EFFECT OF SEASON ON BIOCHEMICAL PROFILE AND OXIDATIVE STRESS MARKERS IN PREOVULATORY FOLLICULAR FLUID IN LOCAL SHEEP OF JAMMU

By

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(J-14-MV-407)

Thesis submitted to Faculty of Postgraduate Studies
in partial fulfilment of the requirements
for the degree of

MASTER OF VETERINARY SCIENCE

IN

VETERINARY PHYSIOLOGY

Division of Veterinary Physiology and Biochemistry
Sher-e-Kashmir University of Agricultural Sciences & Technology of Jammu
R.S. Pura, Jammu-181102
2016

Certificate – I

This is to certify that the thesis entitled **"Effect of season on biochemical profile and oxidative stress markers in preovulatory follicular fluid in local sheep of Jammu"** submitted in partial fulfillment of the requirements for the degree of **Master of Veterinary Science** in subject of **Veterinary Physiology** is a record of bonafide research, carried out by **Miss Uzma Sehrish, Registration No. J-14-MV-407** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma. It is further certified that help and assistance received during the course of thesis investigation have been duly acknowledged.

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ACKNOWLEDGEMENTS

"IN THE NAME OF ALLAH THE MOST GRACIOUS, THE MOST BENIFICIENT AND THE MOST MERCIFUL"

If it comes to formal and traditional word acknowledgement, it will project the volcano of feelings in order to express the deep sense of gratitude to many known and unknown hands who helped me and blessed me with limitless favourable circumstances.

*I don't have words to express my gratitude for my venerated and esteemed major advisor, **Dr. Puspendra Saswat Mahapatra,** Associate Professor and Head, Division of Veterinary Physiology and Biochemistry, for his expert and inspirational guidance, keen interest, critical appreciation in the execution of my work, meticulous supervision, unreserved help rendered a meticulous guidance and constant encouragement during my entire degree programme and critical evaluation of manuscript. I simply feel myself blessed and greatly privileged being provided with an academic advisor like him.*

*I owe special gratitude and feel highly esteemed to thank members of my advisory committee, **Dr. Pratiksha Raghuwanshi,** Assistant Professor (SS), Division of Veterinary Physiology and Biochemistry, **Dr. Sanjay Agarwal,** Assistant Professor (SS), Div. of Veterinary Gynaecology & Obstetrics, **Dr. Utsav Sharma,** Associate Professor and Head, Division of Veterinary Gynaecology & Obstetrics, for their constructive suggestions and unconditional support to complete this valuable project.*

*I am highly thankful to Hon'ble Vice Chancellor of SKUAST-Jammu for allowing me to undertake the study and for providing necessary facilities. I owe my gratitude towards **Dr. T. A. S. Ganai,** Director Education, for being kind enough to provide all the facilities and academic support. I am thankful to **Prof. M. M. S. Zama,** Dean F.V.Sc & A.H and Associate Dean, **Dr. M. S. Bhadwal,** F.V.Sc. & A.H for their courteous & indulgent moral, extraordinary help, timely and technical academic support throughout the course of the study.*

*I express my utmost regards to **Dr. Jonali Devi,** (Assoc. Professor, Div. of Veterinary Physiology and Biochemistry), **Dr. Jafrin Ara Ahmed,** (Asstt. Professor, Div. of Veterinary Physiology and Biochemistry), **Dr. Kawardeep Kour,** (Asstt. Professor, Div. of Veterinary Physiology and Biochemistry) and **Dr. Aditi Lal Koul,** (Asstt. Professor, Div. of Veterinary Physiology and Biochemistry) for their unstinted help, valuable advice and generosity during my entire research work.*

*I take my humble opportunity to express my sincere thanks to **Dr. Rajeev Singh,** Professor and Head, Division of Veterinary medicine (VMD) for providing the necessary facilities and for his kind suggestions.*

*I owe special gratitude and feel highly esteemed to thank **Dr. Tanveer**, Incharge, Municipal Slaughter house, Gujjar Nagar, Jammu for always providing me the necessary help.*

*I shall fail in my duty, if I don't thank to the non teaching staff of the Division of Veterinary Physiology and Biochemistry, **Mrs. Sonika Sawhney, Mr. Fayaz Ahmed Geelani, Mr. Harwinder Singh, Mr. Manzar Masood, Mr. Pushap Kumar and Mohd. Sultan** who were ever ready to help me. I wish to thank the staff members for their cooperation.*

*I am highly thankful to my friends **Dr. Shaweta Dutta, Dr. Rabia Zaman and Dr. Poonam** for being there with me at each and every time and supporting me directly or indirectly.*

*I record with immense pleasure and extend my sincere thanks to **Dr. Shital Dogra, Dr. Samreen Kaur** for her wholehearted support and always being around me with a smile and helping hand during my research period. The constant support, inspiration and help received by my seniors, thanks to **Dr. Rizwana, Dr.Pallavi, Dr. Sumeet, Dr. Juneet,** for helping me during the course of study.*

*With heartiest reverence to my family, I admire the confidence bestowed on me by my parents. My indebtedness to my parents, **Mr. Farooq Iqbaal Qazi** and **Mrs. Shahida Qazi**, whose impalpable antiphlogistic affection, love, moral help and support during every phase of my life actuated me to make this chimeral expectancy* *true, indeed my indebtedness to my parents is beyond expression, as next to Allah, I owe everything of my life to them and without their blessings it would have been an impossible task to complete this study. Where emotions are involved, it is never expressed in words, my brother, **Er. Abu Aamir Qazi** and my sister, **Mrs. Asma Khan** who always stood like a shadow, supporter, inspirers in my life from childhood till now, my warmest love and thanks to them. To give words to the untiring painstaking dedicated help, affection, silent wishes and blessings received from them time to time to bring me to this level is totally impossible for a woman like me. Whatever I am today, I owe it to them. I would also like to express my thanks towards my brother-in-law, Advocate **Asif Rashid Khan** and the little flowers of my family, **Adan Fatima Khan** and **Alyaan Asif Khan**.*

*I am also thankful to my uncle and aunt, **Dr. Muzzaffer Ahmed Beig and Mrs. Shams-un-nisa** and also to my cousins, **Mutayib Rushan Zargar, Tasmiya Tasneem and Mantasha Mirza Beig.***

*All the words in the lexicon will be futile and meaningless, if I fail to divulge my extreme sense of regards to my aunt and uncle, **Mrs. Zarina Zargar** and **Mr. Waseem-ul-Haq Zargar** whose love, encouragement and blessings have always been a bacon light to me in all my undertaking and enabled me to complete my educational endeavours, is beyond expression.*

*Once again, Thanks **Papa and Mummy** for your emotional, inspirational and financial support and for being my best teachers and my backbone since my childhood. Neither verbally nor materialistically can I pay for your unconditional love, affection and for standing by my side at every thick and thin moment.*

Needless to say, all omissions and errors are mine.

5.7.16.

Place: R.S. Pura, Jammu — ***Uzma Sehrish***

Dated: 5.7.2016. — *Regd. No. J-14-MV-407*

ABSTRACT

Title of Thesis	:	**"Effect of season on biochemical profile and oxidative stress markers in preovulatory follicular fluid in local sheep of Jammu"**
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Degree to be awarded	:	Master of Veterinary Science in Veterinary Physiology
Year of award of degree	:	2016
Name of University	:	Sher-e-Kashmir University of Agricultural Sciences & Technology of Jammu (J&K)

ABSTRACT

The investigation was undertaken in ovaries of local sheep of Jammu region to study the effect of season (autumn, winter and summer) on concentration of biochemical metabolites, minerals and oxidative stress markers in follicular fluid (FF) of preovulatory follicles. Sheep ovaries were procured from municipal slaughter house. The diameter of surface follicles was measured and grouped into small (< 6 mm), large (> 6 mm) and pooled (both small & large). From these follicles the follicular fluid was aspirated and after processing, the concentration of biochemical metabolites (glucose, total protein, albumin, globulin, total cholesterol, LDL-cholesterol and nitric oxide), minerals (Fe & Mg), and oxidative stress markers (SOD & TAA) were estimated by spectro-photometric method. But the concentrations of Cu & Zn were estimated using atomic absorption spectrophotometer. It was found that season had significant ($P < 0.05$) effect on concentration of glucose, total protein, total cholesterol, LDL-cholesterol, copper, zinc, SOD activity and TAA in follicular fluid harvested from small and large follicles. In addition to above parameters, season had also significant ($P < 0.05$) effect on nitric oxide content in follicular fluid harvested from pooled follicles, but no significant difference observed in NO content of FF of either large & small follicles during different seasons. It was also observed that studied biochemical profile, mineral content of small & large follicle differed significantly ($P < 0.05$) in all the three seasons. The observed alterations in biochemical composition of FF during different seasons might be controlling the folliculogenesis & oogenesis process in the breed.

Keywords: Season, follicular fluid, biochemical parameters, small follicle, large follicle, local sheep, Jammu

05.07.16

Signature of Major Advisor

5.7.16

Signature of student

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ABBREVIATIONS

%	Percent
<	Less than
>	Greater than
±	Plus or minus
ANOVA	Analysis of Variance
AAS	Atomic Absorption Spectrophotometer
ATP	Adenosine Triphosphate
Abs.	Absorbance
Approx.	Approximately
BOD	Biological Oxygen Demand
Conc.	Concentration
^{O}C	Degree centigrade
CCs	Cumulus cells
Cu	Copper
COC	Cumulus oocyte complex
cGMP	Cyclic Guanosine Monophosphate
DNA	Deoxyribonucleic acid
et al.	And other people
etc.	Et cetera
Fig.	Figure
FF	Follicular Fluid

F.V.Sc & A.H	Faculty of Veterinary Science and Animal Husbandry
Fe	Iron
FRAP	Ferric Reducing Ability of Plasma
GC’s	Granulosa cells
g or gm	gram
g/dl	gram per deciliter
g%	gram percent
HDL	High density lipoprotein
i.e.	That is
IU	International Unit
KL	*kit*-ligand
kDa	Kilo Daltons
L	Litre
LF	Large follicle
LH	Luteinizing hormone
LDL-C	Low Density Lipoprotein Cholesterol
ml	milliliter
mm	millimeter
mM	millimolar
max.	maximum
min.	minimum
/min.	Per Minute
mg/dl	milligram per deciliter

mg/ml	milligram per milliliter
M-II	Metaphase II
Mg	Magnesium
Mn	Manganese
µg/ml	Microgram per milliliter
µmol/l	Micromole per liter
µl	Microliter
No.	Number
nm	nanometer
NO	Nitric oxide
NS	Non Significant
NSS	Normal Saline Solution
NADPH	Nicotinamide Adenine Dinucleotide Hydrogen
NOS	Nitric Oxide Synthase
OCM	Oocyte Collection Media
PCNA	Proliferating Cell Nuclear Antigen
PGs	Primordial Germ Cells
RH	Relative Humidity
rpm	Revolution per minute
RBC	Red Blood Cells
ROS	Reactive Oxygen Species
SE	Standard Error
SOD	Superoxide Dismutase

SF	Small follicle
SBP	Steroid Binding Protein
SBA	Steroid Binding Albumin
Temp.	Temperature
TAA	Total Antioxidant Activity
TAC	Total Antioxidant Capacity
TGF β	Transforming Growth Factor β
UV	Ultra Violet
U/mg	Unit per milligram
U/ml	Unit per milliliter
VLDL	Very Low Density Lipoprotein
µm	Micrometer
Vis	Visible
Zn	Zinc

INTRODUCTION

CHAPTER-I

INTRODUCTION

Sheep and goat are chief livestock species mainly reared for meat and wool/ hair production. Sheep can be reared in areas where no other animal could be raised because of adverse climatic condition and minimal feed availability. Sheep are very efficient in converting feed into meat. Mutton has a great demand in the state of Jammu & Kashmir, but mutton production in the state is not up to mark as various constraints like grazing limitation and production losses due to infertility pose serious threat for population growth of sheep. Sheep production can be enhanced by increasing the efficiency of its reproductive functions through application of upstream reproductive technology.

The female reproduction system is designed to carry out several functions; production of the female egg cells/oocytes and sex hormones that maintain the reproductive cycle. The major function of the female gonad is the differentiation and release of the mature oocyte for fertilization and successful propagation of the species. Ovarian follicles, a spheroid cellular aggregate found in the ovarian cortex are the basic units of female reproduction biology. Each of them contains a single oocyte (immature ovum or egg cell), granulosa cells and theca cells. A cluster of granulosa cells (cumulus oophorus/cumulus cells) surround the oocyte in the follicle. Theca cells (theca interna and theca externa) present outside the basal lamina enclose the follicular cells. During folliculogenesis only one follicle assumes dominance while the other follicles undergo atresia. The major ovarian cell type undergoing this process is the granulosa cell (GC; Hsueh *et al*., 1994). During the maturation process the follicle grows and goes through the primordial, primary, secondary and preantral stage before it acquires an antral cavity. At the antral stage most follicles go through atresia, but a few of them reach the pre-ovulatory phase under the influence of gonadotrophins. Following the Luteinizing hormone surge, one dominant follicle will release a mature oocyte ready for fertilization (Gougeon, 1996; McGee and Hsueh, 2000). The factors those trigger the transformation of the follicle from the primordial to primary stage are yet to be identified. Studies in bovine, baboon and in human indicate that *in vitro* culture of ovarian tissue relieve the inhibition present *in vivo* and transform the primordial follicles into the growing pool (Hovatta *et al*., 1997; Wandji *et al*., 1997; Wandji *et al*., 1996). Once recruited into the growing phase, primordial follicle transforms to a primary follicle and produces proliferating cell nuclear antigen (PCNA) which indicates proliferation of the granulosa cells. The secondary follicle is surrounded by two or more layers of granulosa cells. When there are three to six layers of cells, the theca cells are recruited from the surrounding stroma cells forming theca interna and theca externa with blood vessels in between. At this stage the follicles start to gain a blood supply and can thereby be exposed to any factor circulating in the blood (Bassett, 1943; Reynolds *et al*., 1992). After this, a fluid filled antrum starts to develop. Since then, under the influence of gonadotrophins and growth factors, the follicle grows and the granulosa cells (GC's) differentiate to mural GC's in the periphery of the follicle and cumulus cells (CCs) closest to the oocyte. At the antral stage, the antrum gets filled by a fluid called liquor folliculi. This follicular fluid is a complex mixture of water and solute derived from plasma and metabolites of follicular cells. Its chemical composition is important as it bathes the developing oocytes. Most follicles undergo atretic degeneration, whereas a few of them reach the preovulatory stage under the gonadotropin stimulation after puberty. Follicular development in ewes, from primordial to preovulatory stage, requires about six months (Gougeon, 1986), with 2-3 follicles leaving the pool of primordial follicles to begin growth each day (Cahill and Mauleon, 1980). The Graafian follicles are the major source of the cyclic secretion of ovarian estrogens in animals of reproductive age. In response to preovulatory gonadotropin surges during each reproductive cycle, the dominant Graafian follicle ovulates to release the mature oocyte for fertilization, whereas the remaining theca and granulosa cells undergo transformation to become the corpus luteum. The communication between the oocyte and its surrounding granulosa cells result in maturation of a fertilizable oocyte. Growth and maturation (oogenesis) starts in embryonic life with the formation of primordial germ cells (PGs) in the embryonic epiblast. Under the influence of cytokines like *kit*-ligand (KL) and transforming growth factor β (TGFβ), PGs migrate to the genital ridge to develop into oogonia. Here they expand through mitotic cell division and pass through one last round of DNA replication before they enter meiosis and become oocytes. Coinciding with the initiation of meiosis the oocyte become enclosed in one layer of pregranulosa cells surrounded by a basal membrane to be referred as a primordial follicle (Gosden, 1995). As the follicle grows the oocyte grows and matures from a primordial germ cell to a fertilizable Metaphase-II oocyte. During this time the diameter of the oocyte increases from 35 to 120 µm and the volume increases a 100-fold (Gougeon, 1996; Picton *et al*., 1998). There are two components of oocyte maturation which consist of a long imperceptible cytoplasmic maturation and the short, dramatic and discernible nuclear maturation. Both the processes are crucial for the achievement of a fully mature and competent oocyte.

Ovarian follicular fluid (antral fluid), a secretory product of follicular cells and exudates of serum, has an important role in ovarian physiology including steroidogenesis, growth of follicle, oocyte maturation, ovulation and its transport to the oviduct (Edwards, 1974; Wise, 1987; Gerard *et al*., 2002). Therefore, the composition of follicular fluid is similar, but not identical to that of the plasma. Follicular fluid consists of various nutrients, growth factors, hormones, electrolytes and enzymes that help in folliculogenesis. It protects the oocyte from factors that induce premature resumption of meiosis, guards the oocyte from the proteolytic attack, facilitates its extrusion during ovulation. Biochemical characteristics of follicular fluid influence quality of oocytes and their developmental competence. The ovine ovary is sensitive to the environmental temperature and a seasonal variation existed in ovarian activity (Kaushish, 1994). Ewes start to show breeding activities when days are shortening (short-day breeders). The length of breeding seasons differs among the breeds. In general, the hill temperate breeds had short breeding seasons, whereas lowland temperate breeds have longer seasons (Gordon, 1997). Acute changes in external environment have profound effect on metabolic activity of cell types including follicular cells in an organism. Hence, a changed biochemical (the metabolites, ionic and enzymatic characteristics) composition of the follicular fluid may be expected in different seasons. Although biochemical profiles of follicular fluid in cattle (Wise, 1987), buffalo (Ahmed *et al*., 1997), goat (Mishra *et al*., 2003), and pig (Huang *et al*., 2002) are available, the information on biochemical composition of follicular fluid of ewes (both exotic and Indian breeds) according to changed environment (season) are ill defined.

It is expected that the characteristic of the intra follicular environment in which the preovulatory oocyte grows and matures differs in relation to follicle size, nutritional status, oxidative stress, and changed environment (season). Therefore, to gain more insights related to its reproduction and fertility to improve its proliferation, the present study is undertaken with the following objectives:

1. To measure the concentration of certain metabolites (glucose, total protein, albumin, globulin, total cholesterol, LDL cholesterol and NO), and minerals (Fe, Mg, Cu & Zn) within follicular fluid of local ewes of Jammu region in relation to different seasons.

2. To measure the concentration of oxidative stress marker superoxide dismutase (SOD) and total antioxidant activity (TAA) within follicular fluid of local ewes of Jammu region in relation to different seasons.

REVIEW OF LITERATURE

CHAPTER-II

REVIEW OF LITERATURE

Mammalian reproduction is a highly specialized process. Although the microenvironment of the uterus is very much important for successful fetal development. The process begins with oocyte growth inside the follicle. The follicle is an ovarian structure that not only provides the shelter to oocyte but also influences the quality of oocyte. The differentiation events of the oocyte i.e, the final growth and maturation occur in the days prior and at the time of ovulation. At this time, the follicle is comprised of a fluid-filled chamber (antrum) and several distinct somatic cell types. Each component of the antral follicle contributes to the microenvironment essential for successful oocyte differentiation and subsequent fertilization. Antral follicles are characterized by the presence of an antrum, which is filled with follicular fluid derived both from the bloodstream and from components secreted by somatic cells inside the follicle. It contains a variety of molecules including steroid and protein hormones, anticoagulants, enzymes and electrolytes. This rich fluid bathes the oocyte as it develops and mediates communication between various cell types within the follicle. Two types of granulosa cells, the mural granulosa cells and a specialized cell type called cumulus cells, present in the internal structure of the follicle. The cumulus cells surround the oocyte, forming a cumulus-oocyte complex (COC), while the granulosa cells form the inner lining of the follicle. Each follicle is enveloped by a basal lamina, a specialized sheet of extracellular matrix that separates the internal follicle from the third somatic follicular cell type, the theca cells. Theca cells are a vascularized cell layer that defines the outer boundary of the antral follicle.

In mammals, each estrous or menstrual cycle (primates) produces a single mature oocyte for ovulation and fertilization. However, the oocyte cannot reach this point of development without the support of its follicle. Folliculogenesis is an intricate process by which one follicle per cycle is selected to develop fully and thereby create a mature oocyte. The latter phase of folliculogenesis is termed antral folliculogenesis, a process entirely dependent on gonadotropic hormones. Antral folliculogenesis occurs in waves of growth and regression. Circulating levels of follicle stimulating hormone (FSH) support the growth of a group of antral follicles until the largest follicle begins producing estradiol - an FSH suppressor and transitions to dependence on luteinizing hormone (LH). This follicle is termed the dominant follicle, and this event is called follicle selection. As FSH level decline, the rest of the antral follicles regress in a process called atresia and the dominant follicle attains the capability of reaching ovulation. The chance of ovulation of dominant follicle depends on the frequency and amplitude of LH pulses from the pituitary. These LH pulses increases in intensity as more time elapses from the previous cycle's ovulation and eventually produce a surge sufficient to induce ovulation. However, within a typical estrous or menstrual cycle, the selection of a dominant follicle can happen two or three times before LH surge facilitates ovulation. Dominant follicles that do not encounter the LH surge regress through atresia, allowing FSH levels to rise and support the growth of a new group of antral follicles. Atresia is characterized by cell death within a follicle. The process by which all follicles regress if they do not reach ovulation is the apoptosis. An early atretic follicle have only few dying cells, but, as atresia progresses into its latest stages, the granulosa cell and cumulus cell populations die, followed by resorption of follicular fluid and oocyte death.

The composition of follicular fluid (FF) is distinct from that of serum, that naturally tailored to suit the needs of developing oocytes. Although much remains unknown regarding the complex compositional profile of follicular fluid, whose various contents influence the growth and differentiation of the oocyte. The components of follicular fluid, reactive oxygen species (ROS), antioxidants, hormones, and metabolites have a significant influence on oocyte differentiation. The balance between ROS and anti-oxidants in follicular fluid is critical to the production of a competent oocyte. Maintaining appropriate levels of ROS within the follicular fluid requires the presence of antioxidants: enzymatic and non-enzymatic molecules that neutralize the ROS that they encounter.

The pattern of breeding have been developed through natural selection through several generations resulting in some species that have a series of estrous cycle limited to a portion of the year (seasonally polyestrous). True seasonal breeding is inherent in ewes and does. Most breeds of sheep and goats exhibit seasonal breeding pattern. However, those in the tropics are as an exception who cycle throughout the year. Sheep are short day breeders with their breeding season initiated with decreasing length of daylight and ends when increasing day length reach a ratio of nearly equal daylight and darkness. Ewe-lambs and yearling ewes have shorter breeding seasons than the older ewes. The role of photoperiod in regulating seasonal breeding activity is well established. As breeding season approaches there is an increase in frequency and amplitude of episodic surge of LH. The sensor of photoperiodic response change in mammals is the retina of the eye. The nerve impulse from these photic signals is transmitted from the retina along the retino hypothalamic tract to suprachiasmatic nuclei, located near base of the brain from which arise the sympathetic nerves that innervate the pineal gland. The diurnal rythm of secretory activity of the pineal gland is generated by these suprachiasmatic nuclei. Darkness causes increased sympathetic activity of pineal activity which increases the secretion rate of melatonin whose secretion has been demonstrated only in the seasonal breeders like ewes.

As follicular fluid is in part exudates of serum and is also partially composed of locally produced substances, which are related to the metabolic activity of the follicular cells (Blaszczyk *et al*., 2006). This metabolic activity, together with the barrier properties of the follicular wall, is changing significantly during the growth phase of the follicle (Brantmeier *et al*., 1987). Therefore, a different biochemical composition of the FF in different sized follicles can be expected. Shabankareh *et al*. (2011) reported that the metabolic changes in blood serum might be reflected in the biochemical composition of FF.

In India, sheep is raised primarily for the production of meat. The contribution of sheep to total meat production in the country is around 14 percent. Live sheep are also exported for meat purpose. In Jammu and Kashmir, the non descript breeds of sheep are reared mostly. The importance of baseline information on the effect of season on the serum-biochemical and the haematological values for a meaningful assessment of the health status as well as an intelligent interpretation of arising pathological conditions has been documented in various species like cattle (Schalm *et al*., 1975), in camel (Banerjee *et al*., 1962), in sheep (Grunsell, 1955), in goats (Millson *et al*., 1960), in pigs (Copland, 1976) and lambs (Iyiola-Tunji *et al*., 2015). But there are very few reports which dealt with the seasonal variations of the biochemical parameters and the oxidative stress markers of the non descript breeds of sheep in India. This study was, therefore, an attempt to establish, seasonal comparison of the biochemical parameters, mineral content and the oxidative stress markers in FF of the non descript breeds of sheep in Jammu region. Therefore, for this reason a fundamental understanding of the reproductive pattern of ruminants is essential for their sound reproductive management. Follicular fluid, surrounding the cell layers permit the free diffusion of proteins of up to 500 kDa (Payer, 1975). It is a complex of restricted components of serum and follicular synthesized secretions (Wise, 1987). The growth and development of the follicles is regulated by various biochemical parameters like glucose, total protein, albumin, globulin, total cholesterol and LDL-cholesterol, mineral contents like iron, magnesium, copper, zinc etc. This study will give a better insight into the mechanisms of control of production of these regulatory substances as well as the interactions of these components at their cellular level during folliculogenesis, oocyte maturation and ovulation. One of the factors controlling ovarian physiology in animals is the alteration that occurs in the biochemical composition of follicular fluid. The dynamic constituents of follicular fluid reflected both biochemical and oxidative activity of the follicle, thereby facilitating its role as a conductor of growth and development through the reproductive cycle (Edwards, 1974). The results of such studies can be utilized to develop better strategies for increasing reproductive efficiency in ewes to have a better utilisation of these animals in terms of meat production. The constituents of follicular fluids are considered as a regulating factor in follicular development and steroidegenesis (Gosden *et al*., 1988; Thakur *et al*., 2003). The biochemical composition of FF studied by various workers is reviewed as below:

2.1 Biochemical parameters of follicular fluid

2.1.1 Follicular fluid glucose concentration

Glucose plays an important role in ovarian metabolism since it is the major energy source for the ovary, possibly metabolized by the ovary through the anaerobic pathways, leading to the lactate formation in FF as a result of glycolysis taking place in granulosa cells and influx of same molecules from the plasma into the follicular fluid. The principal source of FF glucose is blood and very little glucose, is synthesized locally by the granulosa cells of follicles. Glucose level in FF can be used as an important marker in the diagnosis and management of various reproductive disorders.

Tripathi *et al.* (2015) reported that, as the follicles became larger, the concentration of glucose increased in FF of ruminants.

Deka *et al*. (2014) studied the glucose concentrations in small (2-4 mm), medium (4-6 mm) and large (6-9 mm) follicles of cows and observed the level of glucose in follicular fluid of large follicles significantly ($P < 0.01$) higher than follicular fluid of small and medium size follicles.

Rufai *et al.* (2013) reported the glucose level was significantly higher ($P < 0.05$) in the follicular fluid of cyclic sheep than that of acyclic sheep.

Jassim *et al.* (2012) reported the glucose concentration in small follicles (4-8 mm) was significantly higher ($P < 0.05$) than the large follicles (> 8 mm) in buffalo follicular fluid.

El-Nasser and Mohammed (2011) reported as the follicles became larger (> 8 mm), the concentration of glucose was significantly increased ($P < 0.05$) in ruminants (cattle, buffalo, sheep and goat) follicular fluid.

Tabatabaei and Mamoei (2011) reported that the glucose concentration in small follicles was significantly lower than that measured in medium and large sized follicles of buffalo.

Nandi *et al.* (2007) determined the concentration of glucose (1.42 ± 0.20 mM) in ovine follicular fluid and observed that the glucose concentration in follicular fluid significantly increased ($P < 0.05$) according to increase in the size of the follicles.

Rahman *et al.* (2005) reported the glucose concentration of small and large follicles in buffalo follicular fluid. He observed the concentration of glucose in small follicles as (30.75 ± 5.50 mg/dl) whereas in large follicles it was observed to be (42.95 ± 4.54 mg/dl).

Leroy *et al.* (2004) reported an increase in glucose concentration in the follicular fluid of cattle according to the size of follicles.

Thakur *et al.* (2003) reported that the glucose concentration in large follicles of goats was non-significantly higher as compared to small ones (26.31 ± 2.15 mg/dl vs. 19.44 ± 2.18 mg/dl).

No difference in the glucose concentration of follicular fluid of pig was observed among various sized follicles by Huang *et al*. (2002). The average glucose concentrations were 86, 86 and 83 mg/dl in follicular fluid of large, medium and small follicles, respectively.

Ahmed *et al*. (1997) and Jindal *et al*. (1997) reported that the follicular fluid of buffalo contained lower concentration of glucose as compared with the blood plasma.

Boryczko *et al.* (1995) reported the glucose level in cystic follicles was lower than that of preovulatory follicles in the follicular fluid of cattle.

Biochemical analysis revealed that the glucose concentration was ~75% in the total carbohydrate content of bovine follicular fluid with fructose being present as only a minor fraction (Lutwak-Mann, 1954).

2.1.2 Follicular fluid total protein concentration

Follicular fluid contains several proteins that derive from blood plasma or are secreted from granulosa or theca cells. The concentration of proteins in FF can be used as a marker to access the quality of folliclular development in order to optimize oocyte selection process. Proteins in the living cells are intimately associated with various phases of activity that constitute the life of the cells. A number of proteins reside in follicular fluid and surrounding the granulosa cells. Developing follicles need amino acids for their activity which are provided to them through the protein content present in the FF.

Tripathi *et al*. (2015) reported that, as the follicles became larger, the concentration of total protein significantly decreased in ruminants.

Deka *et al*. (2014) measured the mean total protein concentration in follicular fluid of small (2-4 mm), medium (4-6 mm) and large (6-9 mm) follicles of cows and found it as (59.94 ± 1.45), (57.10 ± 3.52) and (53.76 ± 2.99) mg/ml respectively. He also observed a non-significant decreasing trend of total protein concentration with increase in size of the follicles.

Rufai *et al.* (2013) reported a non-significant difference of total protein between the cyclic and non-cyclic ovine follicular fluid.

Jassim *et al.* (2012) reported the concentration of total protein in large follicles (> 8 mm) was significantly higher (P < 0.05) than the small follicles (4-8 mm) in buffalo FF.

El-Nasser and Mohammed (2011) reported as the follicles became larger (> 8 mm), the concentration of total protein decreased (P < 0.05) in ruminants (cattle, buffalo, sheep and goat) follicular fluid.

Nandi *et al.* (2007) determined the concentration of total protein (6.17 ± 0.03 g/dl) in ovine follicular fluid and observed that the concentration of total protein in follicular fluid significantly decreases (P < 0.05) according to the increase in the size of follicles.

Rahman *et al.* (2005) reported the total protein concentration of buffalo follicular fluid of small and large follicles. He observed the concentration of total protein in small follicles as (6.30 ± 0.32 g/dl), whereas in large follicles it was observed to be (6.41 ± 0.30 g/dl).

Leroy *et al.* (2004) found no significant change in the total protein concentration of small, medium and large sized follicles in cattle.

Thangavel and Nayeem (2004) reported a declining trend of protein concentration from small to large follicles (small: 6.5 ± 0.19, medium: 6.48 ± 0.22, large: 6.28 ± 0.23 g/dl) in buffalo follicular fluid.

The protein concentration in pig was found to decrease as the follicle size decreases. The average protein concentration was (73.97 ± 9.47), (56.82 ± 7.19) and (59.87 ± 6.86) mg/dl in large, medium and small follicles, respectively (Huang *et al.*, 2002).

Singh *et al*. (1999) found an increase in protein concentration as follicular diameter increased in ewe.

Mohan *et al.* (1997) reported in buffalo follicular fluid that the total protein concentration in the small, medium and large follicle of the left ovaries were 6.9, 6.25 and 6.6 g% respectively while the values for the right ovaries were 7.2, 7.0 and 7.0 g% respectively.

Balakrishna *et al.* (1994) observed the total protein concentration in sheep follicular fluid was decreasing according to an increase in size of the follicles (6.86, 6.05 and 5.71 g/dl, in small, medium and large follicles respectively).

Parmar and Mehta (1991) did not found any difference in the total protein concentration in follicular fluid of various sized follicles in ruminants.

Wise (1987) reported a decrease in total protein concentration as follicular diameter increased in cattle.

No difference in the total protein concentration in follicular fluid was observed among various sized follicles in goat, buffalo and cattle by Sidhu *et al*. (1985).

2.1.3 Follicular fluid albumin concentration

Albumin is a major plasma protein which is synthesised in the liver from amino acids which are absorbed from the ileum. Its function includes regulation and distribution of extracellular fluid, transportation of various hormones, vitamins and trace elements.

Ovary is one of the most active tissue in catabolizing albumin. There is an active inward transport of albumin from blood into follicles which may be required for binding some chemicals as well as minerals inside the follicular fluid for various physiological functions including growth and maturation of the follicles.

Rufai *et al.* (2013) reported a non-significant difference of albumin concentration between the cyclic and non-cyclic ovine follicular fluid.

Rahman *et al.* (2005) reported the albumin concentration of buffalo follicular fluid of small and large follicles. He observed the concentration of albumin in small follicles as 3.94 ± 0.26 g/dl, whereas, in large follicles it was observed to be 4.10 ± 0.14 g/dl.

2.1.4 Follicular fluid globulin concentration

Globulin has a significant importance in the body due to its immunity producing activity. The globulin present in FF, though in small quantity, might be necessary for protecting the follicle from external environments.

Rufai *et al.* (2013) reported a non-significant difference of globulin concentration between the cyclic and non-cyclic ovine follicular fluid.

Rahman *et al.* (2005) reported the globulin concentration of small and large follicles in buffalo follicular fluid. He observed the concentration of globulin in small follicles as 2.82 ± 0.31 g/dl, whereas, in large follicles it was observed to be 2.43 ± 0.24 g/dl.

2.1.5 Follicular fluid total cholesterol concentration

Cholesterol, is an essential part of cells in the body and is used to make certain hormones and digest fats. Cholesterol is the precursor for all the steroid hormones synthesised in all the tissues including ovaries. 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.

Hozyen *et al*. (2016) reported the follicular fluid cholesterol concentration decreased significantly ($P < 0.01$) with increase in the follicle size. Also, cholesterol overall means were significantly ($P < 0.01$) lower during summer and autumn than winter and spring.

Tripathi *et al*. (2015) reported the trend which shows as the follicles became larger, the concentration of cholesterol increased in the ruminant follicular fluid.

Deka *et al*. (2014) measured the mean concentrations of cholesterol in follicular fluid of small (2-4 mm), medium (4-6 mm) and large (6-9 mm) follicles of cows as (2.57 ± 0.41 mg/ml), (2.73 ± 0.46 mg/ml) and (2.36 ± 0.62 mg/ml) respectively with no significant difference in cholesterol levels of different categories of follicles.

Rufai *et al.* (2013) reported a non-significant difference of cholesterol level between the cyclic and non-cyclic ovine follicular fluid.

Jassim *et al.* (2012) reported the cholesterol concentration in small follicles (4-8 mm) was significantly higher ($P < 0.05$) than the large follicles (> 8 mm) in buffalo follicular fluid.

El-Nasser and Mohammed (2011) reported as the follicles became larger (> 8 mm), the concentration of cholesterol decreased ($P < 0.05$) in ruminants (cattle, buffalo, sheep and goat) follicular fluid.

Nandi *et al.* (2007) determined the concentration of cholesterol as 1.96 ± 0.29 mM in ovine follicular fluid and observed that the concentration of cholesterol in follicular fluid significantly increases ($P < 0.05$) according to increase in the size of the follicles.

Rahman *et al.* (2005) reported the cholesterol concentration of small and large follicles in buffalo follicular fluid. He observed that the concentration of cholesterol in small follicles as (102.12 ± 4.11 mg/dl) whereas, in large follicles it was observed to be (108.19 ± 5.25 mg/dl).

Leroy *et al.* (2004) reported an increase in the total cholesterol concentration from small (55.9 ± 3.39 mg/dl) to medium (62.7 ± 2.91 mg/dl) and large (63.7 ± 3.23 mg/dl) follicles in cattle.

Thangavel and Nayeem (2004) reported in buffalo follicular fluid that the cholesterol concentration to be significantly higher in medium sized follicles (80.77 ± 3.96 mg%) than in small sized follicles (74.93 ± 3.53 mg%) and large follicles (74.90 ± 3.39 mg%).

It has been reported that the mean concentration of cholesterol in the follicular fluid of goats was significantly higher in the large follicles as compared with the small follicles (Mishra *et al.*, 2003).

Thakur *et al*. (2003) observed the mean concentration of cholesterol in the follicular fluid of goat was significantly higher in the large follicles as compared with the small follicles (61.50 ± 1.03 vs. 54.40 ± 1.38 mg%).

Huang *et al*. (2002) observed no difference in the cholesterol concentration in follicular fluid of pig was observed among various sized follicles. The average cholesterol concentrations were (153, 161 and 157 mg/dl) in large, medium and small sized follicles respectively.

2.1.6 Follicular fluid LDL-cholesterol concentration

Low Density Lipoproteins (LDLs) are synthesized in the liver by the action of vari- ous lipolytic enzymes on triglyceride-rich Very Low Density Lipoproteins (VLDLs). Specific LDL receptors exist to facilitate the elimination of LDL from plasma by liver parenchymal cells. Granulosa cells *in vitro* are able to utilise LDLs for the production of cholesterol.

Sesh and Meur (2013) ascertained the transfer process of LDL-C from blood into various compartments (Follicular wall, Granulosa cells, Follicular fluid) of the buffalo ovarian follicles across the blood follicle barrier. The follicular fluid from non-atretic and early atretic follicles of pre-ovulatory and ovulatory stages of buffalo ovaries showed that the concentration of the LDL-C decreased progressively which indicated that the origin of LDL-C is from serum only. Whereas, from the non-atretic and atretic status of the follicles, the concentration of LDL-C 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.

Rahman *et al*. (2008) investigated ovarian follicular fluid biochemical profile in female dromedary camel. The concentration of low-density lipoproteins in small follicles (2-6 mm) and large follicles (7-20 mm) did not differ.

De Placido *et al*. (2005) reported that the cholesterol, present in FF, is bound to the high-density lipoprotein fraction (HDL) because the only other cholesterol-containing lipoprotein fraction, the low-density lipoprotein fraction (LDL), is too large to pass the blood–follicle barrier.

2.1.7 Follicular fluid nitric oxide (NO) concentration

"A molecule of the millennium" nitric oxide (NO) has now been found to be an important mediator involved in regulating the reproductive and immune functions in mammals. Nitric oxide is an inorganic, short lived (a few seconds) free radical gas and a versatile signalling molecule regulating a variety of cellular processes. *In vitro,* it is a stable, colourless gas, moderately soluble in water.

The free radical is synthesized by oxidation of L-arginine to NO plus L-citrulline by one of the three isoenzymes of nitric oxide synthase (NOS). The NO in an oxygen-containing aqueous solution which has a short half- life that is attributed to rapid oxidation to nitrite (NO^{2-}) and nitrate (NO^{3-}) and requires the presence of oxyhemoprotein (Ignarro *et al.*, 1993).

Khan and Das (2011) reported the FF NO concentration in buffalo. Small follicles had a higher ($P < 0.05$) NO concentration as compared to the medium and large follicles.

Orsi (2006) reported effect of apoptosis in bovine embryos when exposed to high concentration of NO.

Low concentrations of NO in follicular fluid were associated with follicles containing mature oocytes that eventually became fertilized (Barrionuevo *et al*., 2000).

The role of NO synthase has been determined by NO synthase expression or by plasma nitrate concentrations. Studies suggest that concentrations of follicular NO increase in the secretory phase and peak at mid-cycle (Lee *et al*., 2000).

Nitric oxide in high concentrations binds to enzyme heme groups in the respiratory chain as well as cytochrome oxidase and inhibits mitochondrial activity causing cell death by hypoxia (Clementi *et al*., 1999; Sarti *et al*., 1999).

Li *et al.* (1999) demonstrated NO causes apoptosis in high concentration in human epithelial endometrial cells.

In cows, other non-steroidal, intra-ovarian factors are synthesized by granulosa cells (GCs) which are involved in ovarian physiology, among these, a potent factor is nitric oxide (NO) (Basini *et al.*, 1998).

Nitric oxide (NO) is a local factor involved in the autocrine and paracrine modulation of ovarian folliculogenesis and steroidogenesis. At low concentrations (< 1 µmol/L), the transduction effect of NO is mediated by activation of soluble guanyl cyclase and mediated through cyclic guanosine monophosphate (cGMP) (Rosselli *et al.*, 1998; Hanafy *et al.*, 2001).

The majority of studies regarding the role of NO in follicular development have been realized in the mouse, rat, and human (Jablonka-Shariff and Olson, 1997).

It has been demonstrated that NO in high concentrations induces apoptosis and/or necrosis in rat ovaries (Ellman *et al.*, 1993).

Bush *et al.* (1992) reported that NO is synthesized from L-arginine via an oxygen- and NADPH- dependent reaction that yields nitric oxide and L-citrulline.

2.2 Follicular fluid minerals

2.2.1 Follicular fluid iron concentration

Approximately 60% of body iron is present as haemoglobin of erythrocytes. The major function of iron in the body is transportation of oxygen to the cells and cellular oxidation. Iron is absorbed in the small intestine and bound to a globulin in the plasma called transferrin. It is then transported to bone marrow where RBC generation takes place. Iron deficiency results in normochromatic anaemia which affects the response of ovarian receptors to the estrogen. Its increased or decreased levels in the FF can be linked to its role in folliculogenesis and some diseased conditions in the body of the animal.

Chakraborty *et al*. (2012) reported that with increase in the follicular size, iron concentration increased significantly ($P < 0.05$) in the follicular fluid of pigs.

Nandi *et al.* (2012) reported that there is significantly higher ($P < 0.01$) concentrations of iron in the small follicles than the large follicles of ewes.

Bordoloi *et al.* (2001) reported higher concentration of iron in small follicles of caprine follicular fluid.

Sharma and Vats (1998) reported an increase in the concentration of iron in normal developing antral follicles of goat.

Sangha *et al*. (1993) reported iron concentration in follicular fluid of house rat was significantly higher ($P < 0.01$) in large follicles than small and medium follicles.

2.2.2 Follicular fluid magnesium concentration

Magnesium is an essential intracellular cation necessary for a great number of enzymatic and metabolic processes. It is a co-factor of all the enzymatic reactions that involve ATP. Though, much role of magnesium has not been established in FF still some of the clinical conditions can be linked to its increased or decreased level.

The higher concentrations of Mg in the small follicles could help the mitosis of the follicular cells through the formation of thrombin, a potent mitogen. Magnesium could substitute for calcium in thrombin formation under low Ca:Mg ratio conditions, that exist in small follicles. As magnesium is antagonistic to calcium, the decreased magnesium with follicular development facilitated the calcium action in large follicles. Magnesium acts as a cofactor in enzymatic reaction, and induces meiotic maturation of xenopus oocyte (Belle et al., 1986).

Tripathi *et al*. (2015) reported the trend which shows as the follicles became larger, the concentration of magnesium significantly decreased.

The levels of magnesium did not differ significantly between small and large follicles (Nandi *et al.*, 2012).

Magnesium was higher in the large follicles of goat as compared to the small ones; however, the differences were not significant (3.05 ± 0.26 vs. 2.50 ± 0.22 mEq/L) (Thakur *et al*., 2003).

The concentration of magnesium increased significantly in small follicles of goat as compared to the large ones (Bordoloi *et al*., 2001).

Concentration of magnesium was significantly lower in large follicles (10.4 ± 2.5 µg/ml) than in small (25 ± 5.8 µg/ml) and medium (26.9 ± 4.9 µg/ml) follicles of buffalo respectively (Kaur *et al*., 1997).

The magnesium concentration in follicular fluid of sheep was found to be (0.89 mmol/ml) (Gosden *et al*., 1988).

Wise (1987) reported that the concentration of magnesium increases significantly in small follicles as that of large follicles in cattle.

2.2.3 Follicular fluid copper concentration

Copper deficiency affects the physiological functions in general and reproduction in particular. Copper plays an important role in haemoglobin formation and iron transport. Reduced conception rates and disrupted oestrus activity are found in animals suffering from primary copper deficiency.

Chakraborty *et al*. (2012) reported that with increase in follicular size, the copper concentration increased significantly in the follicular fluid of pigs.

The levels of Copper did not differ significantly between small and large follicles (Nandi *et al.*, 2012).

Sharma and Vats (1998) did not reveal any specific trend but maximum concentration for copper was found in large antral follicles of goat.

Increased Copper concentration in large follicles could be correlated with the increased estrogen levels that were related to increases in ceruloplasmin synthesis (Sharma and Sharma, 1997).

2.2.4 Follicular fluid zinc concentration

Zinc is a micronutrient which serves as a cofactor for more than 80 metalloenzymes involved in DNA transcription and protein synthesis. As DNA transcription is a major part of germ cell development, zinc is likely to be important for reproduction. Zinc ions are involved in the process of cell division, development and differentiation and in the control of gene expression. Zinc also has anti-apoptotic and anti oxidant properties. The role of zinc in reproduction is well documented as it is essential for proper sexual maturity, reproductive capacity, onset of oestrus and has a critical role in the repair and maintenance of the uterine lining following parturition of the animal. But the exact role of Zn in FF is still to be discovered (Leonhard, 2000).

Nandi *et al.* (2012) reported significantly higher concentrations of zinc in small follicles than large follicles of ewes.

Arshad *et al*. (2005) reported zinc contents in the fluid from small and large follicles differed non significantly in buffalo.

Bordoloi *et al.* (2001) found that in caprine follicular fluid the concentration of zinc is higher in smaller follicles.

2.3 Follicular fluid oxidative stress markers

The term antioxidant, also called as antioxygen, in the 19th and early 20th century, has been referred specifically to a chemical that prevented the consumption of molecular oxygen. Antioxidants were then subjected to extensive research as they protect cells against the damaging effects of reactive oxygen species (ROS), such as singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals and peroxynitrite. An imbalance between antioxidants and ROS results in an oxidative stress (OS), leading to cellular damage. OS has been linked to various pathological conditions. There are two types of antioxidants: enzymatic and non- enzymatic. Enzymatic antioxidants are also known as natural antioxidants, which act by neutralizing the excessive ROS, and prevent it from damaging the cellular structure. One such example of it is superoxide dismutase, which is a natural antioxidant present in organisms which eliminate the ROS and rapidly scavenge the attacking radical and thereby terminate its destructive pathways. Accumulation of superoxide radicals and a decline in superoxide dismutase (SOD) levels are involved in apoptotic cell death, whereas antioxidants including SOD can inhibit apoptosis.

Elimination of reactive oxygen species is catalysed by certain enzymes such as superoxide dismutase (SOD), catalase and peroxidase. Antioxidants (including vitamins C and E) and antioxidant cofactors (such as selenium, zinc, and copper) are capable to dispose, scavenge, or suppress ROS formation. “Oxidative stress” rises when due to some reasons the steady state ROS concentration is increased, leading to oxidative modification of cellular constituents, resulting disturbance of cellular metabolism and regulatory pathways (Lushchak, 2011).

The concentration of SOD tended to be higher in large follicles obtained at the follicular phase than that at the luteal phase (56.7 ± 3.7 vs 28.1 ± 6.7 U/ml respectively). Significantly higher concentration ($P < 0.05$) of SOD was recorded in small follicles (≤ 3 mm) as compared to the medium (4-9 mm) and large follicles ($\geq$ 10 mm) collected at the luteal phase (El-Shahat and Kandil, 2012).

Combelles *et al*. (2010) reported the levels of superoxide dismutase (SOD), an enzymatic antioxidant in bovine follicular fluid decrease with increasing follicle size.

Cu–Zn-SOD and Mn-SOD are well expressed in the oocyte metaphase II stage, that suggests that these enzymes are markers for oocyte maturation (El Mouatassim *et al.,* 1999).

Levels of SOD activity were higher in follicular fluid from oocytes that failed to fertilize than in those that did (Sabatini *et al.,* 1999).

The expression of Cu-Zn-SOD and Mn-SOD is closely related to steroidogenesis in the human ovary (Suzuki *et al.,* 1999).

Total antioxidant capacity (TAC) was reported to be highest in the follicular fluid of larger follicles (Gupta *et al*., 2011).

Lower levels of total antioxidant capacity (TAC) in the follicular fluid are predictive of decreased fertilization potential (Oyawoye *et al*., 2003).

MATERIALS AND METHODS

CHAPTER-III

MATERIALS AND METHODS

In order to study the effect of season on biochemical profile and oxidative stress markers in preovulatory follicular fluid in local sheep of Jammu, the experiment was conducted from September, 2015 to May, 2016 and the observations were recorded during specific months starting from September-October (Autumn: control), December-January (Winter) and April-May (Summer). The experiment was carried out at the Division of Veterinary Physiology and Biochemistry, Faculty of Veterinary Sciences and Animal Husbandry, SKUAST-Jammu, R. S. Pura.

3.1 Working environment

All the procedures were carried out in sterile conditions to avoid any bacterial or fungal contamination. The stocks of media were stored at 4^0C and were used within 15 days of preparation. Prepared oocyte collection media (OCM) was kept at 37^0C prior to work.

3.2 Collection and processing of ovaries

Ovaries of the local breed of sheep were collected from the municipal slaughter house and transported to laboratory in icebox within one hour of slaughter. In laboratory, tissues attached to the ovaries were trimmed out with scissor and then washed with normal saline solution (NSS) fortified with gentamicin (50 mg/ml) at 37^0C. The cleaned ovaries were preserved in NSS within a BOD incubator at 37^0C till collection of follicular fluid.

3.3 Follicular fluid collection and processing

All visible non-atretic follicles on the surface of ovaries were categorized according to their size as small (< 6 mm) and large (> 6 mm) (Evans *et al.*, 2000) using a slide calliper. Follicular fluid along with oocyte was aspirated from all visible non-atretic follicles by a 22 gauge needle attached to 5 ml syringe lubricated with OCM. The cumulus oocyte complex (COC) along with follicular fluid (FF) was pooled into 15 ml sterile plastic tube according to categorized follicles from which FF was collected and allowed to settle for 10 minutes in BOD incubator at 37^0C. The

Photograph showing sheep ovary with small follicles on its surface

Photograph showing a large follicle on surface of a sheep ovary

supernatant was collected gently into another 15 ml centrifuge tube. To remove the oocyte and other fine cellular debris present in the collected supernatant, follicular fluid was centrifuged at 3000 rpm for 30 minutes. Then the supernatant was collected and filtered through 0.22 µm syringe filter. Filtered follicular fluid was aliquoted and stored in sterile 2 ml eppendorf tubes at -20^{0}C for analysis of metabolites and minerals. Immediately after collection of processed follicular fluid, glucose concentration, superoxide dismutase (SOD) and total antioxidant activity (TAA) of FF was estimated as per standard methods using Double Beam uv/visible Spectrophotometer (UV5704SS, Electronic Corporation of India Ltd.).

3.4 Follicular fluid biochemical parameters

3.4.1 Total glucose concentration in follicular fluid

Follicular fluid glucose concentration was estimated by GOD-POD end point colorimetric method (Trinder, 1969) using the diagnostic kit of Transasia Bio-medicals Ltd.

The assay procedure for estimating glucose content was as follows:

	Blank	**Standard**	**Test**
Working Reagent	1000 µl	1000 µl	1000 µl
Distilled water	10 µl	-	-
Standard (100 mg/dl)	-	10 µl	-
Follicular fluid	-	-	10 µl

Contents of tubes were mixed well and incubated for 15 minutes at 37^{0}C. The absorbance of standard and each test tube against reagent blank at 505 nm was recorded using uv/visible spectrophotometer. The glucose content of follicular fluid was calculated using the formula

$$\text{Glucose (mg/dl)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \text{ x Concentration of Standard (100 mg/dl)}$$

3.4.2 Total protein concentration in follicular fluid

Total protein content of follicular fluid was estimated by using the diagnostic kit of Transasia Bio-medicals Ltd., following the Biuret method described by Tietz, (1986) as referenced in manufacturer's catalogue. The procedure in tabular form is as below:

	Blank	Standard	Test
Working Reagent	1000 µl	1000 µl	1000 µl
Distilled water	20 µl	_	_
Standard (6.0 g/dl)	_	20 µl	_
Follicular fluid	_	_	20 µl

Contents of the tubes were mixed well by vortexing and incubated for 10 min. at 37^0C. Absorbance of the standard and each test was noted down at 546 nm against reagent blank using uv/visible spectrophotometer. Total protein content of follicular fluid was estimated using following formula

$$\text{Total Protein (g/dl)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \text{ x Concentration of Standard (6.0 mg/dl)}$$

3.4.3 Albumin concentration in follicular fluid

Albumin concentration of follicular fluid was estimated by Bromocresol green method as described by Doumas *et al.* (1972) using the diagnostic kit of Transasia Bio-medicals Ltd. The procedure followed as per the product catalogue, briefly as follows in tabular manner

	Blank	**Standard**	**Test**
Albumin Reagent	1000 µl	1000 µl	1000 µl
Distilled water	10 µl	-	-
Standard (3.6 g/dl)	-	10 µl	-
Follicular fluid	-	-	10 µl

Contents of each tube were mixed well. The absorbance of standard and each test were recorded at 630 nm against reagent blank, after one minute incubation at 37^0C using uv/visible spectrophotometer. Albumin content of follicular fluid was calculated as below

$$\text{Albumin (g/dl)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \text{ x Concentration of Standard (3.6 g/dl)}$$

3.4.4 Globulin concentration in follicular fluid

The Globulin concentration of follicular fluid was estimated by subtracting the estimated total albumin concentration from total protein concentration of follicular fluid.

3.4.5 Total cholesterol concentration in follicular fluid

Total cholesterol content of follicular fluid was estimated as per the modified procedure of Roeschlau, (1974) using the diagnostic kit of Transasia Bio-medicals Ltd. The assay procedure followed

	Blank	Standard	Test
Working Reagent	1000 µl	1000 µl	1000 µl
Distilled water	20 µl	-	-
Standard (200 md/dl)	-	20 µl	-
Follicular fluid	-	-	20 µl

The contents of each tube, i.e, blank, standard and test were mixed well and incubated at 37^0C for 10 minutes. The absorbance of standard and each test tube were recorded against blank at 505 nm using a uv/visible spectrophotometer. The concentration of total cholesterol in test samples were calculated using following formula

$$\text{Cholesterol (mg/dl)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \text{ x Concentration of Standard (200 mg/dl)}$$

3.4.6 LDL-Cholesterol concentration in follicular fluid

Total LDL-Cholesterol concentration of follicular fluid was estimated by using the diagnostic kit of Transasia Bio-medicals Ltd. The procedure of Pisani *et al.* (1995) as cited in manufacturer's catalogue was followed for estimation of LDL-cholesterol as below

	Blank	**Standard**	**Test**
Reagent 1	375 µl	375 µl	375 µl
Distilled water	3 µl	-	-
Standard (107.3 mg/dl)	-	3 µl	-
Follicular fluid	-	-	3 µl
Contents of each tube were mixed well and incubated for 5 min. at 37^{o}C			
Reagent 2 was added @	125 µl	125 µl	125 µl
The contents of tubes incubated for 5 min. at 37^{0}C after vortexing			

The final absorbances at the specified wavelength (600 nm) against reagent blank was taken with the help of a uv/visible spectrophotometer and concentration of LDL-C calculated out using the following formula

$$\text{LDL - C (mg/dl)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \text{ x Concentration of Standard (107.3 mg/dl)}$$

3.4.7 Nitric oxide (NO) concentration in follicular fluid

Nitric oxide concentration in follicular fluid was determined by the method described by Green *et al*. (1982).

Principle

Nitrite (NO^{3-}) in a sample needs no reduction, and therefore reacts with Griess reagent at 37^{o}C. The concentration of nitrite is directly proportional to the nitric oxide (NO) concentration. After incubation of the sample for ten minutes, its absorbance is taken at 546 nm which is detected by a uv/visible spectrophotometer.

Reagent

Griess reagent- 1 part 0.1% NEDD (N-(1-Naphthyl)-ethylenediamine dihydrochloride) in distilled water plus 1 part 1% sulphanilamide (or sulphanilic acid) in 5% concentrated phosphoric acid (H_3PO_4), the two parts being mixed together within 12 hour of use and kept chilled. Each part may be stored refrigerated for upto 2 months.

Procedure

1 ml of Griess reagent was added to 1 ml of centrifuged FF. Then the volume was made up to 3 ml by adding double distilled water. The absorbance of the solution was read at 540 nm with a uv/visible spectrophotometer after incubation for 10 minutes at room temperature. The concentration of NO in the follicular fluid samples were expressed in µM as described by Green *et al*. (1982).

3.5 Micro mineral concentration in follicular fluid

3.5.1 Iron concentration in follicular fluid

The iron concentration of follicular fluid was estimated by using the diagnostic kit of Agappe Diagnostics Ltd. as per the procedure described by Tobacco *et al*. (1981). The procedure for the estimation of the iron content in follicular fluid was given below as described in the manufacturer's catalogue

	Blank	**Standard**	**Test**
Reagent	1000 µl	1000 µl	1000 µl
Distilled water	40 µl	-	-
Standard (200µg/dl)	-	40 µl	-
Follicular fluid	-	-	40 µl

The contents of each tube, i.e, blank, standard and test were mixed well and incubated at 37^{o}C for 10 minutes. The absorbance of standard and each test tube were recorded against blank at 630 nm using a uv/visible spectrophotometer. The concentration of iron in test samples were calculated using following formula

$$\text{Iron } (\mu\text{g/dl}) = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \text{ x Concentration of Standard } (200\,\mu\text{g/dl})$$

3.5.2 Magnesium concentration in follicular fluid

The Magnesium concentration of follicular fluid was estimated following the procedure of Brutis *et al*. (1999) as cited in the manufacturer's catalogue using the diagnostic kit of Agappe Diagnostics Ltd. The procedure in brief was as follows

	Blank	**Standard**	**Sample**
Reagent 1	1000 µl	1000 µl	1000 µl
Standard	-	10 µl	-
Follicular fluid	-	-	10 µl

The contents of tubes were mixed well and incubated for 5 min. at 37^0C. Absorbance of the standard and each test was noted down at 546 nm against reagent blank using uv/visible spectrophotometer. Magnesium content of follicular fluid was estimated using the following formula

$$\text{Magnesium (mg/dl)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \text{ x Concentration of Standard (2.0 mg/dl)}$$

3.5.3 Zinc and Copper concentration in follicular fluid

To analyse Zn and Cu concentration, the follicular fluid (1 ml) was digested using concentrated nitric acid (10 ml) for two days and the digested product (approx. 0.5 ml) were diluted to 10 ml using double distilled glass water. The concentration of micro-elements Zn and Cu were measured by Atomic absorption spectrophotometry using Polarised Zeeman Atomic Absorption Spectrophotometer (Z-2300, HITACHI).

The procedure followed briefly as below

- Digestion of sample (FF) in concentrated nitric acid for zinc and copper was done in a flask. 1 ml of sample (FF) was taken in the flask, to it, 10 ml of concentrated nitric acid was added.

- The flasks with the samples in it were kept on the hot plate (at temperature 100^0C) until its volume reduced to 0.5 ml. Precaution was taken to avoid strong fume emission and formation of bubbles in it. Emission of little fumes was indicating proper acid digestion.
- The change in colour of sample indicated completion of acid digestion.
- The double glass distilled water was added to the remaining sample to make the final volume to 10 ml.
- Finally, the samples were kept in 15 ml tubes and stored in the deep freezer (-20^0C) until the measurement of sample absorbance with a Polarised Zeeman Atomic Absorption Spectrophotometer (Z-2300, HITACHI).

3.6 Oxidative stress markers in follicular fluid

3.6.1 TAA in follicular fluid

Total antioxidant activity was measured by Ferric Reducing Antioxidant Power (FRAP) (Benzie and Strain, 1999). FRAP assay uses antioxidants as reductants in a redox-linked colorimetric method, employing easily reduced oxidant system present in stoichiometric excess.

Principle

At low pH, reduction of ferric tripyridyl triazine (Fe III TPTZ) complex to ferrous form (which has an intense blue colour) can be monitored by measuring the change in absorption at 593 nm. The reaction is nonspecific, in that any half reaction that has lower redox potential, under reaction conditions, than that of ferric ferrous half reaction, will drive the ferrous (Fe^{3+} to Fe^{2+}) ion formation. The change in absorbance is therefore, directly related to the combined or total reducing power of the electron donating antioxidants present in the reaction mixture.

Reagents

1. **FRAP Reagent:**

   A. Acetate Buffer 300 mM, pH 3.6: 3.1 gm sodium acetate trihydrate was added to 16 ml of glacial acetic acid and the final volume made to 1.0 litre with distilled water.

   B. Ferric chloride hexahydrate ($FeCl_3.6H_2O$) (M.W-270.30) 20 mM.

   C. Tripyridyl triazine (TPTZ) (MW: 312.34) 10 mM in 40 mM HCl (MW:36.46).

   The working FRAP reagent was prepared by mixing A, B & C in the ratio of 10:1:1, at the time of use.

2. **Standard:** Ascorbic acid (MW:176.13) 1000 µM

Procedure

Follicular fluid (100 µl) was mixed with 3 ml of working FRAP reagent and absorbance at 593nm was measured at 0 minute after vortexing. Again the absorbance of solution was measured at 593 nm wavelength after incubation for 4 minutes in a water bath at 37^{o}C. Ascorbic acid standards (100 µM-1000 µM) were processed in the same way.

	Blank	**Standard**	**Test**
Sample (FF)	-	-	100µl
Standard (Ascorbic acid- 1000µM)	-	100µl	-
Working FRAP Reagent	3000µl	3000µl	3000µl

Contents of all the tubes were mixed well. Spectrophotometer was standardised with blank solution. Then the optical density (OD) of standard and test samples were measured at zero minute and again after 4 minutes at 593 nm. Results were calculated using following formula

$$\text{FRAP value of sample}(\mu M) = \frac{\text{Change in absorbance of sample from 0 to 4 min.}}{\text{Change in absorbance of standard from 0 to 4 min.}} \text{ x FRAP value of Ascorbic acid Standard } (1000\,\mu M)$$

Note: The FRAP value of Ascorbic acid Standard is 2.

3.6.2 SOD activity in follicular fluid

The activity of SOD in FF was determined following the method of Marklund and Marklund (1974) with minor modification. The procedure followed briefly as follows

Reagents

A. Tris HCl buffer 100 mM, pH 8.2

B. Pyrogallol 4 mM, 50 µl (Solution was prepared fresh just prior to use and stored in brown bottle)

Procedure

In a cuvette 50 µl of FF and 900 µl of 100 mM Tris HCl buffer were added. The solution was mixed thoroughly by vortexing. The solution was incubated at 37^{0}C for one hour during which there was an increase in the optical density of the solution. After one hour when the solution turned turbid, 50 µl of 4 mM pyrogallol solution was added to it. The solution was mixed properly for some time (approx. 5-6 minutes). The rate of auto-oxidation of pyrogallol was taken from the increase in absorbance at 420 nm with a spectrophotometer. The activity of SOD was calculated using the formula given below and expressed as SOD units/ mg protein.

Calculation

$$\text{SOD activity (U/mg of protein)} = \frac{(\Delta EO - \Delta E)}{\Delta EO} \times \frac{1}{\text{mg of protein in 0.01ml of FF}} \times 0.5$$

ΔEO = Change of absorbance of pyrogallol

ΔE = Change of absorbance of sample

Statistical analysis

The mean values with standard error for concentrations of various biochemical parameters, mineral concentration and oxidative stress markers of FF from small, large as well as pooled follicles were computed. Differences between the biochemical constituents among the different sized follicles in a particular season (autumn, winter and summer) as well as difference in concentration of a particular sized follicle during different seasons were analyzed, using standard procedures described by Snedecor and Cochran (1994) for unpaired t tests and ANOVA using the software SPSS-16.

RESULTS

CHAPTER-IV

RESULTS

In the present study, it was assumed that the composition of follicular fluid (FF) in which the growing oocyte present differ in relation to the changed environment or season. To gain insight of the composition of the FF in different seasons i.e, Autumn (September, October), Winter (December, January) and Summer (April, May); the ovaries were collected from municipal slaughter house, follicular fluid harvested and analysed according to season.

4.1 Climatic parameters

During the experimental period (September, 2015 to May, 2016), the climatic parameters of Jammu region is given in Table 4.1. During Autumn, the maximum temperature observed was 31.9^{0}C (22.5-35.8^{0}C), minimum temp. 19.4^{0}C (12-25.6^{0}C), Relative Humidity (R.H) was 69.75% (60.0-97.5%) and average Rainfall was 2.8 mm (0.2-73.0 mm). The number of rainy days observed during the experimental period was only 9 days.

Table 4.1: Climatological parameters of Jammu region during experimental period in different seasons

Parameters	Autumn	Winter	Summer
Max. Temp. (^{0}C)	31.90 ± 0.38	18.90 ± 0.52	36.12 ± 0.40
Min. Temp. (^{0}C)	19.40 ± 0.44	5.60 ± 0.34	18.32 ± 0.44
R. H (%)	69.75 ± 0.86	77.11 ± 1.14	49.09 ± 1.16
Rainfall (mm)	2.80 ± 1.38	0.65 ± 0.42	0.21 ± 0.09

Similarly, in winter the max. temp. observed was 18.9^{0}C with a range of 17.4-25.5^{0}C; the min. temp. was 5.6^{0}C (0.6-11.0^{0}C); R.H was 77.11% (62.0-98.5%). The number of rainy days during this period observed was 6 days with an average rainfall of 0.65 mm (1.8-25.0 mm).

The max. temp. observed in summer (experimental period) was 36.12^{0}C (28.0-42.8^{0}C). The min. temp. and R.H were 18.32^{0}C (13.2-26.2^{0}C) and 49.09% (34.0-

72.0%) respectively. The number of rainy days during the period was 7 days with average rainfall of 0.21 mm (0.2-2.6 mm).

4.2 Sample collection

The ovaries were collected from the municipal slaughter house, Gujjar Nagar, Jammu and the FF collected from different sized follicles (Small and Large follicles) were analysed separately. During the experiment a total of 574 ovaries were used to collect FF in autumn season, 293 and 318 no. of ovaries during winter and summer season respectively as shown in Table 4.2.

Table 4.2: Number of ovaries and follicles harvested during different seasons

Parameters	No. of ovaries used	No. of SF harvested	No. of SF harvested/ ovary	No. of LF harvested	No. of LF harvested/ ovary	Total no. of follicles
Autumn	574	1863	3.25	868	1.51	2731
Winter	293	810	2.76	313	1.07	1123
Summer	318	899	2.83	334	1.05	1233
Total during experiment	1185	2673	-	1515	-	5087

In Autumn, the FF was harvested from 1,863 no. of SF and 868 LF (out of 574 no. of ovaries). Similarly, in winter, FF was collected from 810 no. of SF and 313 no. of LF present on the surface of 293 ovaries. A total of 1233 (SF: 899 & LF: 334) no. of follicles were used to harvest the FF for determining the concentration of different metabolites during the summer season as shown in Table 4.2.

4.3 Biochemical parameters of ovarian follicular fluid

4.3.1 Glucose concentration in follicular fluid

Follicular fluid glucose concentration (Mean ± SE) of large follicle (> 6 mm diameter), small follicle (< 6 mm diameter) and pooled follicular fluid (small and large follicles) are shown in Table 4.3.a, 4.3.b and 4.3.c respectively. Perusal of Table 4.3.a reveals that the glucose concentration in follicular fluid of small follicle during autumn season (121.91 ± 3.13) mg/dl was found to be significantly higher ($P < 0.05$) than those during winter (88.81 ± 3.22) mg/dl and summer (84.08 ± 1.12) mg/dl season. Similar trends were observed for glucose concentration in follicular fluid of large and pooled follicular fluid samples i.e, significantly ($P < 0.05$) higher concentration during autumn compared to winter and summer seasons. It was also observed that the follicular fluid glucose concentration of small, large and pooled follicles during summer and winter were not differing significantly with each other. But the glucose content of follicular fluid of small and large follicles differ significantly ($P < 0.05$) with each other during autumn and summer, but non-significant during winter (Table 4.4.a, 4.4.b, 4.4.c).

4.3.2 Total protein content in follicular fluid

The concentration of total protein in FF of SF, LF and pooled follicles are shown in Tables 4.3.a, 4.3.b and 4.3.c. The values observed as 7.14 ± 0.36 g/dl in SF during autumn, 6.52 ± 0.11 g/dl during winter and 6.30 ± 0.08 g/dl during summer season. Whereas, values observed in LF were (5.18 ± 0.18) g/dl during autumn, (5.30 ± 0.11) g/dl during winter, (4.63 ± 0.22) g/dl during summer and in pooled follicles (6.16 ± 0.35) g/dl, (5.91 ± 0.19) g/dl, (5.46 ± 0.27) g/dl during autumn, winter and summer respectively. The total protein concentration in FF of SF during autumn was significantly ($P < 0.05$) higher than that during summer. But the total protein content in FF of SF during winter did not vary significantly either with its concentration in SF during autumn or summer. The total protein content in FF of LF during autumn did not vary significantly ($P < 0.05$) with that in winter. But concentrations during autumn and winter were significantly ($P < 0.05$) higher than that during the summer season as shown in Table 4.3.a. The total protein concentration in FF of pooled follicles were found to be similar and not varying significantly with each other. Comparison of total protein content in FF of LF with that of SF in different seasons revealed that those are varying significantly ($P < 0.05$) with each other i.e, SF having higher total protein concentration than LF as shown in Table 4.4.a, 4.4.b and 4.4.c.

Table 4.3.a: Effect of season on biochemical parameters of follicular fluid in small follicles

Parameters	Autumn	Winter	Summer
Glucose (mg/dl)	121.91 ± 3.13^{a}	83.81 ± 3.22^{b}	84.08 ± 1.12^{b}
Total Protein (g/dl)	7.14 ± 0.36^{a}	6.52 ± 0.11^{ab}	6.30 ± 0.08^{b}
Albumin (g/dl)	4.60 ± 0.12	4.33 ± 0.09	4.40 ± 0.09
Globulin (g/dl)	2.52 ± 0.35	2.22 ± 0.13	1.90 ± 0.07
Total Cholesterol (mg/dl)	147.23 ± 3.79^{a}	131.03 ± 4.42^{b}	147.93 ± 4.37^{a}
LDL-Cholesterol (mg/dl)	91.56 ± 1.90^{a}	84.69 ± 3.98^{a}	114.44 ± 2.96^{b}
NO (µM)	0.33 ± 0.01	0.36 ± 0.02	0.35 ± 0.004

Mean ± SE bearing different superscript (a, b) in a row differ significantly ($P < 0.05$)

4.3.3 Albumin concentration in follicular fluid

The concentration of albumin in FF of SF, LF and pooled follicles are shown in Tables 4.3.a, 4.3.b and 4.3.c. The values recorded for SF were (4.60 ± 0.12) g/dl during autumn, (4.33 ± 0.09) g/dl during winter and (4.40 ± 0.09) g/dl during summer season. Whereas, the values observed in LF were (3.98 ± 0.11) g/dl during autumn, (4.05 ± 0.16) g/dl and (3.87 ± 0.11) g/dl during winter and summer respectively. In pooled follicles, albumin content in FF was (4.29 ± 0.12) g/dl, (4.19 ± 0.09) g/dl and (4.14 ± 0.10) g/dl during autumn, winter and summer respectively. The albumin concentration in FF of any type of follicles did not vary significantly ($P < 0.05$) according to seasons. The comparison of albumin content in FF of SF and LF during autumn and summer seasons showed significant difference ($P < 0.05$) with each other i.e SF were having higher albumin content as that of LF as shown in Table 4.4.a and 4.4.c. But the albumin values recorded in winter season for both type of follicles did not vary significantly ($P < 0.05$) with each other as shown in Table 4.4.b.

Table 4.3.b: Effect of season on biochemical parameters of follicular fluid in large follicles

Parameters	Autumn	Winter	Summer
Glucose (mg/dl)	165.10 ± 4.77^{a}	90.82 ± 3.19^{b}	94.46 ± 0.70^{b}
Total Protein (g/dl)	5.18 ± 0.18^{a}	5.30 ± 0.11^{a}	4.63 ± 0.22^{b}
Albumin (g/dl)	3.98 ± 0.11	4.05 ± 0.16	3.87 ± 0.11
Globulin (g/dl)	1.20 ± 0.17	1.24 ± 0.22	0.76 ± 0.13
Total Cholesterol (mg/dl)	283.00 ± 8.34^{a}	150.38 ± 8.09^{b}	143.03 ± 2.81^{b}
LDL-Cholesterol (mg/dl)	102.78 ± 0.81^{a}	83.73 ± 1.68^{b}	117.60 ± 2.79^{c}
NO (μM)	0.34 ± 0.01	0.37 ± 0.01	0.36 ± 0.01

Mean ± SE bearing different superscript (a, b, c) in a row differ significantly ($P < 0.05$)

4.3.4 Globulin concentration in follicular fluid

The observed globulin concentration in SF during autumn was (2.52 ± 0.35) g/dl, (2.22 ± 0.13) g/dl and (1.90 ± 0.07) g/dl during winter and summer respectively. The concentration observed in LF was (1.20 ± 0.17) g/dl during autumn, (1.24 ± 0.22) g/dl and (0.76 ± 0.13) g/dl during winter and summer respectively. The concentration recorded for that of pooled follicles were (1.86 ± 0.27) g/dl, (1.73 ± 0.19) g/dl and (1.33 ± 0.18) g/dl during autumn, winter and summer respectively. The values observed during different seasons in either type of follicles did not vary significantly with each other as shown in Table 4.3.a, 4.3.b and 4.3.c. The comparison of globulin concentration in FF of LF with SF in different seasons shows that the values were varying significantly ($P < 0.05$) with each other i.e, SF having higher globulin concentration than that of LF as shown in Table 4.4.a, 4.4.b and 4.4.c.

Table 4.3.c: Effect of season on biochemical parameters of follicular fluid harvested from pooled follicles

Parameters	Autumn	Winter	Summer
Glucose (mg/dl)	143.50 ± 7.06^{a}	87.31 ± 2.40^{b}	89.27 ± 1.68^{b}
Total Protein (g/dl)	6.16 ± 0.35	5.91 ± 0.19	5.46 ± 0.27
Albumin (g/dl)	4.29 ± 0.12	4.19 ± 0.09	4.14 ± 0.10
Globulin (g/dl)	1.86 ± 0.27	1.73 ± 0.19	1.33 ± 0.18
Total Cholesterol (mg/dl)	215.11 ± 20.94^{a}	140.71 ± 5.26^{b}	145.48 ± 2.58^{b}
LDL-Cholesterol (mg/dl)	97.17 ± 1.95^{a}	84.21 ± 2.06^{b}	116.02 ± 1.99^{c}
NO (µM)	0.34 ± 0.005^{a}	0.37 ± 0.01^{b}	0.36 ± 0.002^{ab}

Mean ± SE bearing different superscript (a, b, c) in a row differ significantly ($P < 0.05$)

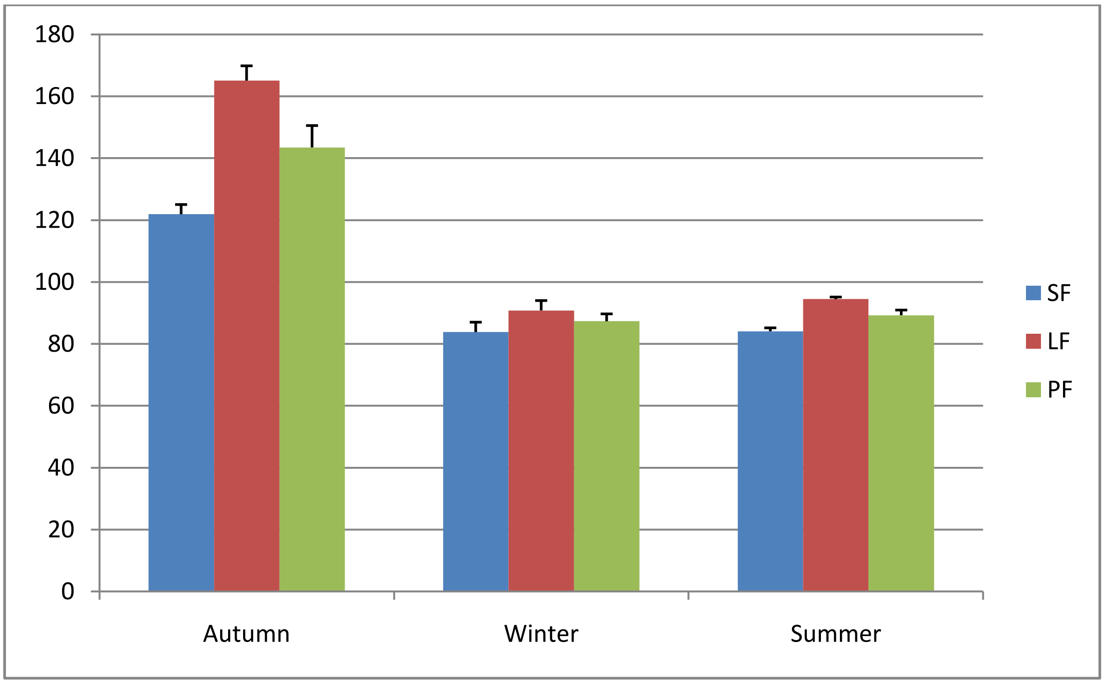

Fig. 4.1: Bar diagram showing effect of seasons on glucose concentration in pooled follicular fluid

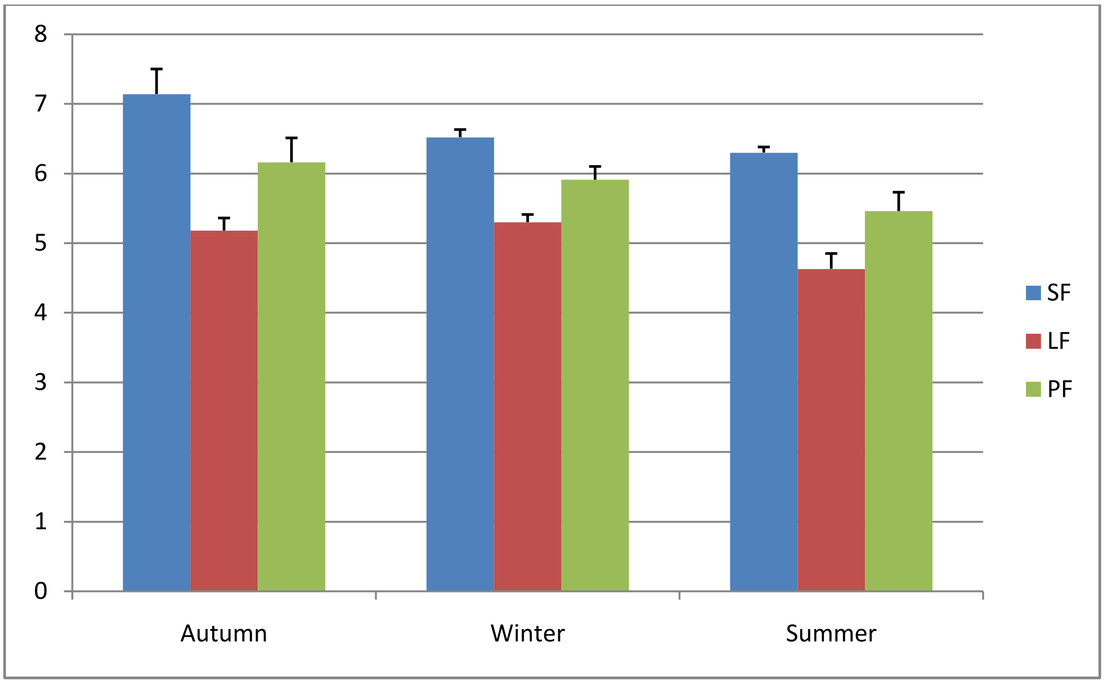

Fig. 4.2: Bar diagram showing effect of seasons on total protein concentration in pooled follicular fluid

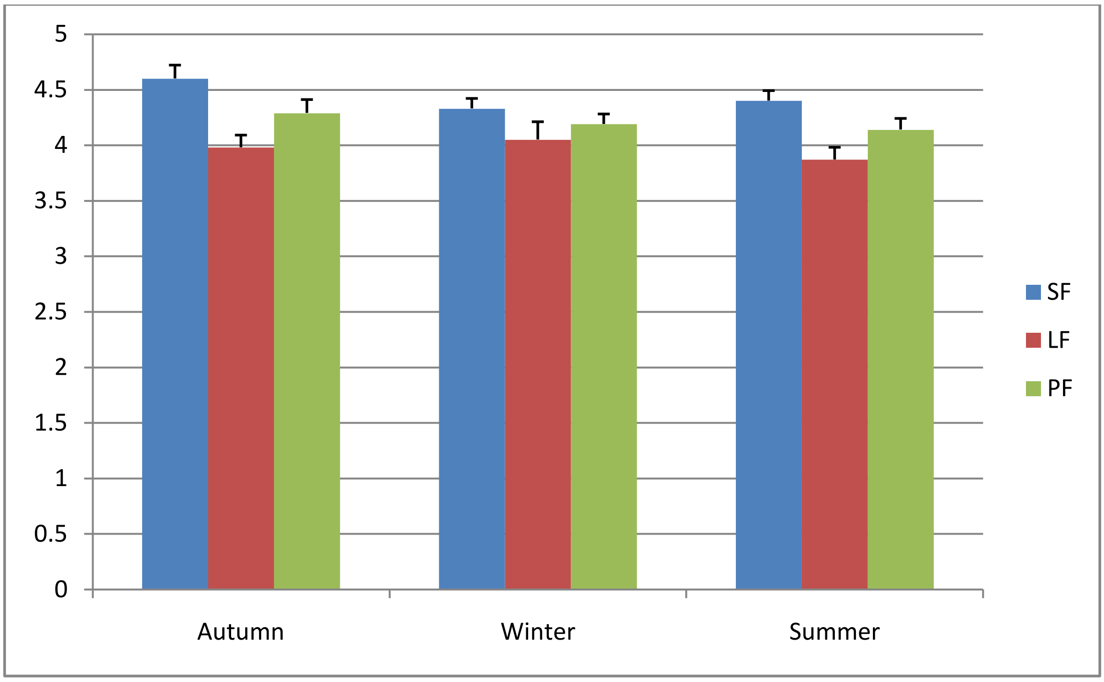

Fig. 4.3: Bar diagram showing effect of seasons on albumin concentration in pooled follicular fluid

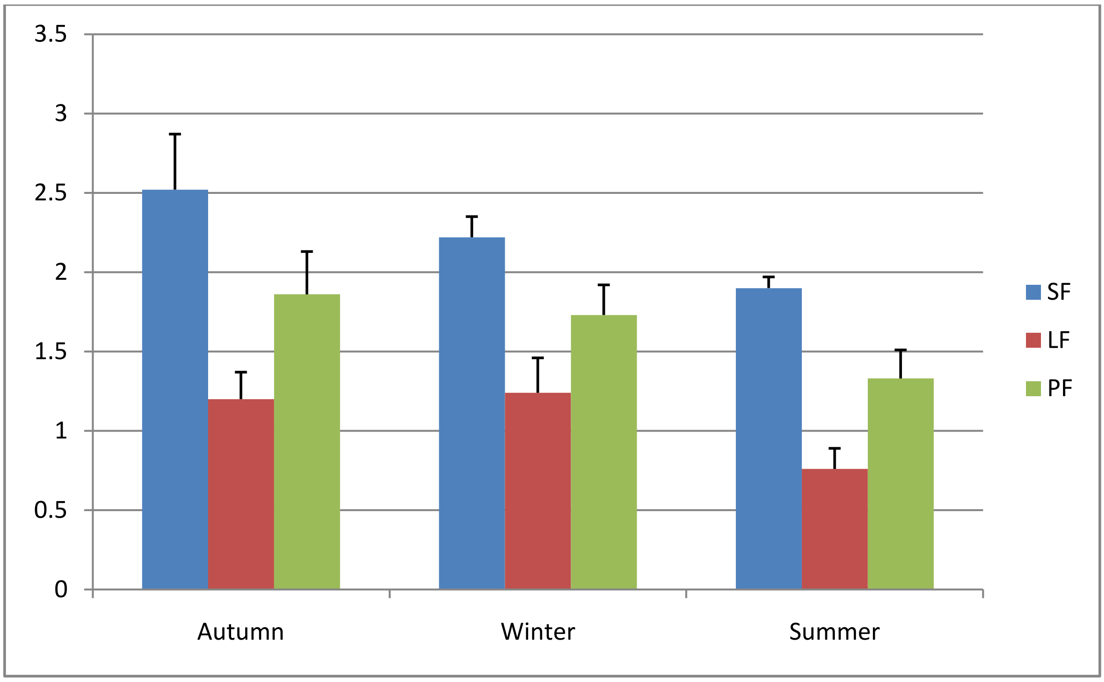

Fig. 4.4: Bar diagram showing effect of seasons on globulin concentration in pooled follicular fluid

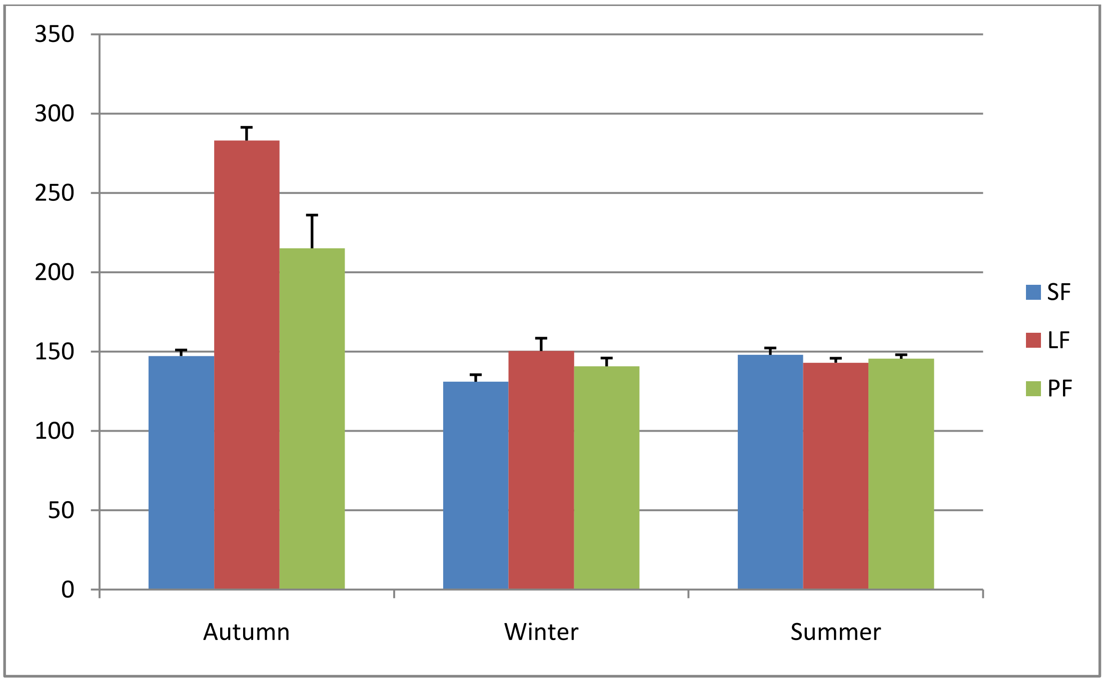

Fig. 4.5: Bar diagram showing effect of seasons on total cholesterol concentration in pooled follicular fluid

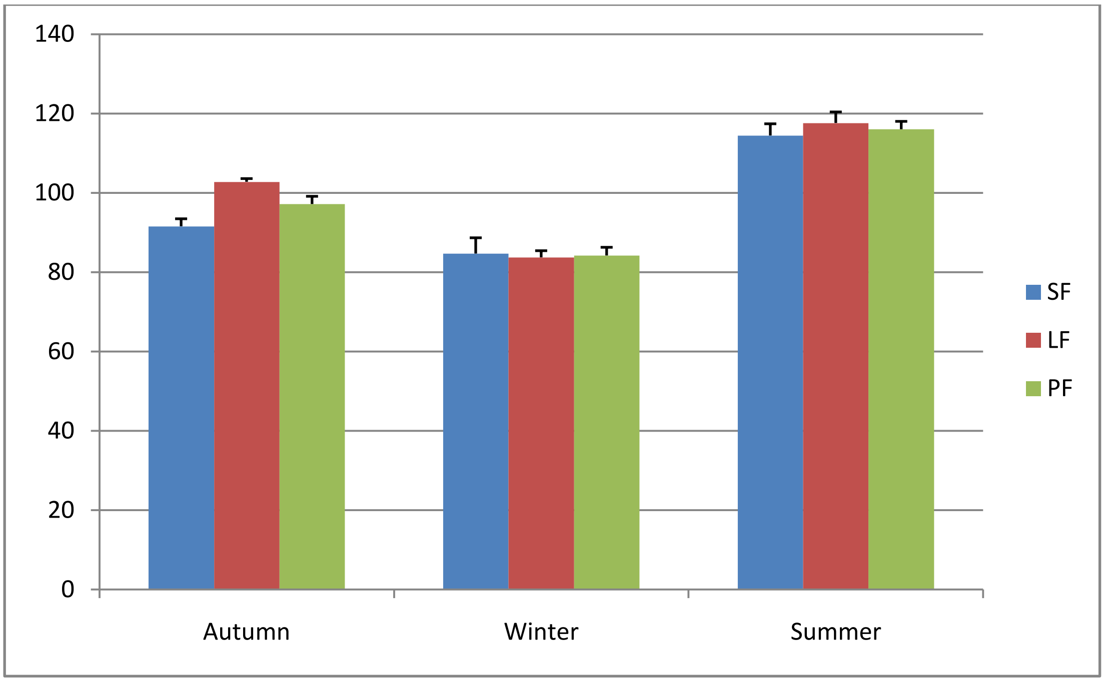

Fig. 4.6: Bar diagram showing effect of seasons on LDL-cholesterol concentration in pooled follicular fluid

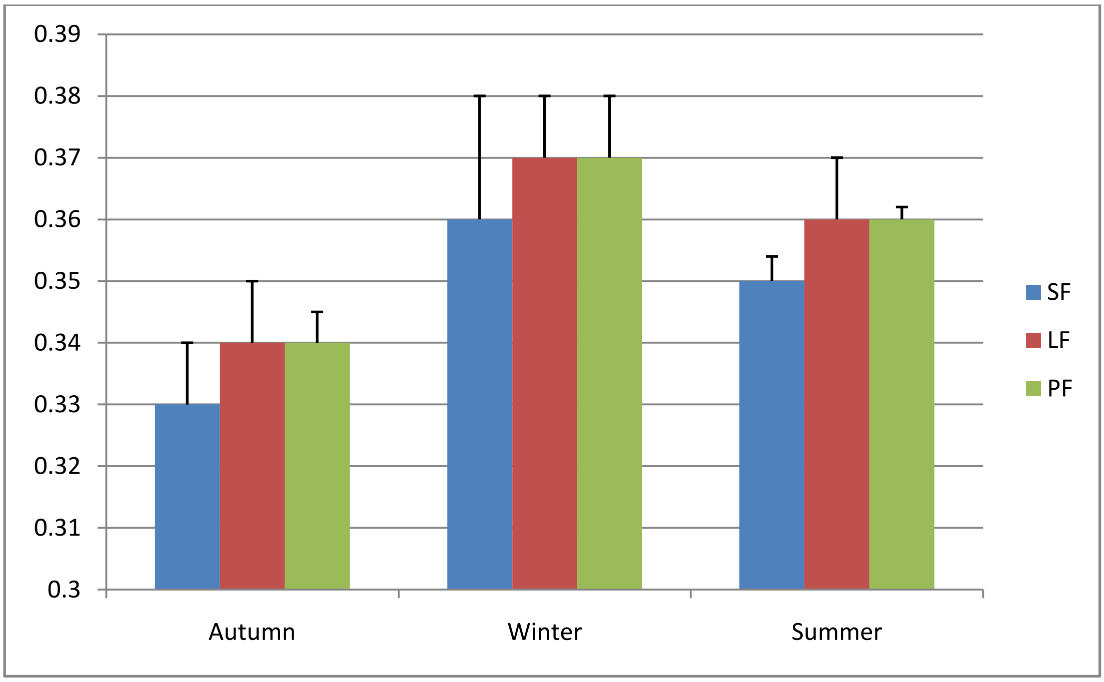

Fig. 4.7: Bar diagram showing effect of seasons on NO concentration in pooled follicular fluid

4.3.5 Total cholesterol content in follicular fluid

Total Cholesterol content of FF of SF in different seasons tabulated in Table 4.3.a. and those of L.F and pooled follicles in Table 4.3.b and 4.3.c respectively. Table 4.3.a reveals that the total cholesterol content in FF of small follicles during autumn (147.23 ± 3.79) mg/dl which was significantly ($P < 0.05$) differing with that during winter, but not with summer. Concentration of total cholesterol in FF of SF during autumn and summer were found to be at par.

The concentration of total cholesterol in follicular fluid of SF during autumn (147.23 ± 3.79) mg/dl was significantly ($P < 0.05$) lower than that of LF (Table 4.4.a). But the concentration of total cholesterol in FF of SF and LF were not varying significantly with each other either during winter or summer (Table 4.4.b and 4.4.c).

Table 4.4.a: Biochemical parameters of follicular fluid in small and large follicles during autumn

Parameters	SF	LF
Glucose (mg/dl)	121.91 ± 3.13^{a}	165.10 ± 4.77^{b}
Total protein (g/dl)	7.14 ± 0.36^{a}	5.18 ± 0.18^{b}
Albumin (g/dl)	4.60 ± 0.12^{a}	3.98 ± 0.11^{b}
Globulin (g/dl)	2.52 ± 0.35^{a}	1.2 ± 0.17^{b}
Total cholesterol (mg/dl)	147.23 ± 3.79^{a}	283 ± 8.34^{b}
LDL Cholesterol (mg/dl)	91.56 ± 1.9^{a}	102.78 ± 0.81^{b}
NO (µM)	0.33 ± 0.01	0.34 ± 0.01

Mean ± SE bearing different superscript (a, b) in a row differ significantly ($P < 0.05$)

4.3.6 LDL-Cholesterol concentration in follicular fluid

LDL-Cholesterol content in FF of SF varied from 84.69 ± 3.98 mg/dl during winter to 114.44 ± 2.96 mg/dl in summer with intermediate concentration of 91.56 ± 1.90 mg/dl in autumn (Table 4.3.a). Concentration during summer was found to be significantly higher (P < 0.05) than the values obtained during autumn and summer. The concentration of LDL-Cholesterol in FF of LF observed to be highest 117.60 ± 2.79 mg/dl during summer, intermediate 102.78 ± 0.81 mg/dl during autumn and lowest value of 83.73 ± 1.68 mg/dl during winter. The concentrations during all the seasons were varying significantly with each other (Table 4.3.b). A similar trend as in LF was observed in LDL-Cholesterol. Concentration in FF of pooled follicles during different seasons, i.e, all were differing significantly (P < 0.05) among each other with highest value during summer.

Table 4.4.b: Biochemical parameters of follicular fluid in small and large follicles during winter

Parameters	SF	LF
Glucose (mg/dl)	83.81 ± 3.22	90.82 ± 3.19
Total protein (g/dl)	6.52 ± 0.11^{a}	5.30 ± 0.11^{b}
Albumin (g/dl)	4.33 ± 0.09	4.05 ± 0.16
Globulin (g/dl)	2.22 ± 0.13^{a}	1.24 ± 0.22^{b}
Total cholesterol (mg/dl)	131.03 ± 4.42	150.38 ± 8.09
LDL Cholesterol (mg/dl)	84.69 ± 3.98	83.73 ± 1.68
NO (µM)	0.36 ± 0.02	0.37 ± 0.01

Mean ± SE bearing different superscript (a, b) in a row differ significantly (P < 0.05)

4.3.7 Nitric oxide content in follicular fluid

Measured NO metabolite concentration in follicular fluid of small, large and pooled follicles during different seasons are shown in Table 4.3.a, 4.3.b and 4.3.c. respectively. Perusal of Table 4.3.a reveals that NO concentration in FF of small follicles in different seasons were not varying significantly ($P < 0.05$) with each other and it quantitatively varies from 0.33 ± 0.01 µM in autumn to 0.36 ± 0.02 µM in winter. Similar concentration was observed in FF of large follicles during different seasons, i.e, autumn: 0.34 ± 0.01 µM, winter: 0.37 ± 0.01 µM and 0.34 ± 0.01 µM in summer. NO concentration in FF of pooled follicles during autumn was significantly ($P < 0.05$) lower than the concentration during winter. Table 4.4.a, 4.4.b and 4.4.c shows the comparison in concentrations of NO in follicular fluid of SF and LF during autumn, winter and summer respectively. These tables reveal that the concentration of NO in FF in SF and LF were not varying significantly in any of the observed seasons.

Table 4.4.c: Biochemical parameters of follicular fluid in small and large follicles during summer

Parameters	SF	LF
Glucose (mg/dl)	84.08 ± 1.12^{a}	94.46 ± 0.7^{b}
Total protein (g/dl)	6.30 ± 0.08^{a}	4.63 ± 0.22^{b}
Albumin (g/dl)	4.40 ± 0.09^{a}	3.87 ± 0.11^{b}
Globulin (g/dl)	1.90 ± 0.07^{a}	0.76 ± 0.13^{b}
Total cholesterol (mg/dl)	147.93 ± 4.37	143.03 ± 2.81
LDL Cholesterol (mg/dl)	114.44 ± 2.96	117.60 ± 2.79
NO (µM)	0.35 ± 0.004	0.36 ± 0.01

Mean ± SE bearing different superscript (a, b) in a row differ significantly ($P < 0.05$)

4.3.8 Iron content in follicular fluid

Iron content in follicular fluid of small and large sized follicles as well as that of fluid collected from pooled/ mixed follicles were non significantly varying with each other. The Fe content of follicular fluid ranges from 207.68 ± 3.80 µg/dl to 209.20 ± 4.22 µg/dl in SF, 217.70 ± 3.50 µg/dl to 224.15 ± 2.83 µg/dl in LF and 212.68 ± 2.88 to 216.67 ± 3.30 in pooled follicles during different seasons (Table 4.5.a, 4.5.b and 4.5.c). The Fe content in follicular fluid of large follicles was significantly ($P < 0.05$) higher than that of SF during autumn and summer but at par during winter (Table 4.6.a, 4.6.b and 4.6.c).

Table 4.5.a: Effect of season on minerals of follicular fluid in small follicles

Parameters	Autumn	Winter	Summer
Iron (µg/dl)	205.07 ± 3.46	207.68 ± 3.80	209.20 ± 4.22
Magnesium (mg/dl)	2.84 ± 0.03	2.71 ± 0.03	2.83 ± 0.09
Copper (µmol/L)	5.42 ± 0.46^{a}	13.27 ± 0.47^{b}	17.36 ± 0.47^{c}
Zinc (µmol/L)	32.20 ± 0.54^{a}	33.73 ± 0.67^{a}	25.59 ± 1.12^{b}

Mean ± SE bearing different superscript (a, b, c) in a row differ significantly ($P < 0.05$)

4.3.9 Magnesium content in follicular fluid

Mg concentration in follicular fluid of small sized follicles observed as 2.84 ± 0.03 mg/dl, 2.71 ± 0.03 mg/dl and 2.83 ± 0.09 mg/dl during autumn, winter and summer season respectively. In large follicles conc. of mg were 2.51 ± 0.06 mg/dl, 2.38 ± 0.02 mg/dl and 2.46 ± 0.06 mg/dl during autumn, winter and summer season respectively. The concentration of Mg in FF of small, large sized follicles as well as the follicular fluid from pooled follicles did not vary significantly during different seasons (Table 4.5.a, 4.5.b and 4.5.c). But during each and every season, the concentration of Mg in follicular fluid of small sized follicles and large sized vary significantly ($P < 0.05$) with higher concentration in SF (Table 4.6.a, 4.6.b and 4.6.c). Though, the concentration of Mg in follicular fluid of different sized follicles were not varying significantly, the concentration in autumn season was found to be higher than other seasons.

Table 4.5.b: Effect of season on minerals of follicular fluid in large follicles

Parameters	Autumn	Winter	Summer
Iron (µg/dl)	223.64 ± 4.93	217.70 ± 3.50	224.15 ± 2.83
Magnesium (mg/dl)	2.51 ± 0.06	2.38 ± 0.02	2.46 ± 0.06
Copper (µmol/L)	6.73 ± 0.56^{a}	14.28 ± 0.94^{b}	13.72 ± 0.85^{b}
Zinc (µmol/L)	38.04 ± 0.84^{a}	25.66 ± 1.08^{b}	36.26 ± 0.93^{a}

Mean ± SE bearing different superscript (a, b) in a row differ significantly (P < 0.05)

4.3.10 Copper content in follicular fluid

Copper concentration in follicular fluid of small, large and pooled follicles in different seasons viz autumn, winter and summer are shown in Table 4.5.a, 4.5.b and 4.5.c. Perusal of these tables reveal that the concentration of Cu in follicular fluid of S.F varies from 5.42 ± 0.46 µmol/L in autumn to 17.36 ± 0.47 µmol/L in summer season. It was also found that Cu conc. in follicular fluid of SF varies significantly (P < 0.05) among the seasons indicating seasonal influence on it. Similarly, the Cu conc. of follicular fluid collected from large follicles was lowest 6.73 ± 0.56 µmol/L in autumn and highest in winter 14.28 ± 0.94 µmol/L. The Cu concentration in FF of LF during autumn was significantly (P < 0.05) lower than those during winter and summer (Table 4.5.b). The Cu concentration in follicular fluid collected from pooled follicle was also exhibiting same pattern as in LF (Table 4.5.c). The copper concentration of SF and LF were compared with each other statistically according to season and shown in Table 4.6.a, 4.6.b and 4.6.c. It was observed that the Cu concentration of follicular fluid of SF and LF were not varying significantly with each other except during summer season.

Table 4.5.c: Effect of season on minerals in pooled follicular fluid

Parameters	Autumn	Winter	Summer
Iron (µg/dl)	214.36 ± 4.00	212.69 ± 2.88	216.67 ± 3.30
Magnesium (mg/dl)	2.67 ± 0.06	2.54 ± 0.05	2.64 ± 0.07
Copper (µmol/L)	6.07 ± 0.39^{a}	13.77 ± 0.52^{c}	15.54 ± 0.71^{b}
Zinc (µmol/L)	35.12 ± 1.00^{a}	29.69 ± 1.36^{ab}	30.93 ± 1.75^{b}

Mean ± SE bearing different superscript (a, b, c) in a row differ significantly (P < 0.05)

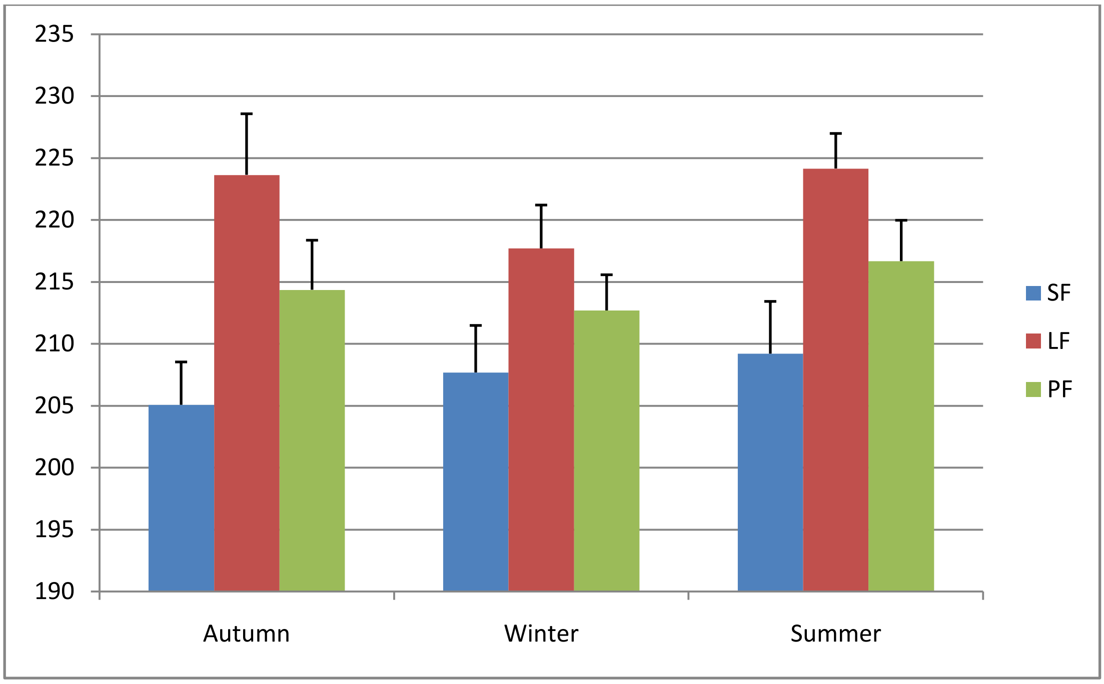

Fig. 4.8: Bar diagram showing effect of seasons on iron concentration in pooled follicular fluid

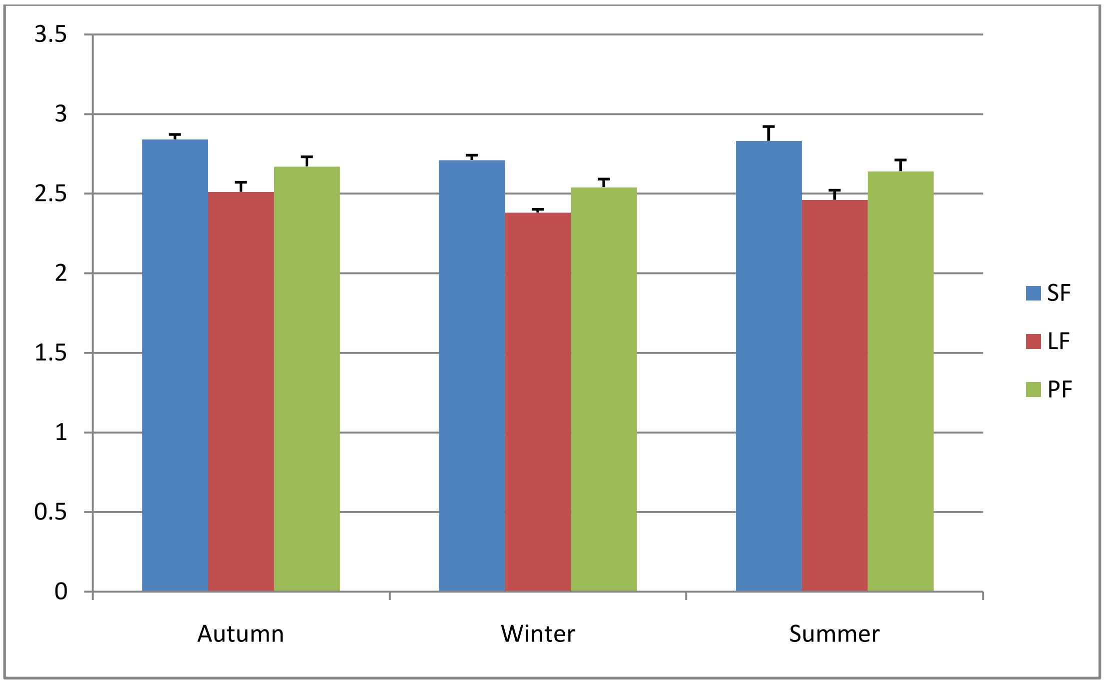

Fig. 4.9: Bar diagram showing effect of seasons on magnesium concentration in pooled follicular fluid

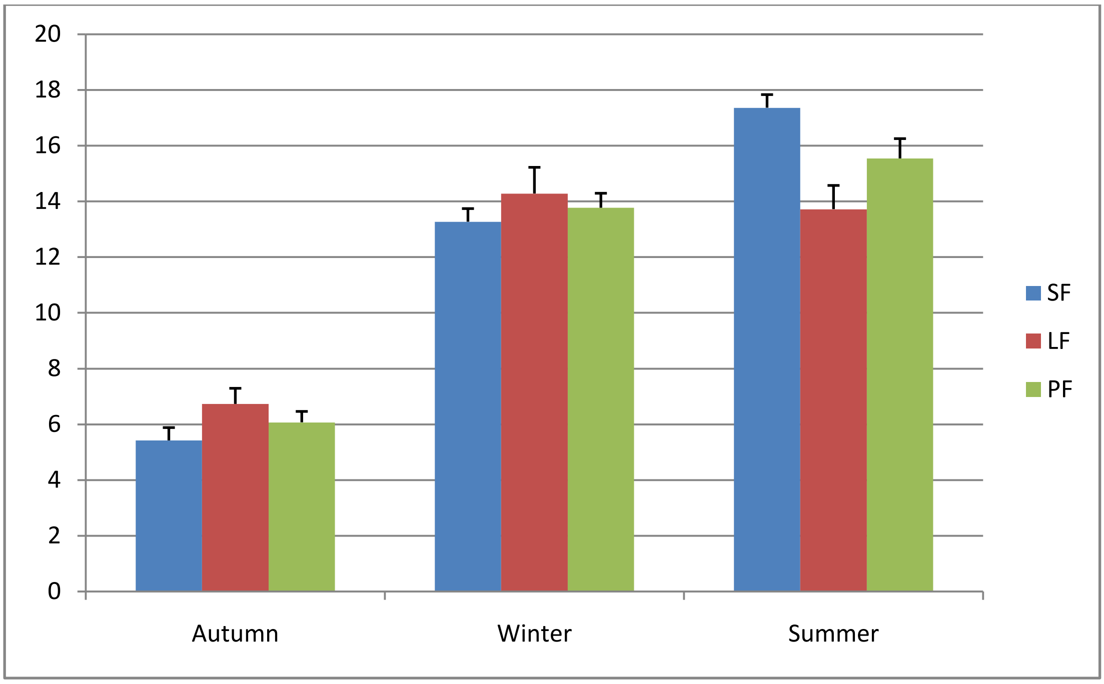

Fig. 4.10: Bar diagram showing effect of seasons on copper concentration in pooled follicular fluid

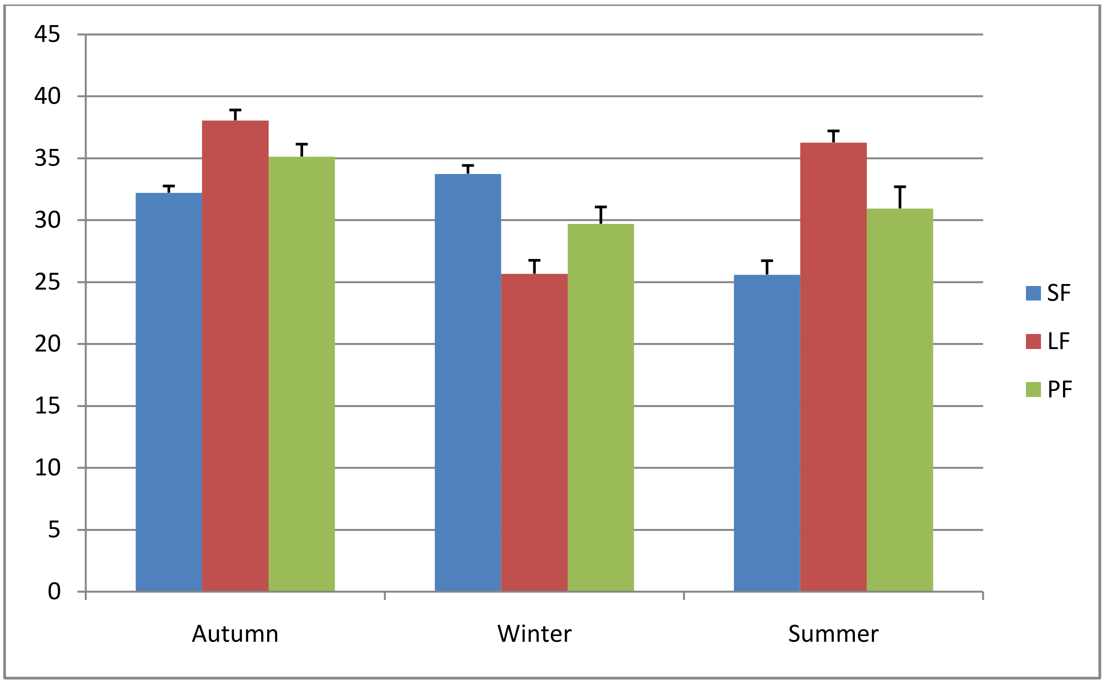

Fig. 4.11: Bar diagram showing effect of seasons on zinc concentration in pooled follicular fluid

4.3.11 Zinc content in follicular fluid

Study of Zn concentration in follicular fluid of small, large and pooled follicles during different seasons revealed a very unsynchronised pattern. Zn conc. in FF of small follicles highest during winter and lowest in summer (Table 4.5.a). The Zn conc. in autumn and winter did not differ significantly ($P < 0.05$) (32.20 ± 0.54 µmol/L vs 33.73 ± 0.67 µmol/L), but both values differ significantly with that of summer. The concentration of Zn in follicular fluid of LF during autumn and summer were non-significant, whereas, both values differ significantly ($P < 0.05$) with that during winter (Table 4.5.b). When study of follicular fluid obtained from pooled follicles, revealed that the concentration Zn during autumn differed significantly ($P < 0.05$) with those during summer and winter (Table 4.5.c) but concentration observed during summer and winter did not vary significantly with each other. The measured concentration of Zn in follicular fluid of SF and LF during autumn were varying significantly ($P < 0.05$) with each other. Similar observations were found in other two seasons also i.e, significant differences in concentration of Zn in follicular fluid of SF and LF were observed in winter and summer (Table 4.6.a, 4.6.b and 4.6.c).

Table 4.6.a: Minerals of follicular fluid in small and large follicles during autumn

Parameters	SF	LF
Iron (µg/dl)	205.07 ± 3.46^{a}	223.64 ± 4.93^{b}
Magnesium (mg/dl)	2.84 ± 0.03^{a}	2.51 ± 0.06^{b}
Copper (µmol/L)	5.42 ± 0.46	6.73 ± 0.56
Zinc (µmol/L)	32.20 ± 0.54^{a}	38.04 ± 0.84^{b}

Mean ± SE bearing different superscript (a, b) in a row differ significantly ($P < 0.05$)

Table 4.6.b: Minerals of follicular fluid in small and large follicles during winter

Parameters	SF	LF
Iron (µg/dl)	207.68 ± 3.8	217.7 ± 3.5
Magnesium (mg/dl)	2.71 ± 0.03^{a}	2.38 ± 0.02^{b}
Copper (µmol/L)	13.27 ± 0.47	14.28 ± 0.94
Zinc (µmol/L)	33.73 ± 0.67^{a}	25.66 ± 1.08^{b}

Mean ± SE bearing different superscript (a, b) in a row differ significantly ($P < 0.05$)

Table 4.6.c: Minerals of follicular fluid in small and large follicles during summer

Parameters	SF	LF
Iron (µg/dl)	209.20 ± 4.22^{a}	224.15 ± 2.83^{b}
Magnesium (mg/dl)	2.83 ± 0.09^{a}	2.46 ± 0.06^{b}
Copper (µmol/L)	17.36 ± 0.47^{a}	13.72 ± 0.85^{b}
Zinc (µmol/L)	25.59 ± 1.12^{a}	36.26 ± 0.93^{b}

Mean ± SE bearing different superscript (a, b) in a row differ significantly ($P < 0.05$)

4.3.12 Total Antioxidant Activity in follicular fluid

Total antioxidant activity of follicular fluid in SF, LF and pooled follicles were measured during different seasons i.e, autumn, winter and summer seasons. In SF this activity was found to be highest during autumn. The value during autumn was also significantly higher ($P < 0.05$) than the values observed during winter and summer (Table 4.7.a). The TAA in follicular fluid of LF was observed to be highest during autumn and lowest during summer, but the values were differing significantly ($P < 0.05$) with each other (Table 4.7.b). A similar trend as that in LF was also observed in follicular fluid collected from pooled follicles (Table 4.7.c). When TAA in follicular fluid of SF and LF were compared, no significant difference ($P < 0.05$) in values were observed during seasons except winter (Table 4.8.a, 4.8.b and 4.8.c).

Table 4.7.a: Effect of season on oxidative stress markers of follicular fluid in small follicles

Parameters	Autumn	Winter	Summer
TAA (µmol/L)	10.84 ± 1.50^{a}	4.82 ± 0.71^{b}	4.07 ± 0.65^{b}
SOD (U/mg protein)	0.19 ± 0.02^{a}	0.16 ± 0.03^{ab}	0.11 ± 0.008^{b}

Mean ± SE bearing different superscript (a, b) in a row differ significantly ($P < 0.05$)

Table 4.7.b: Effect of season on oxidative stress markers of follicular fluid in large follicles

Parameters	Autumn	Winter	Summer
TAA (µmol/L)	15.24 ± 1.62^{a}	8.77 ± 0.77^{b}	4.14 ± 0.50^{c}
SOD (U/mg protein)	0.25 ± 0.02^{a}	0.08 ± 0.02^{b}	0.06 ± 0.008^{b}

Mean ± SE bearing different superscript (a, b, c) in a row differ significantly ($P < 0.05$)

Table 4.7.c: Effect of season on oxidative stress markers in pooled follicular fluid

Parameters	Autumn	Winter	Summer
TAA (µmol/L)	13.04 ± 1.24^{a}	6.79 ± 0.77^{b}	4.11 ± 0.37^{c}
SOD (U/mg protein)	0.22 ± 0.02^{a}	0.12 ± 0.02^{b}	0.08 ± 0.008^{b}

Mean ± SE bearing different superscript (a, b, c) in a row differ significantly ($P < 0.05$)

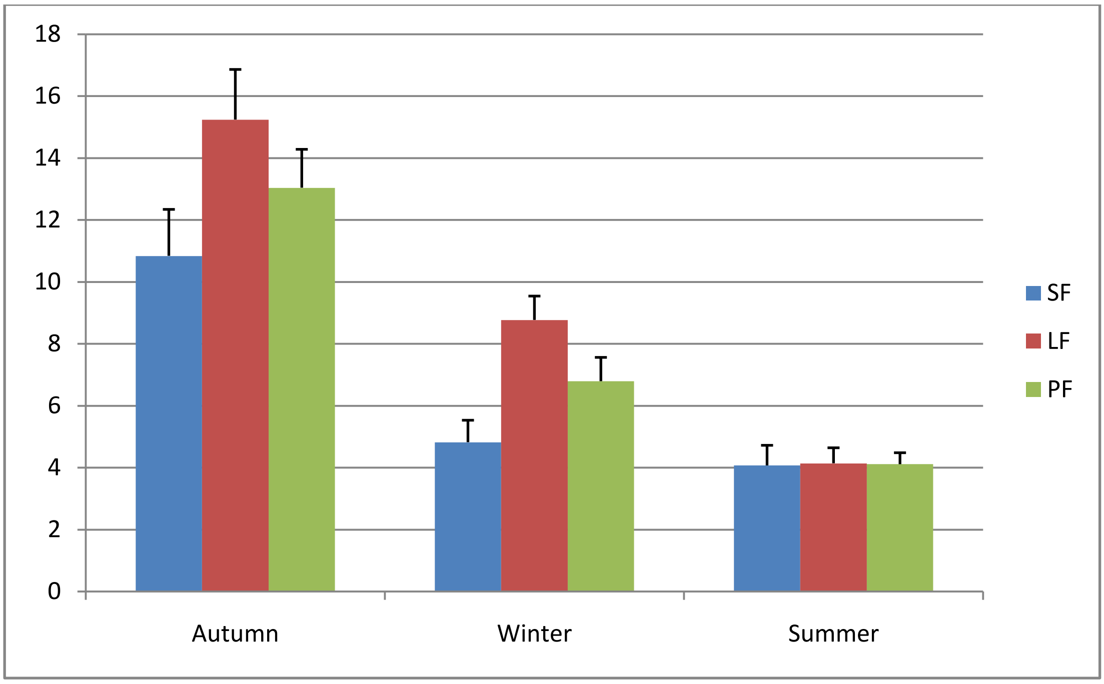

Fig. 4.12: Bar diagram showing effect of seasons on TAA in pooled follicular fluid

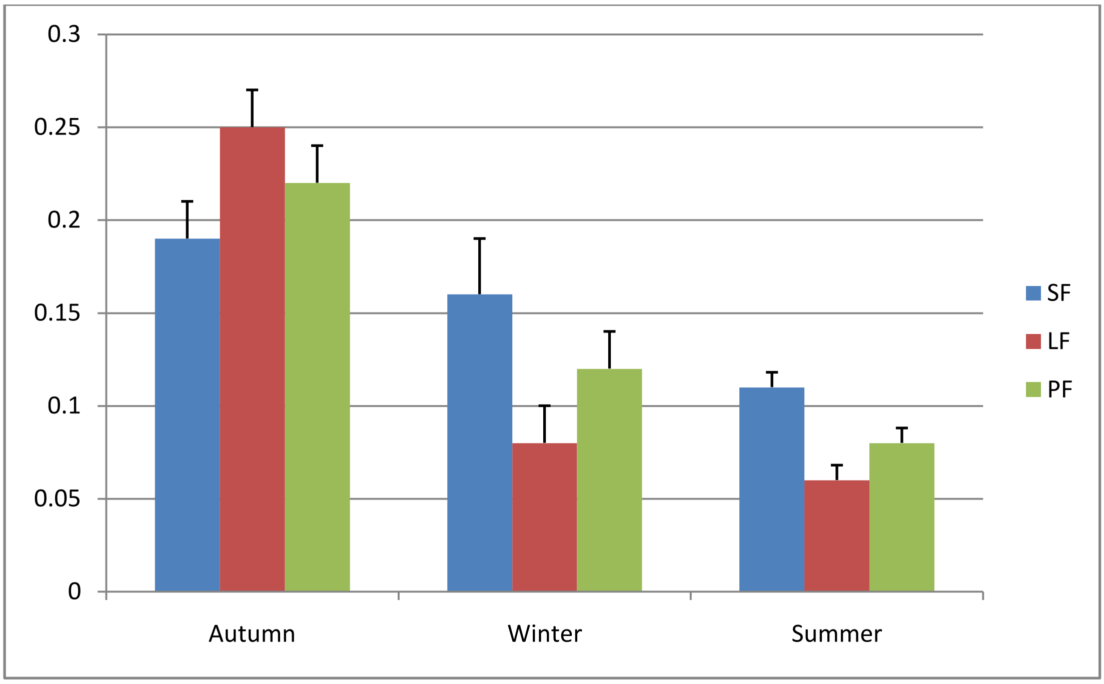

Fig. 4.13: Bar diagram showing effect of seasons on SOD activity in pooled follicular fluid

4.3.13 Follicular fluid SOD

SOD activity in follicular fluid of SF, was observed as 0.19 ± 0.02 U/mg protein in autumn, 0.16 ± 0.03 U/mg protein in winter and 0.11 ± 0.01 U/mg protein in summer. SOD activity in follicular fluid of SF during summer was significantly different ($P < 0.05$) with that during autumn and winter (Table 4.8.a, 4.8.b and 4.8.c). SOD in follicular fluid of LF was observed to be highest during autumn (0.25 ± 0.02 U/mg protein). A significant difference ($P < 0.05$) during autumn as compared to winter and summer was observed in follicular fluid harvested from pooled follicles (Table 4.7.c).

Table 4.8.a: Oxidative stress markers of follicular fluid in small and large follicles during autumn

Parameters	SF	LF
TAA (µmol/L)	10.84 ± 1.5	15.24 ± 1.62
SOD (U/mg protein)	0.19 ± 0.02	0.25 ± 0.02

Mean ± SE bearing different superscript (a, b) in a row differ significantly ($P < 0.05$)

Table 4.8.b: Oxidative stress markers of follicular fluid in small and large follicles during winter

Parameters	SF	LF
TAA (µmol/L)	4.82 ± 0.71^{a}	8.77 ± 0.77^{b}
SOD (U/mg protein)	0.16 ± 0.03	0.08 ± 0.02

Mean ± SE bearing different superscript (a, b) in a row differ significantly ($P < 0.05$)

Table 4.8.c: Oxidative stress markers of follicular fluid in small and large follicles during summer

Parameters	SF	LF
TAA (µmol/L)	4.07 ± 0.65	4.14 ± 0.5
SOD (U/mg protein)	0.11 ± 0.008^{a}	0.06 ± 0.008^{b}

Mean ± SE bearing different superscript (a, b) in a row differ significantly ($P < 0.05$)

DISCUSSION

CHAPTER-V

DISCUSSION

In the present work the effect of season was studied on the concentration of different biochemical metabolites, minerals and stress markers in follicular fluid (FF) of small follicles (SF) and large follicles (LF) present on surface of ovine ovary. During the study maximum average environmental temperature was observed during summer (36.12^{0}C), lowest during winter (18.90^{0}C) and intermediate during autumn (31.90^{0}C). The observed relative humidity was highest during winter (77.11%), intermediate during autumn (69.75%) and lowest during summer (49.09%). So, from temperature and R.H of seasons, it could be concluded that animals faced maximal stress during winter and minimum during autumn. Though, in summer the temperature was highest, but due to low R.H, stress undergone by the animals was lower than that during winter (due to interaction of low temperature and R.H).

5.1 Effect of season on ovarian follicular population

The number of ovarian follicles is an important indicator of the process of folliculogenesis. The average number of SF on surface of ovary in the present study were low in different seasons, i.e, 3.25, 2.76 and 2.83 per ovary during autumn, winter and summer. Similarly, observed LF on surface of ovaries were 1.51, 1.07 and 1.05 per ovary during autumn, winter and summer respectively. In this study the ovaries were categorised as LF which were > 6 mm in diameter and SF which were < 6 mm in diameter. But the previous workers categorised the size much less than the size adopted during present study (Evans *et al*., 2000).

Lower no. of LF observed per ovary might be due to smaller non-descript local breed of sheep with smaller ovary. Moreover, the follicles in the present study were recorded in the slaughtered animals and these animals were usually slaughtered in unreproductive state due to subfertility, poor body condition and ageing. The lower follicular turn over in summer and winter against autumn in the present study was in agreement with the earlier studies in sheep (Ravindra and Rawlings, 1997). Hence, the present study suggested that the ovine ovary was sensitive to climatic condition and a seasonal variation existed in ovarian activity.

5.2 Biochemical profile of sheep follicular fluid in relation to season

The follicular fluid make up the micro environment of the oocyte (Leroy *et al*., 2004). Follicular fluid is a mixture of exudates of serum and secretions of locally present cells. The product of those cells are related to metabolic activity of follicular cells. The metabolic activity and the blood follicle barrier properties change during the growth phase of follicle as well as under the influence of climatic parameters. Most substances present in follicular fluid could diffuse freely in and out of follicle. Though, equilibrium exists, the follicle was never static due to changes in volume and metabolism (Gosden *et al*., 1988).

5.2.1 Effect of season on glucose concentration in follicular fluid

Glucose plays a pivotal role in cellular metabolism as it is the major energy source to them. In ovary, it also acts as the chief energy source and energy derived mainly by anaerobic glycolysis. The present study suggested that the glucose concentration in follicular fluid of large and small follicles present on ovine ovary during autumn season were significantly ($P < 0.05$) higher than the glucose concentration in follicular fluid of similar follicles during summer and winter. The study also indicated that glucose concentration in follicular fluid of large follicles during summer and autumn were significantly ($P < 0.05$) higher than that of SF. But the concentration of glucose in follicular fluid of LF and SF were observed to be similar during winter season. The result suggested that the glucose concentration in follicular fluid of ovarian follicles increased as their size increased which corroborated the finding of previous workers Nandi *et al*. (2007) in sheep; Leroy *et al*. (2004) in cattle; Tripathi *et al*. (2015) in ruminants; Chang *et al*. (1976) in sows and Thakur *et al*. (2003) in goats. This indicated that glucose metabolism by large follicle cells were less intensive than those of small follicles. Moreover, in LF, less number of granulosa cells present per unit volume of follicular fluid in comparison to SF, hence lesser granulosa cells consume less amount of glucose from follicular fluid. Again, increased permeability of follicle blood barrier in growing follicles might be the other reason for increased glucose concentration in large follicles. In winter season, as more energy is required to maintain the body temperature, more glucose might be taken up by other tissues of body and less glucose filtered through capillary membrane into follicular fluid. Hence, no difference in glucose concentration in FF was observed between SF and LF. During autumn, many primordial follicles might left behind their dormancy and entered into the active folliculogenesis. In the process many growing follicles might be selected, formed dominant follicle and converted into the graafian follicle. So, higher metabolic activity during these stages might led to increased capillary permeability and thus increase glucose concentration in FF.

5.2.2 Effect of season on total protein concentration in follicular fluid

The total protein concentration in follicular fluid of LF was observed to be significantly ($P < 0.05$) lower than that of SF during all the seasons, i.e, autumn, winter and summer. This result suggested that the total protein content in follicular fluid significantly decreases according to the increase in size of follicles which is in agreement with the studies reported by Nandi *et al*. (2007) in sheep; Thangavel and Nayeem, (2004) in buffalo; El-Nasser and Mohammed, (2011) in all domestic ruminants. The result of present study also differed from those of Leroy *et al*. (2004) in cattle; Rahman *et al*. (2005) in buffalo; Rufai *et al*. (2013) in sheep and Jassim *et al*. (2012) in buffaloes. Wherein, an indifferent or increasing trend of protein concentration from small to large follicle was also reported by Rahman *et al*. (2005). The follicular fluid provides osmotic pressure, steroid binding proteins (SBPs) and enzymes necessary for development of oocyte. The highest concentration of total protein in follicular fluid of small follicles of sheep in present study might be to satisfy the high requirement of SBP. Moreover, as the antral volume increased with the increase of follicular size, the protein concentration decreased (Singh *et al*., 1999; Mishra *et al*., 2003). It was also reported that continuous protein equilibrium existed between plasma and follicular fluid and the protein concentration was similar in small and large follicle (Andersen *et al*., 1976).

The result of present investigation is also in agreement with the findings of Thangavel and Nayeem, (2004) that SF contained highest concentration of total protein as compared to LF in buffalo ovaries. They suggested the increase in protein concentration in small follicles might be due to action of progesterone during the luteal phase of estrous cycle.

The present investigation revealed that the total protein concentration in SF during autumn was significantly higher ($P<0.05$) than that of LF, but those during summer and winter were observed to be at par. This might be due to increase in enzyme and steroid binding protein during the breeding season of sheep. The result shown in Table 4.3.b had shown significant increase ($P<0.05$) in total protein concentration in follicular fluid of LF during autumn and winter than summer. Those increase in autumn and winter might be due to increase in SBP and loss of equilibrium between FF and plasma respectively.

5.2.3 Effect of season on albumin concentration in follicular fluid

Albumin might be required for binding some chemicals and minerals inside the follicular fluid for various physiological functions including growth and maturation of follicles (Arshad *et al*., 2005). The present investigation revealed that season had no effect on albumin content of small as well as large follicles (Table 4.3.a, 4.4.a and 4.5.a) which supports the findings of Murali Krishnan *et al*. (2005). On comparison of the albumin content of SF and LF with each other during different seasons, it was observed that those varied significantly ($P < 0.05$) with each other during autumn and summer, but not during winter (Table 4.3.a, 4.3.b and 4.3.c). This increase in albumin content in SF might be due to increase in SBA content during period of active folliculogenesis in breeding and early breeding seasons.

5.2.4 Effect of season on globulin concentration in follicular fluid

All types of protein present in follicular fluid were mostly derived from plasma. Follicular total protein was about 75% of that present in serum; albumin and globulin were 72% and 78% respectively of those present in serum. This indicates that a substantial part of the protein content in follicular fluid originates from serum (Edwards, 1974; Wise, 1987). Globulin has a significant importance in the body due to its immunity producing activity. The present study indicated that the mean globulin concentration in small follicles among different seasons did not differ significantly ($P < 0.05$) with that in large follicles. The present study also indicated season had no role in globulin concentration in follicular fluid of small and large follicles (Table 4.3.a, 4.3.b, 4.3.c). But, perusal of Table 4.4.a, 4.4.b and 4.4.c reveal that the concentration of globulin in follicular fluid of SF is significantly higher ($P<0.05$) than that in LF during each and every season indicating follicular size has more impact on its concentration. The finding of present study is in contrary with that of Arshad *et al.* (2005), who reported that the globulin concentration of LF was non-significantly higher than that of SF. Increased presence of globulin in FF of LF might be necessary for protecting the oocyte and other cell types of follicle.

5.2.5 Effect of season on total cholesterol concentration in follicular fluid

Cholesterol plays a significant role in the physiology of the ovary as it is the precursor of steroid hormones secreted by the gonad. In the present study, total cholesterol content in follicular fluid of small follicle during autumn (breeding season of sheep) was significantly higher ($P < 0.05$) than that during winter, but at par with summer. This might be due to increased transportation of cholesterol from blood to follicular fluid during breeding season for its utilisation in steroid synthesis. The same result was observed in LF during autumn season, where the concentration of cholesterol content in follicular fluid of LF was observed much higher than that during summer and winter season. The possible reason might be due to the fact that autumn is the breeding season in local sheep of Jammu.

Comparison of total cholesterol content of LF and SF during different seasons revealed that it was significantly ($P < 0.05$) higher in LF than SF during autumn (283.00 ± 8.34 mg/dl vs 147.23 ± 3.79 mg/dl). This result is in agreement with the observations made by Nandi *et al*. (2007) in sheep; Bordoloi *et al*. (2000); Thakur *et al*. (2003); Mishra *et al*. (2003) in goats; Leroy *et al*. (2004) and Deka *et al*. (2014) in cattle. This increased concentration of cholesterol in large follicles during autumn reflected the increasing demand of cholesterol for steroid synthesis during breeding season. However, the findings of present study differed with the result reported by Gregoraszczuk *et al*. (1996) in pigs and Thangavel and Nayeem, (2004) in buffalo. The decreased cholesterol level in the large follicles in those studies might be attributed to the rapid conversion of cholesterol to steroid hormones.

5.2.6 Effect of season on LDL-cholesterol concentration in follicular fluid

Cholesterol is the precursor of all steroid hormones synthesised in all the tissues as well as ovaries. Cholesterol content increased with growing follicles as evident from present study. Granulosa cells *in vitro* were able to utilise low density lipoproteins for the production of cholesterol and subsequently for the synthesis of progesterone (Savion *et al*., 1981). Studies of Enk *et al*. (1986) on follicular fluid revealed that lower content of low density lipoprotein-cholesterol (LDL-C) and higher content of high density lipoprotein-cholesterol (HDL-C) fraction present in follicular fluid. Hence, HDL-C might have a greater role in steroidogenesis. The concentration of both fractions of cholesterol inside follicle is due to presence of blood follicle barrier. Cholesterol cannot travel alone in the blood and is transported by lipoproteins. Low density lipoproteins take cholesterol from liver and distribute it to the rest of the body. But, Mishra *et al*. (2003) reported that the granulosa cells of the follicles totally depend on the cholesterol from HDL which was derived from the blood by crossing basement membrane of granulosa cells. It was reported by Wise *et al*. (1987) that as the follicles developed, the level of cholesterol also increased. In the present study it was observed that LDL-C levels in follicular fluid of LF was significantly higher ($P < 0.05$) than that of SF during autumn and in other seasons the differences were indifferent statistically. The result indicates that there might be more transportation of cholesterol along with LDL-C into follicular fluid due to more synthesis of LDL-C receptors of follicular membrane/ granulosa cells. The present finding is against the observations of previous workers like (Chang *et al*., 1976; Enk *et al*., 1986) that blood follicle barrier donot allow the passage of particle above 850 KDa like IgM, LDL and VLDL. VLDL is the precursor molecule of LDL. Sesh and Meur, (2013) were not able to detect LDL in follicular fluid of ovulatory, non-ovulatory and atretic follicles. So, the present finding of role of LDL-C in transporting cholesterol into follicular fluid needs further investigation.

The present study also indicates that LDL-C concentration in follicular fluid of both SF and LF varied significantly ($P<0.05$) during different seasons. In SF and LF it was observed to be highest during summer season and lowest during non- breeding season that is winter (Table 4.3.a and 4.3.b). Therefore, more work needs to be done to find out the lipid fractions present in follicular fluid and mechanism of their transportation across blood follicle barrier.

5.2.7 Effect of season on nitric oxide (NO) level in follicular fluid

NO is an inorganic, short lived free radical and a signalling molecule regulating a number of cellular processes. It is a stable colourless gas and moderately soluble in water. NO is one of the intra ovarian factors involved in ovarian physiology. NO is synthesised by oxidation of L-arginine to NO and L-citrulline by enzyme NO synthase (NOS). NO immediately oxidised to NO^{2-} and NO^{3-}. So, NO^{2-}

and NO^{3-} content of biological fluid reflected the concentration of NO in that. NO has a role in folliculogenesis by suppressing follicular apoptosis (Chun *et al*., 1995). Hence, NO acts as a follicle survival factor. But the apoptic suppression activity of NO is cell specific (Kaneto *et al*., 1995). Ben-Shlomo *et al*. (1994) reported that interleukin-1 (IL-1) that stimulates NO production in granulosa cells has anti-gonadotrophic action. But Hurwitz *et al*. (1991) observed NO augmented steroidogenesis in preovulatory hamster follicles. Naafia *et al*. (2011) reported that NO^{2-} and NO^{3-} concentration of the end product of NO was significantly higher ($P < 0.05$) in cyclic sheep ovarian follicular fluid than those in the acyclic ewes. In the present study, the NO content in follicular fluid of SF observed to be non significantly higher ($P < 0.05$) in winter, but concentrations in all the seasons were at par. Similar trend was observed in large ovarian follicular fluid, i.e, no change in concentration of NO during the season was observed. But, in pooled samples, its concentration significantly ($P < 0.05$) varied between autumn (breeding) and winter (non-breeding) seasons. The present study also revealed that the NO concentration of LF and SF were found to be similar in all the seasons. The result of present study is in agreement with the earlier report by Sugino *et al*. (1996) who observed no significant difference of NO^{2-} content in follicular fluid of LF and SF. The study by Faes *et al*. (2007) revealed that NO^{2-} level was not significantly effecting the apoptic process or steroidogenesis activity of granulosa cells of follicles. Similar results were reported by Basini *et al*. (1998) that NO at high concentration inhibit DNA fragmentation but at low concentration stimulate the fragmentation. But, Faes *et al*. (2007) reported that NO concentration in atretic follicles were high. So, he suggested that sudden changes in NO concentration could be detrimental to follicles instead of higher or lower concentration of NO in follicular fluid. The result of present study corroborates this finding and suggested NO concentration of follicular fluid could not be taken as a marker of active folliculogenesis.

5.3 Effect of season on mineral content in follicular fluid

It has been established that minerals present in follicular fluid are regulatory factors in follicular development and steroidogenesis. Minerals are acting as co-factors and activate different enzyme systems for oocyte development and maturation as well as affect ovarian function.

5.3.1 Effect of season on iron content in follicular fluid

Iron (Fe) is an integral part of haemoglobin which is necessary for transportation of oxygen to bodily tissues. Fe deficiency results in anaemia that affects the oxygen level in ovarian tissues and the response of ovarian receptors to the estrogen. So, its variation in follicular fluid might affect the oocyte growth and function. In the present study, it was observed that season had no significant effect on the Fe concentration in follicular fluid harvested from SF, LF or FF collected from pooled follicles. Similarly, the Fe content of follicular fluid of SF and LF did not differ significantly during winter. Whereas, Fe level in follicular fluid of LF observed to be significantly ($P < 0.05$) higher than that in SF during autumn as well as winter. This indicates that LF had a higher Fe quantity during breeding seasons. The result in present study is in agreement with the findings of Chakraborty *et al*. (2012), Sharma and Vats (1998) and Sangha *et al*. (1993) that Fe concentration was higher in large follicles than small follicles. In antral follicles higher amount of steroids stimulate haemodynamic pulses and vascular shunt for the developing follicles (Kor *et al*., 2013). The present result is contradictory to the findings reported by Nandi *et al*. (2012); Bordoloi *et al*. (2001). They have reported that Fe concentration in FF of sheep and goat LF were lower than that of SF. They have suggested that Fe content declined in ovulatory follicle might be due to ischemia. So, the present study suggested that during breeding season there might be increased haemodynamic pulses due to intrafollicular factors leading to increase in Fe concentration in growing follicles.

5.3.2 Effect of season on magnesium content in follicular fluid

Magnesium is a co-factor of all the enzymatic reactions that involve ATP. In the present work, the effect of season was studied on the Mg concentration of different sized follicular fluid of ovine ovary. It was observed that season had almost no effect on the Mg content of follicular fluid in sheep. However, its concentration significantly ($P < 0.05$) differ between LF & SF during all the seasons, i.e, summer, autumn and winter. Higher concentration of Mg in follicular fluid of LF that observed in present study coincides with the previous reports of Tripathi *et al*. (2015) and Thakur *et al*. (2003). This higher Mg content in follicular fluid might be helping in oocyte maturation at later stages of folliculogenesis as it had been established in xenopus oocytes.

The result of present work also contradicts the findings of Nandi *et al*. (2012) in which he claimed that there was no alteration in Mg concentration in different sized follicles. Bordoloi *et al*. (2001) and Kaur *et al*. (1997) also reported that Mg conc. in SFs was higher than that of LF with the suggestion that higher Mg conc. in small follicles helped the mitosis of follicular cells through formation of thrombin, a mitogen.

5.3.3 Effect of season on copper content in follicular fluid

Higher serum copper level in normal animals was observed by workers like Prasad and Rao (1997). Copper (Cu) plays an important role in early growth and development and thus associated with ovarian maturity and ovulation (Kadim *et al*., 2006). The present study reflected that Cu concentration was significantly lower in SF during breeding season, i.e, autumn (5.42 ± 0.46 µmol/L) and highest during early breeding season i.e, summer (17.36 ± 0.47 µmol/L). Similar result was observed for LFs, but highest concentration during winter, i.e, non breeding season. But the follicular fluid collected from pooled ovarian follicles revealed the similar conc. of Cu during different seasons as that of SF. Non-significant difference of Cu conc. was observed in follicular fluid between large and small follicles during seasons except summer. Non-significant difference in Cu in follicular fluid of SF and LF observed in present study is in agreement with that of Nandi *et al*. (2012), but antagonized with the results reported by Chakraborty *et al*. (2012) and Sharma and Sharma (1997). Increased conc. in LFs was correlated with increased estrogen level which could be related to increased ceruloplasmin synthesis. The non-significant decrease in Cu content in follicular fluid of LF in present study might be due to effect of estrogen.

5.3.4 Effect of season on zinc content in follicular fluid

Zinc is an essential micronutrient important for growth and development. It is essential for reproduction in both sexes. Its antioxidant property protects the cells from potential damage caused by free radicals. Zn acts as a co-factor for around 200 enzymes. Zn concentration in FF was significantly ($P < 0.0001$) lower than that of serum (Menezo *et al*., 2007, 2010a). He also found a positive co-relation between serum estradiol and Zn concentration. Zn concentration in FF increases with increase in estradiol (Ng *et al*., 1987). The present study expressed the effect of season on Zn concentration of FF. Zn level in follicular fluid of SF, LF and that obtained from pooled follicles were observed to be significantly higher ($P < 0.05$) during autumn (breeding season) compared to summer and winter. The Zn concentration in follicular fluid of LF observed to be significantly higher ($P < 0.05$) than that of SF in early breeding season (summer). But the condition is reverse in winter which agrees with the findings of Nandi *et al*. (2012) for sheep and Bordoloi *et al*. (2001) for goats. The finding of higher Zn concentration in LF during breeding season might be due to increased estrogen level in FF of LF. At this stage there is expression of Zn transporter proteins like ZnT and ZiP in oocytes and cumulus cells (Cousins *et al*., 2006; Liuzzi and Cousins, 2004). So, the Zn level might be upregulated to prevent the DNA damage in preovulatory or growing follicles due to production of more free radicals during environmental stress. So, Zn level might be increased during the period to counteract the effects of iron and copper on Fenton reaction which involved in free radical formation.

5.4 Effect of season on oxidative stress markers in follicular fluid

Follicular fluid holds considerable biological significance as it buffers the internal environment of follicle against influences of external conditions. Reactive oxygen species (ROS) are some local factors influencing folliculogenesis as well as follicular atresia. It has been proved that ROS are key signals in initiation of apoptosis in granulosa cells of antral follicle and antioxidants protect against their potential damaging effect (Agarwal *et al*., 2005). Oxidative stress occurs at cellular level when reactive metabolites of oxygen are produced faster than that they can be removed by antioxidant defense mechanism that protect ovarian follicle from ROS induced damage (Gupta *et al*., 2011; El-Shahat *et al*., 2012). In the present study, the changes in total antioxidant activity as well as SOD activity during different seasons were evaluated.

5.4.1 Effect of season on TAA in follicular fluid

The total antioxidant activity in SF was recorded highest during autumn (10.84 ± 1.5 µmol/L) that is significantly higher ($P < 0.05$) than the values obtained during winter and summer. Similar trend was observed for LF as that in SF, i.e, highest during autumn and lowest during summer. The result indicated that the increased TAA observed during breeding season of ewe. But on comparison of TAA in follicular fluid of SF and LF during different seasons; significant difference ($P < 0.05$) was observed in winter season. In this season the TAA level was higher in LF than SF. Lower TAA level in SF might be due to either neutralisation of greater amount of TAA molecule generated by ROS molecules or presence of lesser TAA level as has been observed by Jan *et al*. (2014) in acyclic buffalo. The increased TAA activity in LF during breeding season (autumn) might be due to the activation of both enzymatic and non enzymatic components of antioxidant defence mechanism. This finding corroborates the previous findings by El-Shahat *et al*. (2012) and Cassano *et al*. (1999) that the antioxidant activities of follicular fluid varies with follicular size. So, it is evident from the present study that antioxidant activity in follicular fluid altered during breeding and non-breeding season.

5.4.2 Effect of season on SOD activity in follicular fluid

SOD activity present in follicular fluid is the first enzymatic step that protects oocytes against ROS (Li *et al*., 1993). High ROS concentration damage meiotic spindle which result in poor oocyte quality. SOD activity in follicular fluid was higher in spring and do not differ significantly during autumn, winter and summer (Lasota *et al*., 2009) in pigs. SF of sheep exhibited higher SOD activity (Singh *et al*., 1998) which depends on quantity of oxygen supplied to ovary. The activity of SOD in different season varied from 0.06 to 0.25 U/mg protein. On comparison of SOD in follicular fluid of SF, LF and pooled follicles during different season, significant ($P < 0.05$) higher activity observed in autumn (breeding season). The total SOD activity recorded during autumn in SF (0.19 ± 0.02 U/mg protein), LF (0.25 ± 0.02 U/mg protein) and pooled follicles (0.22 ± 0.02 U/mg protein). Many authors ascertained SOD activity in follicular fluid of pigs (Tatemoto *et al*., 2004); ruminants (Singh *et al*., 1998) and rats (Tilly and Tilly, 1995). A clear difference in the SOD activity was ascertained only between FF taken in autumn (the highest activity) and in winter and summer (when the activity was lowest). In autumn (the breeding season of ewe), the SOD activity was high which suggests that the processes leading to production of ROS intensify during this season and a high level of SOD need to neutralise them. Sabatini *et al*. (1999) found that a high SOD activity can inhibit the rupture of follicles, a process which is based on mechanism involving ROS use. But the non significant activity of SOD in winter and summer is not clear. Further studies are needed to clarify it. Significant ($P < 0.05$) SOD activity was seen in summer season as compared to autumn and winter. Higher SOD activity in SF during winter and summer were observed. The findings of this study is in agreement with that of Singh *et al*. (1998) which estimated SOD activity of goat and sheep follicles. Small follicles of both species demonstrated higher activity, whereas, large follicles showed the lowest SOD activity.

SUMMARY AND CONCLUSION

CHAPTER–VI

SUMMARY AND CONCLUSION

The present study was conducted in the Division of Veterinary Physiology and Biochemistry, Faculty of Veterinary Sciences and Animal Husbandry, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu (SKUAST-J), R.S. Pura-181102, Jammu, from September, 2015 to May, 2016. The study was conducted to determine the effect of season on biochemical profile and oxidative stress markers in preovulatory follicular fluid in local sheep of Jammu.

The ovaries of the local breed of sheep were collected from the municipal slaughter house and transported to laboratory in icebox within one hour of slaughter. In laboratory, tissues attached to the ovaries were trimmed out with scissors and then washed with normal saline solution (NSS) fortified with gentamicin (50 mg/ml) at 37^0C. The cleaned ovaries were preserved in NSS within a BOD incubator at 37^0C till collection of follicular fluid. All visible non-atretic follicles on the surface of ovaries were categorized according to their size as small (< 6 mm) and large (> 6 mm).

Follicular fluid along with oocyte was aspirated from all visible non-atretic follicles by a 22 gauge needle attached to 5 ml syringe lubricated with oocyte collection media (OCM). The cumulus oocyte complex (COC) along with follicular fluid (FF) was pooled into 15 ml sterile plastic tube according to categorized follicles from which FF was collected and allowed to settle for 10 minutes in BOD incubator at 37^0C. The supernatant was collected gently into another 15 ml centrifuge tube. To remove the oocyte and other fine cellular debris present in the collected supernatant, follicular fluid was centrifuged at 3000 rpm for 30 minutes. Then the supernatant was collected and filtered through 0.22 μm syringe filter. Filtered follicular fluid was aliquoted and stored in sterile 2 ml eppendorf tubes at -20^0 C for analysis of metabolites and minerals. Immediately after collection of processed follicular fluid, glucose concentration, superoxide dismutase (SOD) and total antioxidant activitiy (TAA) of FF was estimated.

The glucose concentration of follicles of all sizes were observed to be significantly ($P < 0.05$) higher during autumn in comparison to other seasons indicating increased permeability of follicle-blood barrier in growing follicles during breeding season. Glucose content of follicular fluid in LFs were significantly higher ($P < 0.05$) than that of SF except during winter (non-breeding) season which suggested glucose metabolism by large follicular cells were less intensive than those of small follicles and presence of less number of granulosa cells per unit volume of follicular fluid in LF.

The total protein concentration in follicular fluid of LF was observed to be significantly ($P < 0.05$) lower than that of SF during all the seasons. The result suggested that the total protein content in follicular fluid significantly decreases according to the increase in size of follicles. The higher concentration of total protein in follicular fluid of small follicles of sheep in present study might be to satisfy the high requirement of steroid binding protein (SBP) during follicular growth. Moreover, the total protein concentration of follicles of all sizes were observed to be significantly ($P < 0.05$) higher during autumn, due to increase in enzyme and steroid binding protein &/ or loss of equilibrium between FF and plasma during the breeding season of sheep.

The present investigation revealed that season had no effect on albumin content of small as well as large follicles. Albumin content of SF significantly ($P < 0.05$) higher than that of LF except during winter season. This increase in albumin content in SF might be due to increase in steroid binding albumin content during period of active folliculogenesis in breeding and early breeding seasons.

The mean globulin concentration in small follicles among different seasons did not differ significantly ($P < 0.05$) with that in large follicles. Hence season had no significant effect on globulin concentration in follicular fluid of different sized follicles. The concentration of globulin in follicular fluid of SF is significantly higher ($P < 0.05$) than that in LF during each and every season indicating follicular size had more impact on its concentration for protection of the growing oocyte.

Total cholesterol content in follicular fluid of follicles of different size during breeding season of sheep were significantly higher ($P < 0.05$) than that during winter (non breeding season). This might be due to increased transportation of cholesterol from blood to follicular fluid as well as de novo synthesis of cholesterol during breeding season for its utilisation in steroid production. Comparison of total cholesterol content of LF and SF during different seasons revealed that it was significantly ($P < 0.05$) higher in LF than SF during autumn. This increased concentration of cholesterol in large follicles during autumn reflected the increasing demand of cholesterol for steroid synthesis during breeding season.

LDL-Cholesterol (LDL-C) levels in follicular fluid of LF was significantly higher ($P < 0.05$) than that of SF during autumn indicating increased transportation of cholesterol along with LDL-C into follicular fluid might be due to more synthesis of LDL-C receptors of follicular membrane/ granulosa cells. It was surprised to observe the LDL-C concentration in SF & LF significantly higher during early breeding (summer) season than breeding (autumn) and non-breeding (winter) seasons. So, the role of LDL-C in transporting cholesterol into follicular fluid needs further investigation.

The NO content in follicular fluid of SF and LF observed to be non significantly higher ($P < 0.05$) in winter than other seasons. The study also revealed no significant difference between NO concentration of LF and SF in any of the season. Therefore, follicular fluid NO concentration could not be considered as a marker of active folliculogenesis.

Season had no significant effect on iron concentration in follicular fluid harvested from SF, LF or FF collected from pooled follicles. The Fe content of follicular fluid of SF and LF did not differ significantly during winter. But, Fe level in follicular fluid of LF observed significantly ($P < 0.05$) higher than that in SF during autumn and winter. This indicated that LF had a higher Fe quantity during breeding season (autumn). So, this study suggested that during breeding season there might be increased haemodynamic pulses due to intrafollicular factors leading to increase in Fe concentration in growing follicles.

It was observed that season had almost no effect on the Mg content of follicular fluid in sheep. However, its concentration significantly ($P < 0.05$) differ between LF & SF during all the seasons. Higher concentration of Mg in follicular fluid of LF might be helping in oocyte maturation at later stages of folliculogenesis as it had been established in xenopus oocytes.

The Cu concentration was significantly lower in SF and pooled follicles during breeding season (autumn), and highest during early breeding season (summer). That was highest during winter (non-breeding season) in large follicular fluid. No significant difference of Cu conc. was observed in follicular fluid of large and small follicles except during summer. Increased Cu conc. enhances ceruloplasmin synthesis which could be correlated to increased estrogen level in growing follicles.

Zn level in follicular fluid of SF, LF and that obtained from pooled follicles were observed to be significantly higher ($P < 0.05$) during autumn (breeding season) than other seasons. The Zn concentration in follicular fluid of LF observed to be significantly higher ($P < 0.05$) than that of SF in early breeding season (summer). The higher Zn concentration in LF during breeding season might be due to increased estrogen level. Expression of Zn transporter proteins like ZnT and ZiP in oocytes and cumulus cells at growing stage might up regulate the Zn level to prevent the DNA damage from free radicals during environmental stress. Hence, Zn level might be helping follicular cells to counteract the effects of iron and copper on Fenton reaction which involved in free radical formation.

The total antioxidant activity (TAA) in SF, LF and pooled follicles were significantly differing according to the seasons with highest activity during breeding season (autumn) of ewe. TAA in follicular fluid of LF was significantly ($P < 0.05$) higher than that of SF during winter. Lower TAA level in SF might be due to either neutralisation of greater amount of TAA molecule generated by ROS molecules or lesser TAA. The increased TAA activity in LF during breeding season (autumn) might be due to the activation of both enzymatic and non enzymatic components of antioxidant defence mechanism to protect growing oocyte. Hence, it is evident from the present study that antioxidant activity in follicular fluid altered during breeding and non-breeding season.

SOD activity in follicular fluid of SF, LF and pooled follicles was significantly ($P < 0.05$) higher in autumn in comparison to other seasons. In autumn (breeding season of ewe), the SOD activity was high which suggested that the processes leading to production of ROS intensify during this season and a high level of SOD needed to neutralise them.

Conclusion

It may be concluded from present study that season and size of follicle have significant effect on certain biochemical profile (glucose, total protein, total & LDL cholesterol), and micro minerals (Cu & Zn) content of follicular fluid. Though there was increased total antioxidant activity in follicular fluid during breeding season of ewe, SOD (the antioxidant enzyme, first to act against ROS) activity was observed significantly lower. Hence, further studies are needed to explore the cause of lower activity of SOD as well as the concentration of NO in follicular fluid. Similarly, the function of Zn in folliculogenesis and oogenesis need to be studied in detail.

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Certificate – IV

Certified that all the necessary corrections as suggested by the external examiner/ evaluator and the advisory committee have been duly incorporated in the thesis entitled **"Effect of season on biochemical profile and oxidative stress markers in preovulatory follicular fluid in local sheep of Jammu"** submitted by **Miss Uzma Sehrish, Registration No. J-14-MV-407**.

07.09.16

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