Prasad, 2008). Disease outbreak among dairy cows constitutes a problem both in terms of financial losses (value of dead cow, decreased production and extra labour) and compromised animal welfare (suffering before death or euthanasia), (Thomsen and Houe, 2006). The economic implications of animal diseases are becoming increasingly important at both farm and national levels, as diseases represent avoidable waste of scare resources, especially cross breeds, as they stand more susceptible to diseases, hardships and contingencies peculiar climate (Thirunavukkarasu *et al*., 2010a).

Metabolic disorders of cattle are a group of diseases that affect dairy cows immediately after parturition. There are several metabolic disorders identified in dairy cows during the first month after parturition. The metabolic diseases such as ketosis, milk fever and downer cow syndrome are the most common expensive disease entities in lactating dairy animals. This disease condition cause severe economic losses in terms of heavy reduction in milk yield and impaired reproductive performance. The estimation of the effects of these diseases on milk production, fertility and survival is of great importance to assess cost–benefits of diagnosis, treatments and prevention efforts. But the quantifying economic losses of metabolic diseases in dairy farming were rarely addressed in developing countries. Quantification of economic losses due to metabolic disease is overall view of the impact of these diseases and can contribute estimating the extent of the losses to be avoided. The comprehensive reviews of major metabolic diseases in bovines were studied and more focus on incidence, prevalence rate and quantification of economic losses. We have reviewed major achievements as we see them in understanding economic losses of lactation ketosis, milk fever and downer cow complex. References will be limited to economics rather than general focus, with emphasis on the most current reviews.

2. Materials and Methods

All studies on metabolic diseases among dairy animals published in peer–reviewed journals were identified using a number of different literatures. No restrictions regarding year of publication were imposed and worldwide estimation. The literature search was based on the keywords such as metabolic disease, ketosis, milk fever and downer cow. The search was performed with the keywords being part of the title, descriptors and / or appearing anywhere in the reference in order to find as many papers dealing with metabolic diseases as possible. This studies including information solely about incidence, prevalence rate and quantification of economic losses in dairy farms. After the initial search of the literature, it was decided to include studies in other countries also; therefore studies from outside India were included. Hereafter, the publications were analysed for information on metabolic diseases as well as associated with economic losses.

3. Results and Discussion

3.1. Ketosis

Ketosis is simply a condition marked by increased levels of circulating ketone bodies without the presence of the clinical signs of ketosis. Ketosis can cause economic losses through decreased milk production and association with pre parturient diseases (Ardvan Nowroozi *et al*., 2011).

A) **Prevalence and Incidence**

(Dohoo and Martin, 1984a) found that the prevalence of ketosis was 12.10 percent in cows and the incidence rates of ketosis have been reported to be between 11.10 and 12.10 percent (Erb and Grohn, 1988; Rasmussen *et al*., 1999; Ostergaard and Grohn, 2000), further they observed that the peak prevalence of hyperketonemia occurred in the third and fourth week of lactation and on an individual herd basis reported that herd prevalence in cows from 0 to 65 days in milk varied from 0 percent to 33.9 percent in a study where the overall prevalence was 12.1 percent ketosis, the number of cows affected 6570 (5.6 percent), 4409 (7.9 percent) and 6769 (12.7 percent) in the parity 1, 2, 3 respectively. Most authors report that the incidence of ketosis increases with age and that the peak incidence may be in cattle in lactations stage from third to six (Shaw 1956; Overby *et al*., 1974; Erb and Martin, 1978; Lindstram *et al*., 1984; Bendixen *et al*., 1987; Grohn *et al*., 1989). It has been reported that overall prevalence of subclinical ketosis ranges from 6.9 percent to 14.1 percent in the first two months of lactation (Andersson and Emanuelson, 1985; Nielen *et al*., 1994; Duffield *et al*., 1997).

However, prevalence as high as 34 percent has been reported (Kauppinen 1983; Duffield *et al*., 1998; Duffield, 2000). The overall prevalence of ketosis was 9.38 percent in cows and 2.92 percent in buffaloes, observed by (Thirunavukkarasu *et al*., 2010b) in Tamil Nadu in the year 2008, further they observed low prevalence of ketosis in cows of Erode and Coimbatore districts of Tamil Nadu could be attributed to the relatively better feeding management in these districts.

(Emery *et al*., 1964) suggested that 50 percent of all lactating cows go through a stage of subclinical ketosis in early lactation. (Herdt *et al*., 1981) found higher milk yields put cows at an increased risk of developing subclinical ketosis. Increased milk production may be associated with increased fat mobilization and a greater risk of hyperketonemia (Lean *et al*., 1992). (Kauppinen, 1984) reported that subclinical ketotic cows had significantly higher annual milk yields than non ketotic cows. In 507 untreated Holstein cows and heifers from 25 dairy herds, the cumulative incidence over the first 9 weeks of lactation was 59 percent and 43 percent (Duffield *et al*., 1998). Ardavan Nowroozi Asl et al., 2011 studied the prevalence of subclinical ketosis in dairy cattle in the South Western Iran and observed 97 percent of the cows experienced at least one episode of subclinical ketosis during the sixth week post partum period and that there was a statistical relation between the prevalence of sub clinical ketosis and milk production.

B) **Economic Loss**

The economic loss of one cow with subclinical ketosis is estimated to be $78 U.S (Geishauser *et al*., 2001). A five percent and the disease costs $145.00, a 100 cow dairy herd would have a cost of clinical ketosis of $725 in a year. Whereas, an average of 100 dairy cows were suffered with subclinical ketosis and the incidence rate of 41 percent with an annual cost of $3198 (Todd Duffield, 2003).

The loss due to ketosis was estimated by (Thirunavukkarasu *et al*., 2010b) and reported that Rs. 577.09 per affected cow, which included the cost of medicines (Rs. 262.99, 45.57 percent), Veterinarian's fee including additional labour cost (Rs. 224.98, 38.99 percent) and expenses on feed supplements (Rs. 89.12, 15.44 percent). However, the loss per affected buffalo was slightly lesser at Rs. 510.80 of which Rs. 240.80 (47.14 percent), Rs. 187.50 (36.71 percent) and Rs. 82.50 (16.15 per cent) were contributed by medicine cost, veterinarian's fee (including additional labour cost) and cost of feed supplements, respectively.

3.2. Milk Fever

It is an afebrile hypocalcaemia disease of cattle usually associated with parturition and initiation of lactation. This disease has been known by a number of terms including parturition paresis, milk fever, parturient apoplexy, eclampsia and paresis peurperalis (Littledike *et al*., 1981). Further, increasing production of milk after calving places an enormous demand for glucose and minerals at a time when feed intake would not have reached its peak, leading to draining of glucose and calcium from the blood and leaving the milch animal's metabolism under severe stress, as transitions to lactation (Bethard and Smith, 1998). Clinical hypocalcaemia before, during or after calving (Bar and Ezra, 2005). Milk fever is a metabolic disease occurring in dairy animals during per parturient period. Thus, milk fever management is economically most important, as it results in not only reduction in milk production, but also loss of animals (Thirunavukkarasu *et al*., 2010a).

A) **Incidence**

The incidence is highest in the Jersey breed (Littledike, 1974). (Horst, 1986) has also reported occurrence of milk fever at the rate of 5 to 10 percent in the USA. (Rajala-Schultz and Grohn, 1999) reported that 23,416 Ayrshire cows affected by milk fever. The percentage of incidence for milk fever

is 0.0 percent (6570), 0.1 percent (4409) and 2.6 percent (6769) for respectively, 1, 2, and 3 lactation. The total milk loss in third lactation stage is 141.6 kg. (Thirunavukkarasu *et al*., 2010a) has revealed that of the total 3774 cows in five milk shed districts of the State of Tamil Nadu, 516 (13.67 percent) were affected by milk fever and of 342 buffaloes 41 (11.99 percent) suffered with milk fever.

B) **Economic Loss**

The economic loss from milk fever in Sweden was estimated in 1969 to be atleast 10 million Swedish crowns annually (Jonsson, 1960). Payne in 1966 listed the national estimate of depreciation due to milk fever in Great Britain at 1, 61,000 pounds annually. Estimates of losses appear to be much too low it losses had been evaluated as previously reported by (Leeh *et al*., 1964). They reported that cows that have had the disease depreciated by an average of 35 pounds in market value and also suffered a market reduction in productive life. In France, 1, 50,000 cases of milk fever were reported in 1959 and a 10 million franc loss was estimated (Lavor *et al*., 1961). (Rajala-Schultz *et al*., 1999) have reported that reduction in the milk yield of cows affected by milk fever ranged from 1.1 kg/day to 2.9 kg/day, depending on parity and the time taken for diagnosis. (Hutjens, 2003) has reported that the average loss due to milk fever per animal was of $334, due to the loss of 1100 lb of milk and 5 days of extra days open. The economic loss was estimated at $334 per occurrence milk losses were estimated to be 142 kg in cows of parity third or more in a study on Israeli Holstein cows that calved between June 2002 and December 2003 (Bar and Ezra, 2005). (Hutjens, 2003) observed that 8 percent of the affected animals died and 12 percent of them were culled.

The average loss per animal due to the treatment of milk fever was higher at Rs. 618 for a cow than for a buffalo, Rs. 488, the average loss being Rs. 608 (Thirunavukkarasu *et al*., 2010). (Guard, 1996) was estimated 334, based on the cost of treating clinical cases and production losses. The average loss due to reduction in milk yield per affected animal was also higher for a cow (Rs. 346) than a buffalo (Rs. 177).

3.3. Downer Cow Complex

(Fen wick, 1969) in Australia defined a downer as any cow with milk fever that did not get up within 10 min of first treatment with intravenous calcium, (Bjorsell *et al*., 1969) from Sweden considered a downer cow to be one that had not risen within 24 hours after first treatment with calcium for Milk fever. Terms used to describe these various syndromes are ‘downers’, ‘alert downers’, ‘a typical milk fever’ or ‘creeper cows’ and ‘fat cow syndrome’ (Morrow, 1976; Morrow *et al*., 1979). Downer cow diseases are complications resulting from milk fever with demonstrable muscle, tendon or nerve injuries (Littledike *et al*., 1981). Other syndromes in this disease complex are not as easy to characterize indeed, various combinations of diseases may be in the same. Downer cow syndrome has no universal definition. We favour the definition used by (Cox *et al*., 1986), a downer cow is one down for at least 24 hours without apparent reason for being down.

A) **Incidence**

In the Ontario case series, 75 out of 82 downer cows had been treated for milk fever, Seventy out of 82 cases occurred from November to April and 82 (67 percent) downer cows died or were slaughtered (Curtis *et al*., 1970). Low cholesterol concentration in serum (Less than 100 mg/100 ml) 8 weeks before calving was associated with a 70 percent incidence of downer cow in a German study (Sommer, 1975). In a feeding trial, 6 of 8 downer cows failed to respond to treatment (Julien, 1977).

Dry cows fed only on 8 percent crude protein ration had a 7 percent metabolic disease incidence and no alert downers (Littledike *et al*., 1981). A mail survey of 723 Minnesota dairy herds with 34,650 cows per year at risk found a rate of 21.4/1000 cow per year at risk. Unpublished data from Cornell

University using data from the study described in path model of reproductive disorders and peroformance, include 28 downer cases in 7767 lactations from 34 herds (4/1000 cow-year). Inspite of the apparent variety of the syndrome, it is important because of the devastating prognosis for the cow Minnesota farmers reported that of cows who were down (and who neither were slaughtered in less than one day nor died in less than four days), 23 percent were slaughtered, 44 percent died and only 33 percent recovered. Cows that were over conditioned during the dry period and fed a ratio of 15 percent crude protein had a 69.4 percent incidence of metabolic diseases and a 31 percent incidence of the alert downer cows. The only evidence regarding milk yield as a risk factor for downer cow syndrome is the reported impression of Minnesota farmers that their downer cows were 48 per cent high producers, 46 percent average producers and only 6 percent low producers (Cox *et al*., 1986). The incidence rate of downer cows among milk fever cows (4 per cent to 35 percent) is at least 10 times higher than the crude rates estimated for the general population of dairy cows. Unpublished data from 7767 lactations in private herds around Cornell University show that 10 out of 28 downer cows (36 percent) also had a clinical milk fever diagnosis.

B) **Economic Loss**

Treatment of cows severely affected with fat cow syndrome is expensive, time consuming and often ineffective. Practical control of these diseases must be affected through management. There is lack of evidence regarding quantification of economic losses due to downer cow syndrome in dairy cattle.

4. Conclusion

In the literature, there are wide variations in the reported prevalence of bovine ketosis. These variations may be attributed to the differences in both the method and the material of the measurement used in determining the prevalence of bovine ketosis. Milk fever is uncommon before the third parturition and incidence is highest at the fifth or sixth parturition. Thus, cows are most likely to develop milk fever during their most productive years. Economic losses due to milk fever occur due to expenditure on treatment of disease affected animals and reduction in quantity of milk. Losses from this disease are difficult to quantitate because of the many indirect costs. Example, some owners cull older, high producing cows from a herd, because of a history of repeated case of milk fever. There is little documentation of the incidence rate of downer cow syndrome.

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Case Report | Open Access

Surgical Management of an Uncommon Cutaneous Myxoma in a Dog

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Abstract A 5 years old male Pomeranian dog was referred with a history of having tumor mass in the right front paw and occasional bleeding from the growth. On clinical examination, there was a firm, round, ulcerated and reddish the mass found to be located in the palmar region of the right front paw. The tumor mass was successfully removed surgically and histopathological examination of the tumor mass confirmed as myxoma.
Keywords *Cutaneous; Myxoma; Paw; Dog; Surgical Correction*

1. Introduction

Myxoma is a rare tumor in domestic animals [1, 2] especially in dogs [3]. Myxomas arise from fibrocytes or other primitive mesenchymal cells that produce an abundant amount of extracellular matrix composed of mucin rather than collagen [4]. Myxoma can occur in a variety of locations including the heart, bones, subcutaneous and aponeurotic tissue, genitourinary tract, skeletal muscle and at any site where the connective tissue exists [5]. This is a record of a rare case of cutaneous myxoma in a dog and its successful surgical management.

2. Case History and Observations

A 5 years old male Pomeranian dog was referred with a history that the owner noticed a tumor like mass in the right front paw 2 months ago and since then the mass was gradually increasing in size. Anamnesis revealed that the animal showed normal feeding habits and there was an occasional bleeding from the growth when the paw hit on some hard objects while walking. On clinical examination, the growth was found to be located in the palmar region of the right front paw. The mass was firm, round, ulcerated and reddish (Figure 1). The area around the growth was soiled with raw blood and there was no similar lesion in other parts of the body. Animal showed little discomfort while walking due to the growth in the palmar region of the affected paw. The rectal temperature, heart rate,

pulse rate and respiratory rate of the dog were within the normal physiological limits. Radiograph of the chest region revealed no metastatic lesions in the lung. Blood and serum biochemical values of the dog were within the normal values. Based on the clinical and radiological examination the case was tentatively diagnosed as benign tumor. Since the animal was healthy with normal appetite, the surgical excision was decided and prepared for aseptic surgical correction.

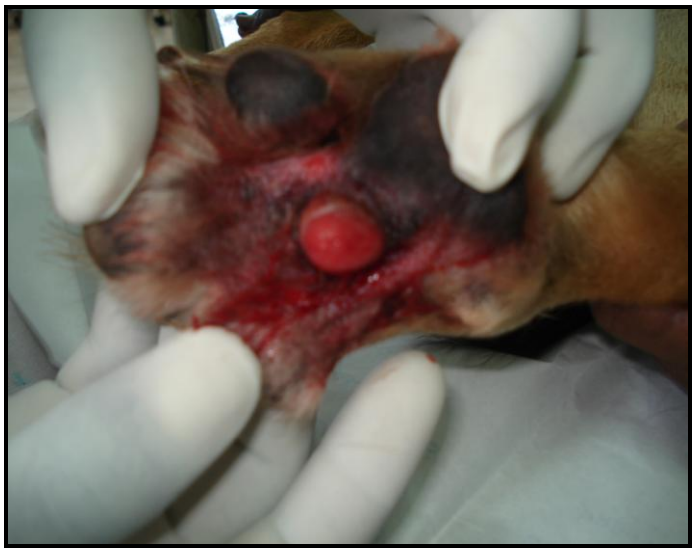

Figure 1: *An Uncommon Cutaneous Myxoma in Palmar Region of the Right Front Paw in a Dog*

3. Treatment and Discussion

The dog was premedicated with atropine sulphate (Inj. Atropine sulphate, BAIF Labs Ltd, Maharashtra, India) @ 0.04 mg/kg body weight s/c and xylazine hydrochloride (Inj. Izine–Intas Pharmaceuticals Ltd., Ahmedabad, India) @ 1 mg/kg body weight i/m and general anaesthesia was induced with ketamine hydrochloride (Inj. Ketmin 50, Themis Medicare Ltd., Mumbai, India) @ 10mg/kg body weight i/m. An elliptical incision was made at the base of the tumor and bleeding vessels were ligated with 2-0 chromic catgut. The involvement of the tumor growth was found to be only with the skin. The growth was carefully excised and the skin was sutured by simple interrupted suture using silk. The tissue sample was preserved in 10% formal saline for histopathological examination. Post operatively the animal was given Ampicillin and Cloxacillin (AC-VET, Intas Pharmaceutical Ltd., Ahmedabad, India) @ 10mg/kg body weight intramuscularly twice daily for 5 days and daily dressing with povidone iodine solution (Pivipol solution, Ar Ex Laboratories Pvt. Ltd., Bombay, India). The sutures were removed on 10th post operative day and the animal recovered uneventfully. There was no recurrence during the follow up period of one year.

Macroscopically the excised mass was 2.5 x 2.5 x 2.0 cm in size and it was circular shaped with a rough surface. The growth was firm, slimy and non encapsulated. Cut surface of the mass was glossy with dark-brown viscous mucin and cystic cavitations. Histologically stellate to fusiform cells distributed in a vacuolated, basophilic, mucinous stroma containing few tiny blood vessels that was partitioned by collagenous connective tissue septae were seen. The cells were scattered appearing singly or in small clusters. The cell nucleus was round, ovoid and elongated with multiple nucleoli (Figure 2). Based on the histopathological examination the case was diagnosed as myxoma.

Myxoma is, as general, a soft tissue tumor [6]. Myxomas are extremely rare neoplasms of the dogs and cats arising from dermal or subcutaneous fibroblasts [7, 8]. Etiologies of these tumors are unknown [5].

Genetics, environmental factors, carsinogenic drugs or miscellaneous toxic substances are reported to be a causative factor for these neoplasms [9]. Myxoma is mostly seen in adult or elderly animals and although there is no sex predilection, it is supposed that Doberman pincher and German shepherd dogs have a racial predisposition [8]. But the present case was a five years old male Pomeranian. Myxomas occur more frequently on the limbs, the back or the groin [10]. In our case, the tumor was on the palmar region of the paw which is a very rare location in respect to literature.

Although, it is reported to be a fibroma by some researchers because it is derived from fibroblasts, cutaneous myxoma is differentiated from fibromas with its extensive intercellular mucine accumulation [11]. In our case, the lesion's content was dark-brown viscous mucin. Dark-brown colour is due to continuous haemorrhage into the tumor which is highly vascularized [12]. Cutaneous myxomas are generally benign in character [1, 8] and may be locally infiltrative or invasive [13]. Treatment of choice for cutaneous myxoma is radical surgical excision and no recurrence is expected after total surgical excision [3]. Cutaneous myxomas are reported to have 20-25% local recurrence rate when excision of the tumor is incomplete [4, 5]. In the present case there was no recurrence even after one year of surgery due to complete surgical excision.

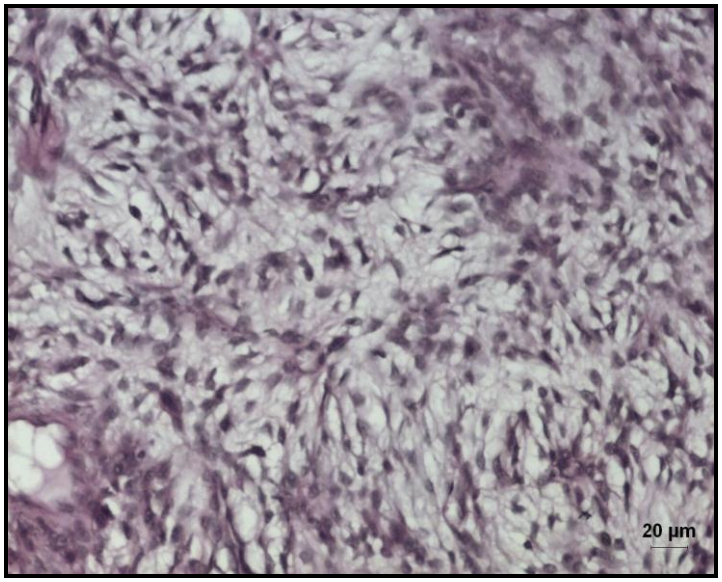

Figure 2: *Histopathological Appearance of Cutaneous Myxoma*

Summary

Myxomas are rare tumours of fibroblastic origin, characterized by a large amount of mucin in the intercellular matrix. These benign neoplasms may occur at any site at which there is connective tissue. This paper describes a cutaneous myxoma in the right front paw of a 5 years old male Pomeranian dog. The tumour was excised surgically and no recurrence was detected during the follow up period of one year.

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Research Article | Open Access

Augmentation of Probiotic Viability in Ice Cream Using Microencapsulation Technique

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Abstract This study was aimed to evaluate the survivability of two proven probiotic strains viz., *Lactobacillus casei (NCDC-298)* and *Bifidobacterium animalis ssp. Lactis* (BB-12*)* in ice cream using microencapsulation technique. Four different types of probiotic ice cream viz., free and encapsulated *Lactobacillus casei (NCDC-298)* and *Bifidobacterium animalis ssp. Lactis* (BB-12*)* were manufactured. Probiotic viability of these strains was monitored during different storage period upto 180 days at -23°C. The viable cell count of *Lactobacillus casei (NCDC-298)* and *Bifidobacterium animalis ssp. Lactis (BB-12)* in the free state in prepared ice cream mixture was $5.3 \pm 0.2 \times 10^9$ cfu/ ml and $4.6 \pm 0.2 \times 10^9$ cfu/ ml at day one and the numbers were decreased to $4.5 \pm 0.2 \times 10^6$ and $2.1 \pm 0.1 \times 10^7$ cfu/ ml in ice-cream after 180 days of storage at -23°C respectively. After the procedure of microencapsulation of *Lactobacillus casei (NCDC-298)* and *Bifidobacterium animalis ssp. Lactis (BB-12)* along with calcium alginate and whey protein concentrate beads, the probiotic survivability raised at the rate of above 30 percent during the same period of storage. The present study revealed that microencapsulation can significantly increase the survival rate of probiotic bacteria in ice cream over an extended period of shelf-life. Further the addition of microencapsulated probiotics in ice cream had no significant effect on the sensory properties.

Keywords *Probiotic Survival; Sodium Alginate; Whey Protein Concentrate*

1. Introduction

Ice cream is a delicious, wholesome, nutritious frozen dairy product, which is widely consumed in different parts of the world and it is very popular among all sections of the people because of the taste delight to nutrient delivery.

Probiotics are live microorganisms, which when administered in adequate amounts confer a health benefit on the host (FAO, 2001). Probiotics have been recently defined as “live microbes which transit the gastro-intestinal tract and in doing so benefit the health of the consumer (Tannock *et al.,* 2000)

Awareness among the consumers on diet related health issues and evidence regarding acquiring health benefits of probiotics have increased the consumer's demand for probiotic foods all over the world. The addition of probiotic micro-organisms to various foods in order to enhance their nutritive value and potential health benefits is currently of great interest.

The genera of *Lactobacillus* and *Bifidobacterium* are most used probiotic micro-organisms which are believed to have beneficial effects on human health (Saxelin *et al.,* 2005). Owing to the perceived health benefits, probiotics have been incorporated into a range of dairy products including ice cream, yoghurt, cheese, milk powder and frozen dairy desserts.

Development of probiotic dairy products is a key research priority for food design and a challenge for both industry and science sectors. Some of the reported nutritional and physiological benefits of probiotic foods are promotion of growth and digestion, setting effect on the gastro intestinal tract, improving bowel movement, suppression of cancer, catering to lactose intolerance and lowering blood cholesterol level etc.

The therapeutic value of any probiotic food normally depends on the viability of these bacteria. International Dairy Federation (IDF) has suggested that a minimum of 10^7 probiotic bacterial cells should be alive at the time of consumption per gram of the product. Some authors have shown that the freezing process affects dramatically the number of live probiotic cells (Hekmat and McMahon, 1992; Kailasapathy and Sultana, 2003). Encapsulation helps to isolate the bacterial cells from the effects of the hostile environment and enhance their viability during processing and also for their targeted delivery in gastrointestinal tract, thus potentially preventing cell loss. Microencapsulation protects probiotic organisms during freezing, freeze drying and also improves the survival of probiotic bacteria in frozen desserts (Kearney *et al*., 1990; Shah and Ravula, 2000).

The objective of this study was to evaluate the survival of microencapsulated and free probiotic culture in ice cream over a period of 180 days storage at $^{-}$23°C by using sodium alginate and whey protein concentrate beads.

2. Materials and Methods

2.1. Activation of Probiotic Cultures

Freeze-dried pure probiotic culture of *Lactobacillus casei (NCDC-298)* and *Bifidobacterium animalis ssp. Lactis (BB-12)* were obtained from NCDC (Karnal) and CHR-Hansen (Horsholm, Denmark) were activated by inoculating in the MRS-broth at 37°C for 24 hour. The probiotic biomass in late-log phase was harvested by centrifugation at 5000X G for 10 min at 4°C and then washed twice in sterile 0.9 per cent saline under the same centrifugation conditions and used in the microencapsulation process.

2.2. Method of Encapsulation of Probiotics

All glass wares and solutions used in the protocols were sterilized at 121°C for 15 min. Alginate beads were produced using a modified encapsulation method (Sultana *et al*., 2000; Krasaekoopt *et al*., 2003; Kebary *et al*., 1998). A probiotic cell suspension was prepared by centrifuging 80 ml of 24 hour old culture at 5000X G for 15 minutes. The cells were washed twice with saline solution (20 ml). The wall materials were sodium alginate (2.0%w/v) + starch (0.5%w/v) and sodium alginate (2.0%w/v) + whey protein concentrate (1.0%w/v) + starch (0.5w/v). To form capsules, a cell suspension was mixed with a 60 ml of wall material solution and the mixture was dripped into a solution containing $CaCl_2$ as the divalent cation. The $CaCl_2$ concentration was at 0.1M and dripping was achieved with a sterile syringe with different size of needles (21G, 26G and insulin syringe). The distance between syringe

and $CaCl_2$ solution was 30 cm. The droplets formed gel spheres instantaneously, entrapping the cells in a three dimensional lattice of ionically cross linked alginate.

2.3. Procedure for Making of Probiotic Ice Cream

Ice cream mix was prepared to contain a final composition of 10 per cent fat, 36 per cent total solids, 15 per cent sugar, 0.5 per cent stabilizer and emulsifier in the ice cream, the mix ingredients were homogenized as described by (Rothwell, 1976; Arbuckle, 1986;) and then heated to 80°C for 30 sec. Mixes were cooled to 5°C and aged for 4 hrs. After ageing the ice cream mix was heat treated to a temperature of 80°C for 30 sec and cooled to 40°C. Two probiotic strains viz., *Lactobacillus casei (NCDC-298)* and *Bifidobacterium animalis ssp. Lactis (BB-12)* in free and encapsulated form were inoculated into ice cream mix at the rate of 4 per cent level and incubated at 40°C until the pH of 5.5 is reached (Hekmat and Mcmahon, 1992). The culture could reach the pH of 5.5 within 4 hours. Then the ice cream mix was freezed at – 4 to – 5°C and stored at – 23°C where the ice cream was hardened.

2.4. Enumeration of Free and Encapsulated Probiotics

Enumeration of probiotic bacteria was achieved as described by (Haynes and Playne, 2002; Lourence, 2002). Probiotic bacterial counts were enumerated before and immediately after freezing as well as at the end of every 30 days until 180 days of storage at ⁻23°C. The samples (10 g) of ice cream mixture prior and after freezing were decimally diluted in 100 ml sterile peptone water (0.1%) and 1 ml aliquot dilutions were poured onto plates of the MRS-agar in triplicate.

All enumerating plates of *L. acidophilus (LA-5)* and *Bifidobacterium animalis ssp. Lactis (BB-12)* were incubated at 37°C for 72 hour under aerobic and anaerobic conditions, respectively. The averages of all results were expressed as colony-forming units per gram of sample (CFU g⁻1). The entrapped bacteria were released from the beads was counted in ice cream as per the procedure described by (Sheu and Marshall, 1993).

2.5. Analysis of Beads

The beads prepared from extrusion method were stored in 0.1M $CaCl_2$ solution and water at 37^0C for one day and observed under light microscope for their size and shape. The size was measured by using stage micrometer, 100 beads were measured for each sample and the average bead size was recorded before and after storage. The calcium alginate beads were stained with safranin and its diameter was measured at 10X. At least 100 randomly selected beads were measured for each sample.

2.6. Analysis of Physico-Chemical Properties

The pH of the ice cream was measured using a digital pH-meter (H1 2211 Ph/ORP Meter, Hanna Instruments). The fat contents of milk and ice cream were determined using the Gerber method. All chemical measurements were done in triplicate. The overrun of the final product was determined using the following formula (Homayouni *et al.,* 2005).

$$\text{Overrun} = \frac{\text{Weight of Unit Mix} - \text{Weight of Equal Volume of Ice Cream}}{\text{Weight of Equal Volume of Ice Cream}}$$

2.7. Analysis of Sensory Properties

Sensory properties of microencapsulated probiotic ice cream samples were organoleptically analysed by 24 panelists using a sensory rating scale of 1–10 for flavor and taste, 1–5 for body and texture and 1–5 for colour and appearance, as described by (Homayouni *et al.*, 2006b).

2.8. Statistical Analysis

The data collected on various parameters were subjected to analysis of variance (ANOVA) procedure. The data were analyzed by approved statistical methods of SPSS (Statistical Package for the Social Sciences).

3. Results

3.1. Chemical and Physical Characteristics

The chemical composition of the cow milk used in the production of probiotic ice cream was: pH 6.57 ± 0.01, titratable acidity 0.22 ± 0.04 and fat 3.90 ± 0.02%. The dry matter and fat content of the ice cream mixture was: 39.31 ± 0.12% and 9.04± 0.03%, respectively. The overrun value was 95 ± 2.0. The respective mean value of fresh extrusion beads in $CaCl_2$ and water were 3.0±0.14, 3.0±0.12mm and 24 hrs stored beads were 2.6±0.10mm, 2.9±0.11mm respectively.

3.2. Survivability of Free and Encapsulated Bacteria in Ice Cream

Survivability of two proven bacteria viz., *Lactobacillus casei (NCDC-298)* and *Bifidobacterium animalis ssp. Lactis (BB-12)* were enumerated at day one and at the end of every 30 days until 180 days of storage. The viable counts were showed in Table 1 and Table 2. Unencapsulated free *Lactobacillus casei (NCDC-298)*, the cell number dropped substantially from $5.3 \pm 0.2 \times 10^9$ to $4.5 \pm 0.2 \times 10^6$ (about 3 log number) from day one to 180 days of storage at −23°C, wherein microencapsulated *Lactobacillus casei (NCDC-298)*, the cell number decreased from $4.8 \pm 0.2 \times 10^9$ to $2.6 \pm 0.3 \times 10^8$ (about a log number). The *Bifidobacterium animalis ssp. Lactis (BB-12)* count showed an average 2 log reduction in free state from $4.6 \pm 0.2 \times 10^9$ to $2.1 \pm 0.1 \times 10^7$ during day one to 180 days, wherein microencapsulated state of the same strains showed a decreased count from $4.5 \pm 0.3 \times 10^9$ to $1.8 \pm 0.6 \times 10^9$ respectively.

Table 1: *Viability of Free and Microencapsulated Lactobacillus Casei (NCDC-298) Strain in Probiotic Ice Cream during Different Storage*

Storage (in days)	Free *Lactobacillus Casei (NCDC-298)* in cfu/ ml	Microencapsulated *Lactobacillus Casei (NCDC-298)* in cfu/ ml
0[b]	$(8.7 \pm 0.1) \times 10^9$	$(5.8 \pm 0.2) \times 10^9$
1	$(5.3 \pm 0.2) \times 10^9$	$(4.8 \pm 0.2) \times 10^9$
30	$(2.4 \pm 0.1) \times 10^9$	$(3.2 \pm 0.4) \times 10^9$
60	$(3.5 \pm 0.1) \times 10^8$	$(6.6 \pm 0.3) \times 10^8$
90	$(2.4 \pm 0.2) \times 10^8$	$(5.4 \pm 0.3) \times 10^8$
120	$(5.6 \pm 0.4) \times 10^7$	$(4.8 \pm 0.1) \times 10^8$
150	$(3.4 \pm 0.2) \times 10^7$	$(3.2 \pm 0.1) \times 10^8$
180	$(4.5 \pm 0.2) \times 10^6$	$(2.6 \pm 0.3) \times 10^8$
S_{30}-value[c] (days)	40.13 ± 0.7	106.06 ± 0.7
S_{180}-value[c] (days)	56.61± 0.8	150.11± 1.8

[a] Mean of three replications ± standard error

[b] Number of alive cells in ice cream mix before freezing

[c] Survival value (S_{30}&S_{180}-value) is the time required to destroy one log cycle of the microorganism after 30 days and 180 days

The probiotic survivability was expressed as the survival value (S-value), this defined as the time required destroying 90% or one log cycle of the organism. The S-values of both free cells and microencapsulated probiotics in ice cream during 180 days storage at −23°C are shown in Table 1 and 2. The S-values of unencapsulated free and microencapsulated *Lactobacillus casei (NCDC-298)* at 30 days were 40.13 ± 0.7 and 106.06 ± 0.7 respectively. Whereas The S-values of unencapsulated free and microencapsulated *Bifidobacterium animalis ssp. Lactis (BB-12)* at 180 days were 61.43 ± 0.6 and 207.42 ± 1.7 respectively.

Table 2: *Viability of Free and Microencapsulated Bifidobacterium Animalis Ssp. Lactis (BB-12) Strain in Probiotic Ice Cream during Different Storage*

Storage (in days)	Free *Bifidobacterium Animalis Ssp. Lactis (BB-12)* in cfu/ ml	Microencapsulated *Bifidobacterium Animalis Ssp. Lactis (BB-12)* in cfu/ ml
0[b]	$(8.6 \pm 0.2)\times10^{9}$	$(6.7 \pm 0.3) \times10^{9}$
1	$(4.6 \pm 0.2) \times 10^{9}$	$(4.5 \pm 0.3) \times10^{9}$
30	$(3.8 \pm 0.1) \times10^{9}$	$(4.3 \pm 0.1) \times10^{9}$
60	$(6.5 \pm 0.4) \times 10^{8}$	$(3.9 \pm 0.3) \times 10^{9}$
90	$(5.7 \pm 0.2) \times 10^{8}$	$(2.6 \pm 0.7) \times10^{9}$
120	$(5.0 \pm 0.1) \times 10^{8}$	$(1.8 \pm 0.5) \times 10^{9}$
150	$(3.6 \pm 0.2) \times 10^{7}$	$(1.9\pm 0.6) \times10^{9}$
180	$(2.1 \pm 0.1) \times 10^{7}$	$(1.8 \pm 0.6) \times10^{9}$
S_{30}-value[c] (days)	82.43 ± 1.4	91.53 ± 0.6
S_{180}-value[c] (days)	61.43± 0.6	207.42± 1.7

[a] Mean of three replications ± standard error

[b] Number of alive cells in ice cream mix before freezing

[c] Survival value (S_{30}&S_{180}-value) is the time required to destroy one log cycle of the microorganism after 30 days and 180 days

3.3. Sensory Analysis

Sensory analysis of probiotic icecream was showed in the Table 3. The overall acceptability in terms of colour, texture and taste of free and microencapsulated *Lactobacillus casei (NCDC-298)* and *Bifidobacterium animalis ssp. Lactis (BB-12)* samples were 17.83 ± 0.09, 18.03 ± 0.11, 18.08 ± 0.09 and 18.03 ± 0.18 respectively.

Table 3: *Sensory Properties of Probiotic Ice Cream*

Ice-cream Samples Contains	Colour and Appearance (1-5)	Flavors and Taste (1-5)	Body and Texture (1-10)	Overall Acceptability
Free *L. acidophilus (LA-5)*	4.21 ± 0.04[a]	4.30 ± 0.03[ab]	9.32± 0.02[a]	17.83 ± 0.09[a]
Microencapsulated *L. acidophilus (LA-5)*	4.40 ± 0.03[ab]	4.40 ± 0.02[a]	9.23 ± 0.06[ab]	18.03 ± 0.11[ab]
Free *Bifidobacterium animalis ssp. Lactis (BB-12)*	4.41 ± 0.03[ab]	4.44 ± 0.02[a]	9.23 ± 0.04[a]	18.08 ± 0.09[a]
Microencapsulated *Bifidobacterium animalis ssp. Lactis (BB-12)*	4.39 ± 0.08[ab]	4.42 ± 0.05[a]	9.22 ± 0.05[a]	18.03 ± 0.18[a]
Without probiotics	4.30 ± 0.03[ab]	4.63 ± 0.02[a]	9.20± 0.02[a]	18.13 ± 0.07[ab]

Means in the same column followed by different superscripts were significantly different ($P < 0.05$).

4. Discussion

4.1. Chemical and Physical Characteristics

There is no significant difference in the bead size of extrusion method with two different wall materials, but increase in size with increasing size of needle was observed in this study, which is similar with the findings of (Ozer *et al*., 2008) he revealed that the bead size ranged from 0.5-1.0 mm diameter when 0.6 mm syringe are used for dripping in extrusion method and bead size 3-4 mm diameter was observed when using 21G needle. (Sheu *et al*., 1993) reported that large beads might cause coarseness of texture in ice milk and ice cream and very small beads did not provide sufficient protection of the probiotic bacteria.

4.2. Survival of Free and Encapsulated Bacteria in Ice Cream

The survivability of *Lactobacillus casei (NCDC-298)* and *Bifidobacterium animalis ssp. Lactis (BB-12)* were recorded between the free and encapsulated states in probiotic ice cream at the end of 180 days frozen storage showed a significant difference ($P < 0.05$). The present results are in accordance with (Shah and Ravula, 2000). Who reported that microencapsulation improved the counts of *Lactobacillus acidophilus* MJLA1 and *Bifidobacterium spp.* BDBB2 compared to free cells in frozen fermented dairy desserts stored for 12 weeks and similarly, in frozen ice milk, 40% more lactobacilli survived when they were entrapped in calcium alginate beads (Sheu and Marshall, 1993).

Comparison of S-value after 30 and 180 days of storage revealed that freezing process had significant ($P < 0.05$) effect on the viability of free cells. Further, microencapsulated cells required longer time to decrease one log cycle in viable counts. Therefore, microencapsulation of probiotic bacteria in beads with diameter between 2-3mm can increase the viability of probiotics.

From this study, the numbers of viable probiotic bacterial cells decreased, when they were added to the ice cream mixture and then frozen in ice cream freezer. Probiotic bacterial cell death was greatest immediately after frozen product exited the freezer and slowed during storage. The major freeze-damage occurred when probiotics were in the ice cream freezer. Further damage to cells inside the ice cream freezer was probably due to formation of ice crystals and by scraping of the cylinder wall by the blades of the ice cream freezer.

Further, it has been found that the resistance to freezing damage was differed between two probiotic strains. The percent average of encapsulated cells found viable after 30 days frozen storage were about 53 and 69 per cent for *Lactobacillus casei (NCDC-298)* and *Bifidobacterium animalis ssp. Lactis (BB-12)* respectively and survival among the free cells were much lower, about 25 and 44 per cent for *Lactobacillus casei (NCDC-298)* and *Bifidobacterium animalis ssp. Lactis (BB-12)* respectively. Microencapsulated cells survived freezing better than free cells ($P < 0.05$) when compared within the same strain. 30 per cent more survival rate was observed when the probiotics were encapsulated in calcium alginate than when they were not encapsulated. Protection by microencapsulation was significant ($P < 0.05$) in the ice cream freezer as well as during frozen storage. These results were in close agreement with findings of (Homayouni *et al*., 2008).

4.3. Sensory Analysis

The scores of sensory analysis of the probiotic ice cream samples for colour, body-texture and taste showed that the addition of free and encapsulated probiotics in ice cream had no effect on sensory properties of probiotic ice cream (Table 3). Overall acceptability in terms of colour, texture and taste of all samples were good and no marked off-flavor was found during the storage period.

5. Conclusions

The study indicates that probiotic survivability in ice-cream can significantly improved by microencapsulation. High fat and solids content of ice cream and other frozen desserts may provide protection to the probiotic bacteria and serve as carrier for delivering the probiotic bacteria into the human gut. In all types of ice-cream the number of viable probiotic bacterial count were between 10^8 and 10^9 cfu/g at the end of three months of storage which is the normal shelf life of ice cream. This viable cell number is higher than that of the International Dairy Federation recommendations (10^7 cfu/g), As the efficient delivery of live cultures represents a major challenge in probiotic product development, the results of the present study demonstrated that the potential of increasing both the technological suitability and expanding the performance of probiotic strains can be done through encapsulation techniques. In addition, dairy foods provide an ideal food delivery system of probiotic bacteria to the human gut to promote growth or support viability of these cultures. It is concluded that the incorporation of encapsulated probiotic strains in dairy products can result in more efficacious and diverse probiotic products in the future, leading ultimately to improved consumer health.

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