SEASONAL DYNAMICS IN ACROSOME REACTION INDUCTION IN RAM SPERMATOZOA

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(Ph.D Thesis)

SUPERVISOR

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ADNAN MENDERES UNIVERSITY
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SAĞLIK BİLİMLERİ ENSTİTÜSÜ MÜDÜRLÜĞÜNE

AYDIN

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PREFACE

Turkey leads in farming of small ruminants throughout Europe. It holds 5th or 6th position in the world in small ruminants' farming. Sheep is the most commonly reared small ruminant of Turkey. Nearly 20% of total meat and 6% of total milk produced in Turkey comes only from sheep. There is an increasing trend of commercial sheep farming; however, until now, it is not considered as a profitable business. The low production performance of the native sheep breeds due to their poor genetic potential might be the reason of unprofitability of this business. The lack of breeding strategy and inadequate information about the productive and physiological traits of native breeds at national level are the contributing factors in this regard.

The genetic improvement in livestock has primary significance in order to get a sustainable production from agri-food industry. In this sense, artificial insemination using cryopreserved semen is a common biotechnology which provides an opportunity to wide-spread use of the genetic of superior males. In contrast to its large scale use in bovine breeding, it is not routinely practiced in sheep due to the poor fertility of frozen-thawed ram sperm. Furthermore, the poor seminal quality during anoestrous season in ram may decrease fertility after AI which is another important limiting factor for the large scale application of AI with frozen semen.

The seasonality in ram is not very well marked as it appears in sheep, however, the environmental factors, such as temperature, humidity, and photoperiod, may alter the testicular size, sexual behaviour, hormonal pattern and semen characteristics. Therefore, it was expected that seasonal changes has a definite impact on ram sperm freezability and fertility. Additionally, the premature acrosome reaction and osmotic stress are the other factors that affect sperm freezability and fertility. In this background, the present study was carried out to identify the exact time period of the year in which ram sperm have higher resistance to premature acrosome reaction and osmotic stress. The study was also the sequel of previous research work carried out by our group regarding the acrosome reaction induction, osmotic tolerance and seasonal effect on ram sperm quality. We hope that our work provides significant contribution in the scientific literature and basis for the future research in the field of animal andrology.

CONTENTS

ABBREVIATIONS

AI	Artificial insemination
ANOVA	Analysis of variance
AR	Acrosome reaction
CaM	Calmodulin
cAMP	Adenosine 3′,5′-cyclic monophosphate
CI	Calcium ionophore
DIC	Differential interference contrast
DMRT	Duncan's multiple range test
ERK	Extracellular signal-regulated kinase
FITC-PNA	Fluorescein isothiocyanate-peanut agglutinin [Lectin from *Arachis hypogaea* (peanut)]
FSH	Follicle stimulating hormone
GLM	General Linear Model
HE	Hypo-osmotic swelling and eosin
HOST	Hypo-osmotic swelling test
LH	Luteinizing hormone
LPC	Lysophosphatidylcholine
PBS	Phosphate buffer saline
PI3K	Phosphoinositide 3-kinase
PKA	Protein Kinase- A
PKC	Protein Kinase- C
PTK	Protein Tyrosine Kinase
TCG	Tris-citric acid-glucose
VDCC	Voltage dependent calcium channels

LIST OF FIGURES

1. INTRODUCTION

1.1. Significance of Sheep in Turkey

Turkey is a leading sheep and goat rearing country in the Europe, ranking 5th or 6th in the world with about 42 million head of small ruminants. Among small ruminants, sheep is commonly reared animal and its contribution to the total red meat and milk production of Turkey is 20% and 6%, respectively (Aytekin and Öztürk 2012). Sheep meat and milk are the main sources of animal origin protein for the inhabitants in the mountainous region. The trend of commercial sheep farming for meat purpose is growing in Aegean region of Turkey. The commercial sheep farming increased perhaps due to low input for extensive production and the income-demand relationship for sheep meat and milk, which attracted the stakeholders for investment. On the contrary, production performance of the native sheep breeds is low due to poor genetic potential. The reason for low production performance of sheep in Turkey is due to lacking of breeding strategy and inadequate mass of information regarding the productive and physiological traits of native breeds at national level.

1.2. Adaptation of AI in Sheep

The genetic improvement in livestock has fundamental significance to get a sustainable production from agri-food industry. In this sense Artificial insemination (AI) using cryopreserved semen is a common biotechnology which provides an opportunity to wide-spread use of the genetic of superior males. In contrast to its large scale use in bovine breeding, it is not routinely practiced in sheep due to the poor fertility of frozen-thawed ram sperm (Maxwell et al 1999, Maxwell and Evans 2009). One of the reasons is that cryocapacitation and damaged plasma membrane render the sperm's lifespan short in the female reproductive tract which consequently lead to reduced fertility in sheep. The poor seminal quality during anoestrus season in ram may decrease fertility results after AI (Santolaria et al 2011) which is an important limiting factor for the large scale application of AI with frozen semen.

1.3. Seasonal Manifestation on Ram Semen Quality

Environmental factors, such as climatic temperature, humidity and photoperiod may alter the testicular size (D'Occhio et al 1984, Kafi et al 2004), sexual behavior (Poulton and Robinson 1987, Martemucci et al 2000), hormonal pattern (Walkden-Brown et al 1994, Paz et al 2012) and semen characteristics (Pérez et al 1997, Wang et al 2014) in mammals, particularly in small ruminants. The seasonal manifestation of reproductive activity is not well marked in ram as it is observed in ewes. In fact, ovulation and oestrus in the ewe is arrested during summer, whereas, spermatogenesis and sexual activity in ram never stop and continue throughout the year. However, the sexual behaviour, hormonal activity, gametogenesis, testicular weight and volume are generally high at the end of summer and in autumn (breeding season) and low at the end of winter and in spring (non-breeding season). Resumption of sexual activity in ram is about 1-1.5 months earlier than ewes, since spermatogenesis need extra time (approximately 45 days) to complete, in contrast to ovulation which can occur within few days after the start of sexual activity. It is reported that LH and FSH levels start rising 2-4 weeks after the decrease in photoperiod (reviewed by Rosa and Bryant 2003). It clearly shows the seasonal change in photoperiod coupled with fluctuation in gonadal steroids has the regulatory impact on semen quality in ram.

The effect of season and/or day length on semen quality has been studied in different sheep breeds (Colas 1980, Boland et al 1985, Amir et al 1986, Ibrahim 1997, Kafi et al 2004, Marti et al 2012, Olah et al 2013). Recently, the seasonal wave like pattern in DNA fragmentation index has been demonstrated in ram sperm which laid down the assumption that the scrotal microenvironment may interfere with the sperm chromatin stabilization process in the epididymis (Malama et al 2013). The variation in semen quality of ram is due to complex hormonal interactions and gonadal endocrine patterns based on the day length, which differ between breeding and non-breeding periods of the year (Langford et al 1987, Lincoln et al 1990, Martemucci et al 2000).

1.4. Seasonal Influences on Ram Sperm Freezability

Ram sperm show great diversity in quality after freezing thawing in different seasons. However, discrepancies exist between different breeds. Awassi rams, as an example, showed better sperm cryosurvival rate throughout the year except summer whereas, Barbados and Suffolk breeds showed best semen freezability in winter and lowest in autumn (Olah et al 2013). In Leccese ram highest post-thaw survival of sperm with minimum damaged acrosomes were recorded in summer and autumn seasons (Alessandro and Martemucci 2003). The Angora rams, however, did not show any seasonal pattern in semen cryosurvival rate (Loubser and van Niekerk 1983). It is clear that breed genetics, environmental and seasonal variations have a significant influence on ram semen freezability.

1.5. Seasonal Variations in Acrosome Reaction Induction

Mammalian spermatozoa must undergo acrosome reaction (AR) at the site of fertilization in response to oocyte-derived stimuli (Roldan et al 1994). The AR is therefore, an essential requirement of successful fertilization and if this occurs well before the spermatozoa reach to oocyte, then the fertilization might not be successful. AR is an irreversible exocytotic process which involves fusion of the outer acrosomal membrane and the overlying plasma membrane. Disruption of the plasma and outer acrosomal membranes exposes the inner acrosomal membrane resulting in the release of hydrolytic enzyme known as acrosin which hydrolyzes the zona proteins and hence enhancing the ability of sperm to penetrate through zona pellucida (Witte and Schafer-Somi 2007, Almadaly et al 2015).

The whole process of AR involves different events including calcium influx, actin polymerization, and rise in intracellular pH, protein activation or inactivation through tyrosine phosphorylation (Siddique and Atreja 2012) along with some modifications in sperm actin cytoskeleton before structural changes of the sperm plasma membrane (reviewed by Breitbart et al 2005). *In vivo* AR occurs after the successful interaction between sperm and zona pellucida and is coupled with capacitation (Yanagimachi 1994). It can also be induced *in vitro* by using different effectors or substances. These effectors including zona pellucida (Yanagimachi 1994),

dilauroylphosphatidylcholine (PC12), calcium ionophore, lysophosphatidylcholine (Purdy and Graham 2004, Gomez-Cuetara et al 2006, Aksoy et al 2010, Ahmad et al 2014), ATP (Luria et al 2002), nitric oxide (Herrero et al 1997, Siddique and Atreja 2012) and progesterone (Harrison et al 2000) have been used to induce AR in several mammalian species. All these substances use different signal transduction pathways and mediating mechanisms which are partially known (Luria et al 2002, Liguori et al 2005, Aquila et al 2011).

In the previous studies in ram the AR has been induced successfully by using calcium agonists (Uçar and Parkinson 2003) and LPC (Ahmad et al 2013). However, the sensitivity of ram sperm to LPC and CI during different seasons and comparative efficacy of these substances to induce AR are yet to be known. The elevated and rapid calcium ionophore mediated AR during summer season (Murase et al 2007) and its association with lower litter size (Holt et al 1997) in boar suggests that the mammalian sperm receptivity to *in vitro* induced AR may fluctuate round the year. Although the AR induced by LPC or CI may be different from the physiological AR induced by the ZP (Liu and Baker 1996, Liguori et al 2005), the ability of ram sperm to respond to these substances may be useful for assessing certain aspects of sperm functions.

1.6. Osmotic Tolerance of Sperm

The ram sperm are highly vulnerable to osmotic stress and exhibit irreversible loss of motility if exposed to osmolality <125 or >600 mOsm/L (Curry and Watson 1994). Therefore, the ram sperm are difficult to cryopreserve like many other species such as bovine (Guthrie et al 2002), porcine (Gilmore et al 1998), murine (Willoughby et al 1996), equine (Ball and Vo 2001), and canine (Songsasen et al 2002), due to their high sensitivity to osmotic stress. It has been reported that the season has a significant influence on the plasma testosterone level (Kafi et al 2004), sperm plasma membrane integrity (Wang et al 2014) and expression of membrane proteins involved in cellular functions (van Tilburg et al 2014) in different species. This indicates that sperm membrane structural composition also changes under the influence of different

seasonal and climatic conditions. Consequently, it is expected that the seasonal variation may affect the plasma membrane osmotic tolerance limit of sperm.

In the ram, published data have demonstrated an adverse effect of experimental heat stress on sperm motility (Arman et al 2006), morphology (Moreira et al 2001) and fertilizing capacity (Mieusset et al 1992). Furthermore, the ram semen freezability (Alessandro and Martemucci 2003), motility and membrane integrity index, morphometric parameters and subpopulations vary significantly between seasons (Marti et al 2012). However, the information on osmotic tolerance limit of ram sperm and its fluctuation between the seasons is still lacking.

1.7. Objectives

Keeping in view of above mentioned lacking in the literature, present study was designed to evaluate the seasonal dynamics in responsiveness to different acrosome reaction inducing agents (LPC and CI) and osmotic tolerance limit of ram sperm.

2. MATERIALS AND METHODS

2.1. Animals and Management

The study was carried out during one year period from December 2013 through November 2014. Six mature regular semen donor Kivircik rams were used in this study. The rams were maintained at the experimental animal pens of the Department of Reproduction and AI, Faculty of Veterinary Medicine, Adnan Menderes University, Aydin, Turkey. The animals were offered hay and water ad libitum on daily basis. The ambient temperature and humidity of the pen was recorded at noon time on daily basis. Approval for use of the experimental animals was obtained from the ethical committee of Adnan Menderes University (Approval No. 64583101/2013/064; Dated: September 02, 2013) before the start of study.

2.2. Chemicals

The chemical used in this study are; Lysophosphatidylcholine (LPC), Calcium Ionophore (CI), Eosine, Nigrosine, Paraformaldehyde, Lectin from *Arachis hypogaea* (peanut) (FITC-PNA), Na_2HPO_4, NaH_2PO_4, Tris, Citric acid, Glucose, Sodium citrate and fructose. All these chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA).

2.3. Preparation of Stock Solutions

Tris-citric acid-glucose (TCG) buffer was prepared by dissolving 300mM Tris base, 95mM citric acid monohydrate, and 28mM glucose in 1000mL of distilled water. The pH of TCG buffer was adjusted 7.0. The 2mM CI stock solution was prepared by dissolving 10mg (w/v) of CI (A23187) in 9.55mL of dimethylsulfoxide. Whereas, the LPC stock solution of was prepared by dissolving 100mg (w/v) of L-α- lysophosphatidylcholine in 10mL of TCG solution. FITC-PNA stock solution was prepared by 5mg (w/v) of Lectin [from *Arachis hypogaea* (peanut) FITC conjugate, lyophilized powder] in 25mL of phosphate buffer saline (PBS). Paraformaldehyde fixative solution was prepared by dissolving 110mM Na_2HPO_4,

2.5mM NaH_2PO_4 and 4% w/v paraformaldehyde in 100mL of distilled water. The eosin-nigrosin stain was prepared by dissolving 1% (w/v) eosin, 3% (w/v) nigrosin, 3% (w/v) sodium citrate in 100mL of distilled water. The stock solutions of LPC and CI were frozen, whereas, all other solutions were stored in refrigerator at 4°C. All the solutions were thawed and brought to 35 °C well before use.
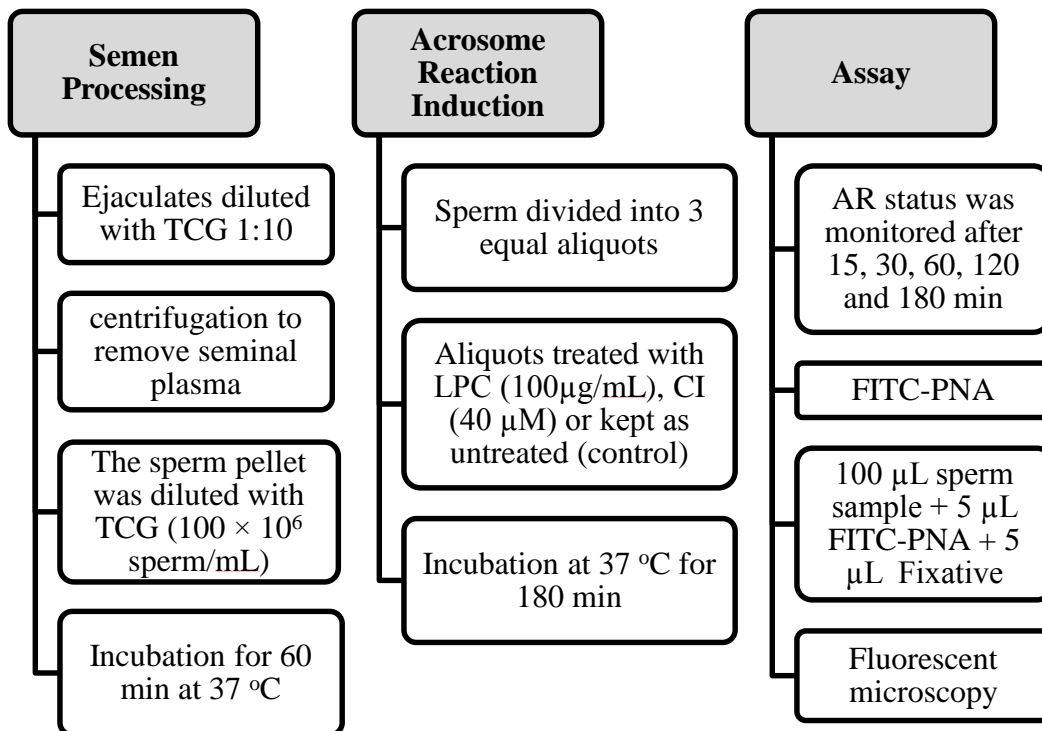
2.4. Semen Collection

Semen was collected using an electroejaculator once in a month from each ram (three ejaculates per ram per season) during winter (Dec-Feb), spring (Mar-May), summer (Jun-Aug) and autumn (Sep-Nov) throughout study period. One ram was died during the month of September; therefore, the data of 70 ejaculates were used for the analysis. The volume of each ejaculate was recorded immediately after collection and diluted 10 folds (v/v) in TCG extender. Sperm motility was assessed at ×200 magnification using a phase contrast microscope (TMS Nikon, Tokyo, Japan) equipped with a heated stage adjusted to 37°C. Sperm concentration was determined by using a Thoma counting chamber after dilution of a portion of semen in tap water (1:200 v/v). The percentage of morphologically abnormal spermatozoa in each ejaculate was determined on wet mount slides using two or three drops of semen diluted in Hancock's solution (Hancock 1952) with use of a phase-contrast microscope at ×1000 magnification. The ejaculates with a progressive motility higher than 60% and abnormal sperm rate lower than 20% were used in all replicates.

2.5. Study 1: Seasonal Changes in Acrosome Reaction: Effect of LPC, CI and Incubation Time after Stimulation

2.5.1. Study Design

The experimental design of this study has been presented in Fig. 2.1. Briefly, after initial evaluation of ejaculates in different seasons a 500µL portion of fresh ejaculates were diluted

with 5mL TCG and centrifuged twice at 800×*g* for 5min to remove seminal plasma. To determine the acrosome-reacted sperm in fresh ejaculate, a 100µL sperm sample was fixed in fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) immediately after centrifugation. The remaining sperm pellet was then diluted with TCG to final concentration $100×10^6$ spermatozoa/mL and incubated in a water bath at 37°C for 60min. After incubation, the sperm sample was divided into three aliquots and were supplemented with one of the acrosome reaction inducing agents like LPC, CI or remained as a control. LPC (from 10mg/ml stock) was mixed into the sample to a final concentration of 100µg/mL. Similarly CI (from 2mM stock) was added to the extended semen to a final concentration of 40 mM. These samples were then incubated in water bath at 37°C. At various intervals (15, 30, 60, 120 and 180min) after stimulation with LPC or CI, the acrosomal status of the samples was monitored as described earlier (Coy et al 2002) with slight modification. Briefly 100 µL sample from each aliquot was taken and exposed to 5 µL (from 200µg/mL stock) of FITC-PNA for 5min at 38°C. The reaction was stopped by adding 5µL of 4% paraformaldehyde (pH 7.4) solution.

| **Semen Processing**	**Acrosome Reaction Induction**	**Assay**
Ejaculates diluted with TCG 1:10	Sperm divided into 3 equal aliquots	AR status was monitored after 15, 30, 60, 120 and 180 min
centrifugation to remove seminal plasma	Aliquots treated with LPC (100µg/mL), CI (40 µM) or kept as untreated (control)	FITC-PNA
The sperm pellet was diluted with TCG ($100 × 10^6$ sperm/mL)	Incubation at 37 °C for 180 min	100 µL sperm sample + 5 µL FITC-PNA + 5 µL Fixative
Incubation for 60 min at 37 °C		Fluorescent microscopy

Fig. 2.1. Flowchart depicting the design of study 1.

2.5.2. Fluorescent Microscopy and Staining Pattern

For fluorescent microscopy 10 µL drop of sperm suspension including FITC-PNA was placed on a glass slide and covered by cover slip (24×50 mm sized). Slides were immediately checked under an epifluorescent microscope (Olympus BX53) equipped with differential interference contrast (DIC), multiple fluorescence filter (U-FBN/FI/BX3) and digital camera (DP26- Olympus 5.0 MP). Acrosome status was assessed and photographed in same field by using both DIC and epifluorescent optics (×1000). At least 5 photographs from 5 different fields in each slide were taken under DIC and epifluorescent optic simultaneously. A total of 200 sperm per each slide were assessed according to staining pattern as; sperm stained with FITC-PNA were classified as acrosome-reacted cells, whereas, the sperm without fluorescence were considered acrosome-intact cells (Fig. 2.2.).
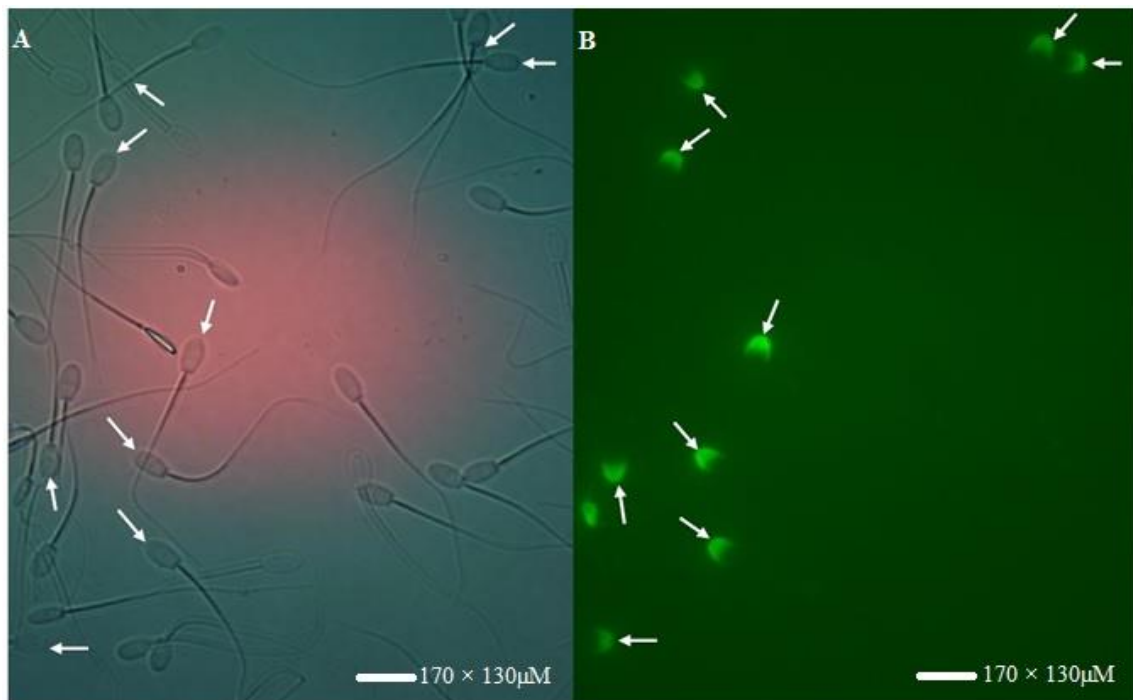
Fig. 2.2. Photographs of ram sperm taken simultaneously from the same field under DIC (A) and epifluorescent optic (B). The sperm with reacted acrosome were get stained with FITC-PNA and showed fluorescence. All acrosome reacted sperm in panel A and B are indicated with arrows. The sperm with intact acrosome did not stain and hence not appeared under epifluorescence optic. All the sperm in panel A of the image without any arrows mark have intact acrosome.

2.6. Study 2: Seasonal Changes in Osmotic Tolerance Limit of Ram Sperm

2.6.1. Study Design

The schematic flowchart (Fig. 2.3.) represents the methodology of this study. In this experiment a 100 µL portion of fresh sperm were diluted with 200 µL TCG and incubated in water bath at 35°C for 15min. After incubation, 25µL sperm sample was exposed to 475µL of fructose-sodium citrate solutions of different osmolalities including 50 (24.98mM fructose, 8.33mM sodium citrate), 100 (49.95mM fructose, 16.66mM sodium citrate), 290 (144.87mM fructose, 48.32mM sodium citrate), 500 (249.78mM fructose, 83.31mM sodium citrate), 750 (374.67mM fructose, 124.96 mMsodium citrate) and 1000 (499.56mM fructose, 166.61mM Sodium citrate) mOsm/L.

The samples were then incubated at 35°C for 15 min. The isosmotic and hyperosmotic incubation samples (290, 500, 750 and 1000mOsm/L) were returned to hypo-osmotic condition by re-suspending the sperm pellet in 100mOsm/L fructose-sodium citrate solution after centrifuging (300×g for 5min) and removing the supernatant. These samples were again incubated at 35°C for 15min.

After osmotic challenges, the sperm viability and membrane integrity were estimated using a combined hypo-osmotic swelling and eosin test (HE-test) as reported earlier (Ducci et al 2002, Ahmad et al 2014). A total of 200 sperm from each slide in five different fields were observed for live (unstained heads of sperm with or without curled tails), live intact (unstained heads of sperm with curled tails only) and total intact (stained or unstained heads with curled tails) using bright field microscope at ×400 magnification.
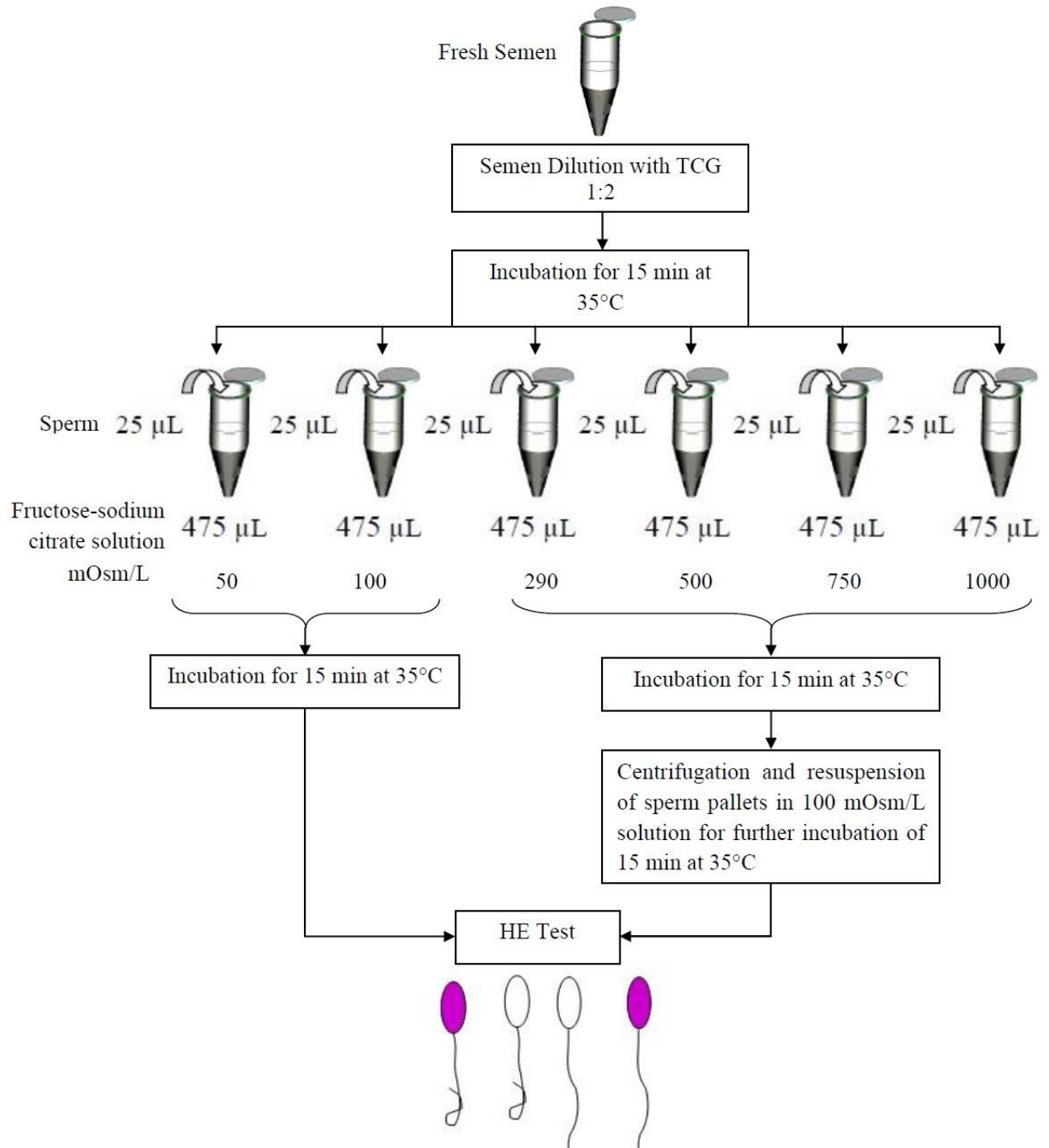
Fig. 2.3. The schematic flowchart depicting the design of study 2.

2.7. Statistical Analysis

All the statistical analyses were performed using statistical software (SPSS; version 17.0.1 Chicago, IL, USA). In study 1, initially the General Linear Model (GLM) with repeated measures was applied to determine the interactions between season × incubation time and between incubation time × inducing agent. The mean percentage of acrosome reacted sperm among different seasons at various intervals (15, 30, 60, 120 and 180min) within and between LPC, CI and control groups were compared by using one-way ANOVA. Similarly, in study 2 the percentages of live, live-intact and intact spermatozoa after exposure to different osmotic conditions were compared among seasons and osmotic challenges by using one-way ANOVA. All the data are expressed as the means ± SEM. Significance between the groups was determined by using Duncan's multiple range test (DMRT). A probability level of $P<0.05$ was set to determine the difference between the groups in both experiments.

3. RESULTS

3.1. Study 1

The seasonal changes in acrosomal status in fresh sperm are presented in Fig. 3.1. The percentage of acrosome reacted sperm stared increasing (P>0.05) from autumn and reached to its peak during winter season where it was significantly higher (P<0.05) than other seasons like spring and winter. The seasonal dynamics in AR at various incubation time intervals after stimulation with LPC, CI or control are presented in Fig. 3.2. Seasonally the acrosome reaction induced in the presence of LPC or CI showed different pattern. The percentage of acrosome reacted sperm was higher (P<0.05) in LPC and control during winter season compared to other seasons at all incubation time intervals (15 to180min). However, CI induced the AR in a time dependant manner (reacted sperm rate gradually increased during incubation time of 180min) and it varied dramatically between seasons. During winter and summer seasons, the CI-induced AR remained higher (P<0.05) at all incubation time points.

When frequency of AR occurrence irrespective of seasons over incubation time of 18 min was compared among LPC, CI and control (Fig. 3.3.), it was found that the AR induced by CI was relatively slow (P<0.05) and the reacted sperm gradually increased (P<0.05) in a linear fashion over incubation period (15 to 180min). In contrast, LPC induced abruptly reached to a plateau within 15min and rate of reacted sperm did not change (P>0.05) over the incubation time (180min). Interestingly, the reacted sperm (%) in CI treated group reached to same extent as that of LPC treated group after incubation of 180min. Conversely, no change was observed in AR status of control group over incubation time. According to GLM with repeated measures model, the interaction between seasons × incubation times was not significant (P>0.05), whereas, the interaction between incubation time × inducing agent (LPC or CI) remained significant (P<0.05).
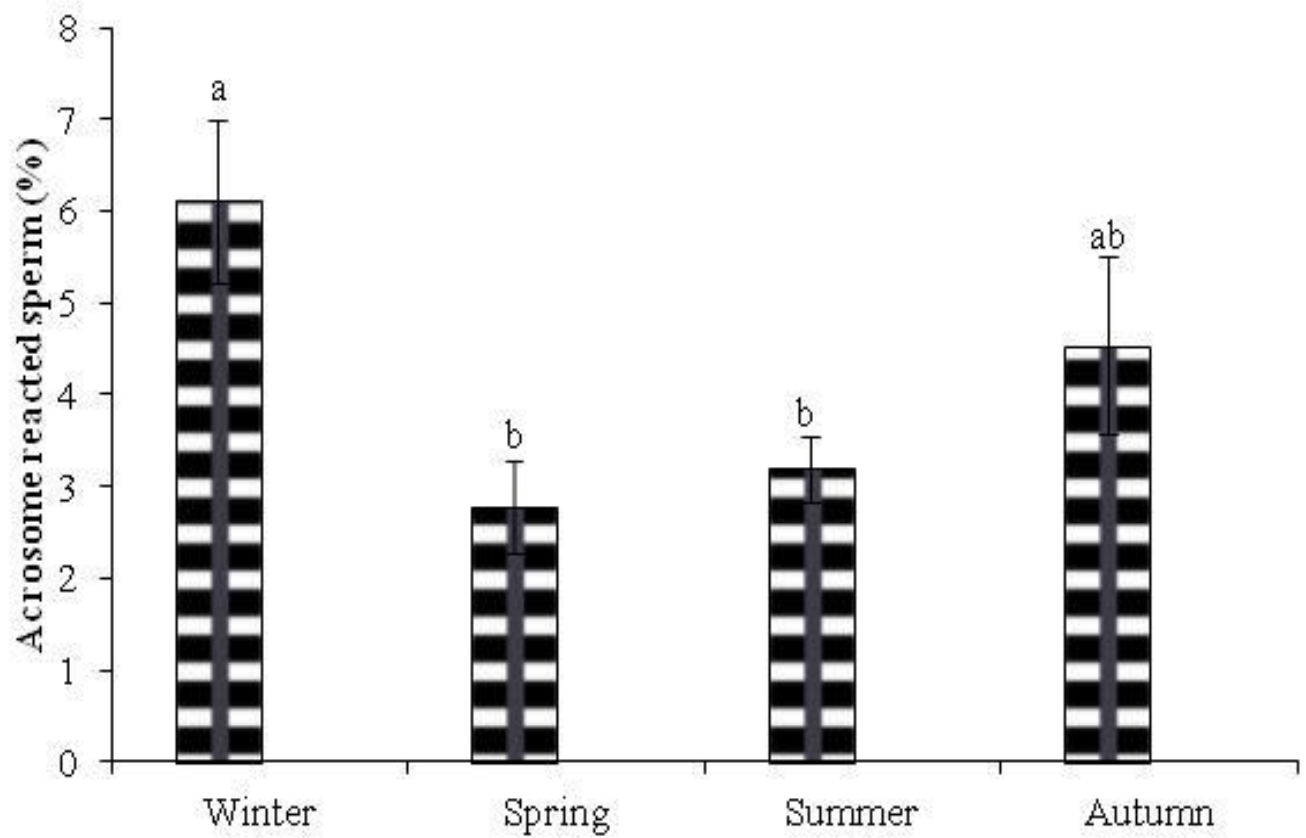
Fig. 3.1. Seasonal changes in acrosomal status in fresh ram sperm. Different superscripts show significance among seasons (P<0.05).
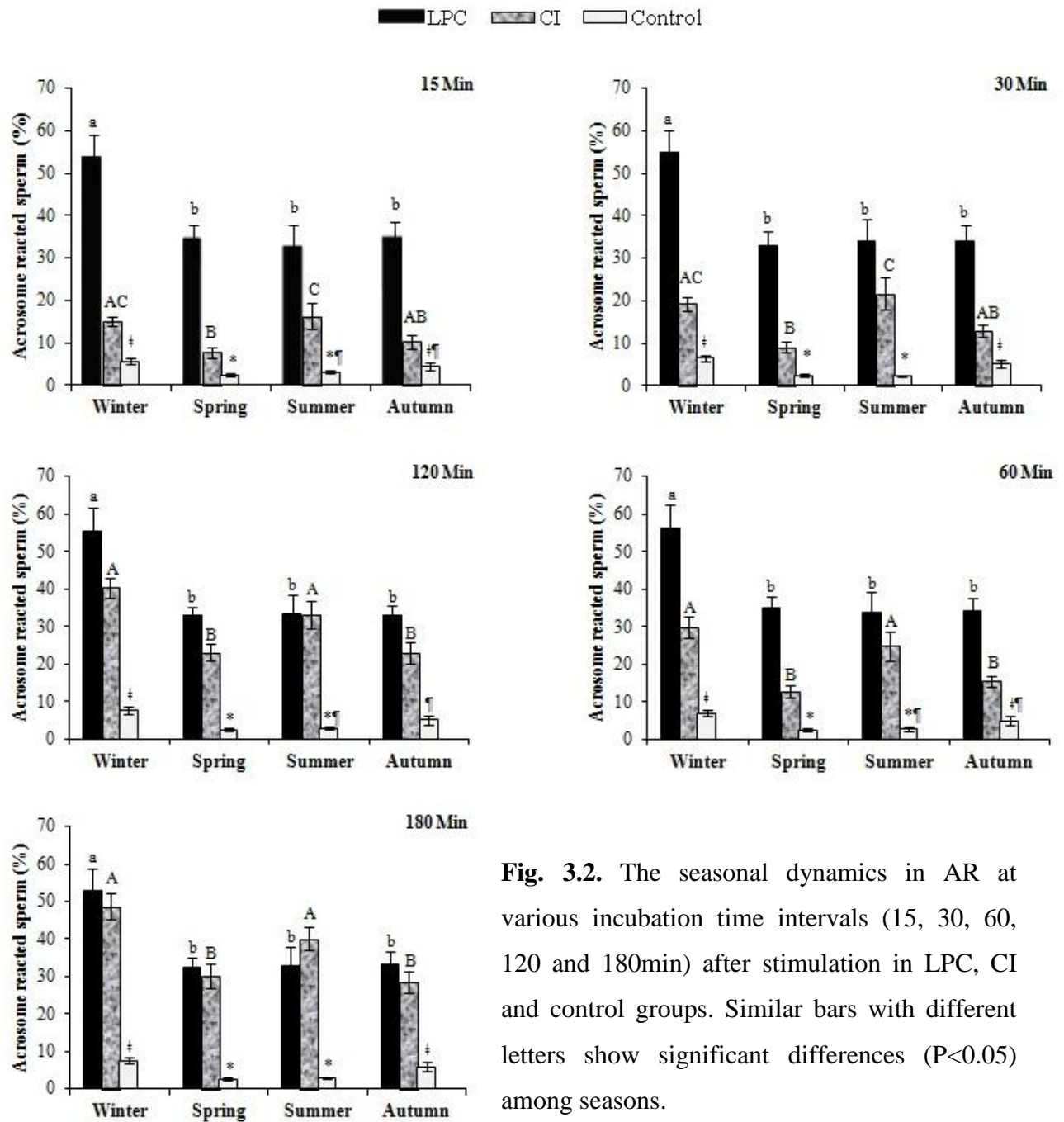
Fig. 3.2. The seasonal dynamics in AR at various incubation time intervals (15, 30, 60, 120 and 180min) after stimulation in LPC, CI and control groups. Similar bars with different letters show significant differences (P<0.05) among seasons.
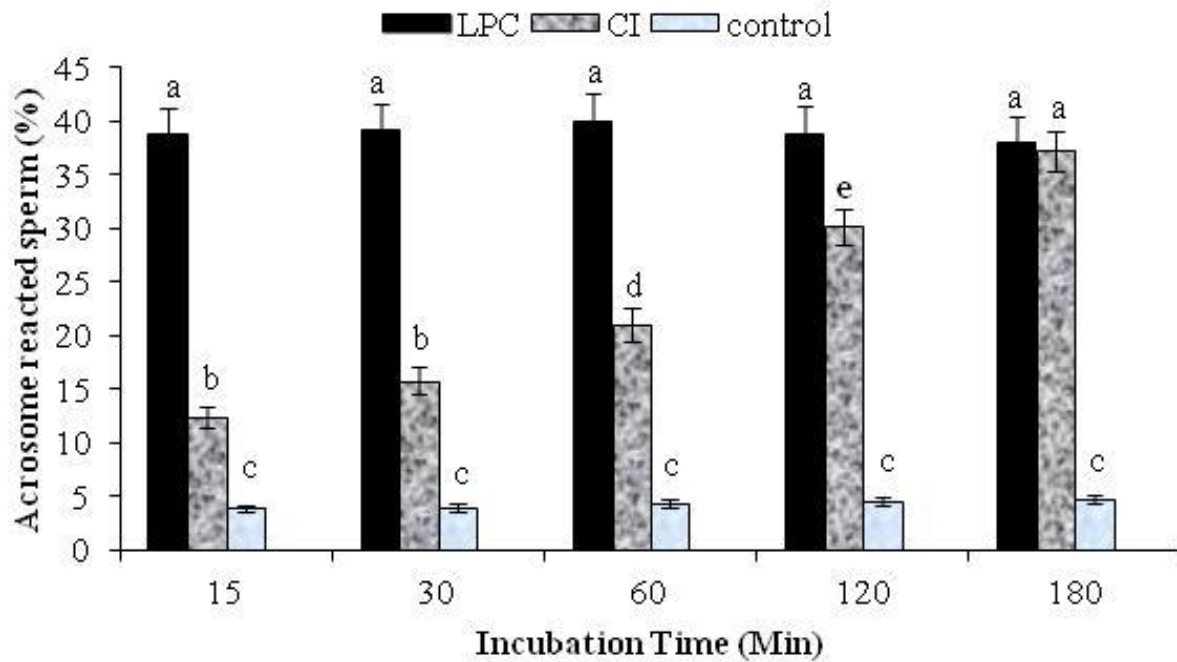
Fig. 3.3. Comparative efficacy of LPC and CI in AR induction over incubation time of 15, 30, 60, 120 and 180min. Values with different superscripts at/between various time points are significantly different (P<0.05) among LPC, CI and control groups.

3.2. Study 2

The data of seasonal changes in osmotic tolerance limit of ram sperm membrane are presented in Fig.3.4. The live sperm percentage was recorded lowest (P<0.05) at all hypo-, iso- and hyper-osmotic conditions during winter season. Similarly lowest (P<0.05) intact and live-intact sperm percentages were observed in 50, 100, 290, 500 and 1000mOsm/L conditions during winter season. However, no difference (P>0.05) was observed in intact and live-intact sperm percentages during hyperosmotic condition (750mOsm/L) among seasons. Although live cell percentages remained high in all osmotic challenge groups, the intact and live-intact percentages abruptly dropped down to 5-10% (overall) after exposure to >500mOsm/L during all the seasons. In contrast these parameters were not decreased to this much extends in hypoosmotic side. Overall the lowest (P<0.05) and highest (P<0.05) resistance to different osmotic challenges was observed during winter and autumn seasons, respectively. Regardless of the season the percentage of live, intact and live-intact sperm significantly (P<0.05) decreased with increase in osmotic pressure from 290 to1000mOsm/L (Fig. 3.5.). Similarly these parameters were also lower in 50mOsm/L compared to 100mOsm/L challenged group. The highest (P<0.05) membrane integrities were observed in 100mOsm/L group which is commonly recommended for performing hypo-osmotic swelling test (HOST).
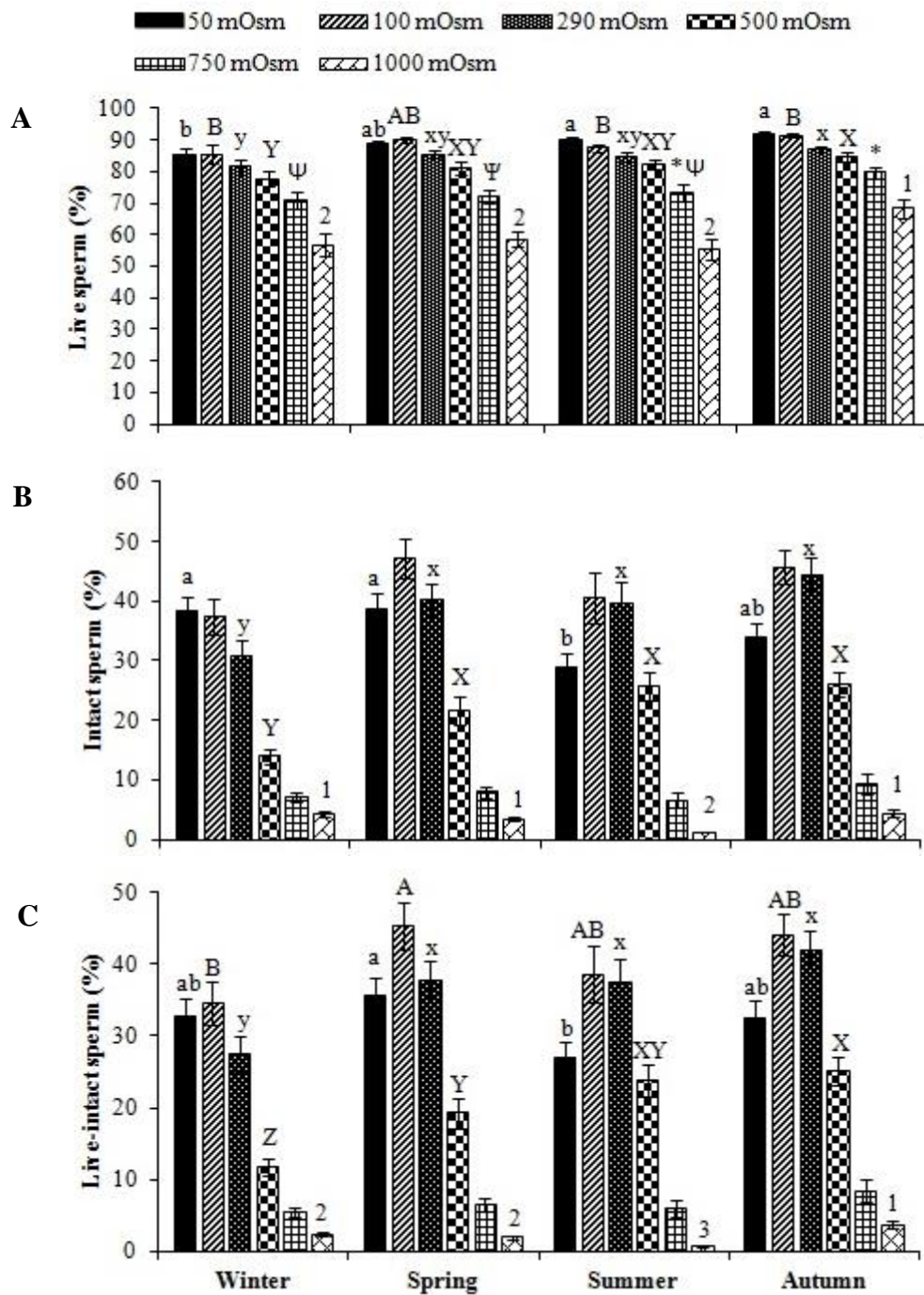
Fig. 3.4. Seasonal changes in percentages of live sperm (A), intact sperm (B) and live-intact sperm (C) during exposure to hypo-, iso- and hyper-osmotic conditions. Similar bars with different letters show significant differences (P<0.05) among seasons.
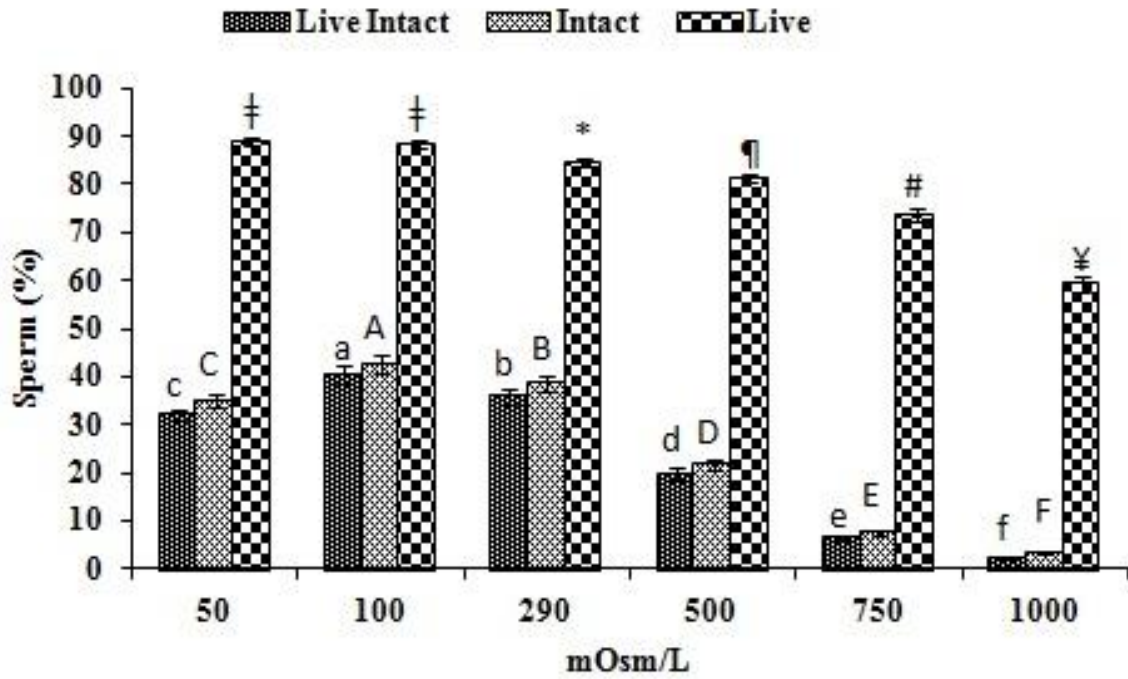
Fig. 3.5. Osmotic tolerance limits of ram sperm after exposure to hypo-, iso- and hyper-osmotic conditions for 15min. Similar bars with different letters indicates significant differences (P<0.05) among osmotic challenges.

4. DISCUSSION

The seasonal differences in behavioral and physiological reproductive activity are not well marked in rams compared to ewes. However, the seasonal fluctuations in sexual behavior, hormonal activity, gametogenesis and testicular size in ram are not the hidden facts (Rosa and Brynat 2003). Similarly, seasonal alterations in sperm quantity, quality as well as freezability in ram have been reported by many authors (Loubser and van Niekerk 1983, Alessandro and Martemucci 2003, Moghaddam et al 2012, Marti et al 2012, Olah et al 2013). Therefore, the seasonal changes in the AR induction by using different stimulating agents and osmotic tolerance limit of ram sperm have been examined in the present study. It reveals that ram sperm receptivity to undergo AR and plasma membrane permeability fluctuates under the influence of different seasons.

In the present study the two different substances (LPC and CI) were used as stimulating agents to induce acrosome reaction. This has been done to better understand that which one of these substances is responsible to provoke signaling transduction pathway efficiently in order to induce AR in a particular season, seeing as both LPC and CI use different pathways for AR induction (Aguirreburualde et al 2012, Tateno et al 2013).

LPC a product of phospholipase A_2 activity, known as fusogenic membrane disturbing lipid and also found in the ampullary fluid of the female genital tract (Ehrenwald et al 1988, Grippo et al 1994). It induces the AR by displacing sperm surface proteins, reorganizing membrane lipid domains and increasing intracellular calcium (Tarin and Trounson 1994, Riffo and Parraga 1997, Purdy and Graham 2004). LPC-induced AR is dependent on the involvement of certain kinases including extracellular signal-regulated kinase (ERK), PKC, PKA, PTK, PI3K and Akt pathway (Liguori et al 2005). Additionally, the LPC provoke the acrosin activity that is independent of calcium influx through voltage dependent calcium channels (VDCC) type L (Aguirreburualde et al 2012).

CI (A23187) has been used extensively as artificial AR inducer in variety of mammalian species (Aitken et al 1993, Yanagimachi 1994, Whitfield and Parkinson 1995, Gomez-Cuetara et al 2006, Sa-ardrita et al 2009, Aksoy et al 2010). Ionophores are lipid soluble substances and act

as a carrier for specific ions to move across the membrane by forming channels in a lipid bilayer. Thus a massive influx of Ca^{+2} into the sperm head can be achieved by avoiding natural intracellular regulatory mechanisms (Neild et al 2005). High influx of Ca^{+2} in turn plays a major role in the signal transduction pathways which ultimately lead to AR (Aitken 1997). Furthermore, the CI-induced acrosome reaction is not/partially independent on protein tyrosine phosphorylation or cAMP mediated capacitation (Grasa et al 2006, 2009). Rather the enhanced calmodulin (CaM) localization in the post-acrosomal region during CI mediated AR suggests a potential role of CaM in the events of AR (Colas et al 2009).

In the present study higher percentage of acrosome reacted sperm were observed during winter season in fresh and LPC treatment groups. Whereas, in CI treatment group a time dependant elevated acrosome reaction was observed in two different seasons (winter and summer). These data suggest that stability of acrosomal membrane might be lower in winter and summer seasons. Furthermore, the positive interaction between incubation time × inducing agent imply that responsiveness of sperm to different inducing agents may differ over incubation time.

It is well known that cholesterol plays a key role in sperm capacitation mechanism (Cross 1998, 2003, Flesch and Gadella 2000, Travis and Kopf 2002) and its efflux from the acrosomal membrane is the first obligatory step during capacitation and AR process. Similar to equine, boar and bovine, the ram sperm membrane is poor in cholesterol: phospholipid ratio compared to other mammalian species like rabbit, human and fowl (reviewed by Moce et al 2010). Since the rate of capacitation depends on the rate of cholesterol efflux from the sperm plasma membrane (Gadella et al 2001) therefore, it can be speculated that, the sperm with low cholesterol content undergo capacitation and AR more easily and faster compare to those with high cholesterol content (Witte and Schafer-Somi 2007). Recently, it was demonstrated that sterol removal stimulates acrosome exocytosis through spermin phospholipase B activation in murine (Asano et al 2013). The cholesterol and total lipid contents of ram sperm membrane are reported to be decreased during summer and winter seasons (Gundogan 2006). This special decrease in cholesterol and lipid contents perhaps made the lipid bilayer of the sperm plasma membrane more fusogenic (Witte and Schafer-Somi 2007) and hence might be the reason of increased sensitivity of ram sperm to the CI mediated AR during winter and summer seasons in the present study. However, the LPC mediated AR was higher only during winter season in this study which

suggest that different signal transduction pathway followed by LPC which might be more provoked during winter season. This may be speculated that LPC is not/partially dependant on seasonal fluctuations in cholesterol and lipids contents in ram sperm.

Plasminogen activators (PA) and Plasminogen activators inhibitors (PAI) are present in the plasma and the outer acrosomal membranes of sperm and are being released during the AR (Tsantarliotou et al 2008). PA activity is modulated by testosterone and melatonin, and it has seasonal variation (Rekkas et al 1993, Zervos et al. 2010). Sperm capacitation and AR is reported to be dependent on local fibrinolysis produced by the PA/PAI system (Taitzoglou et al 1996). The PA localization in sperm membrane and its release during AR indicate the potential participation of these proteolytic enzymes in AR (Tsantarliotou et al 2008) since melatonin influences blood testosterone and in turns testosterone can affect PA activity (Rekkas et al 1991, 1993). In addition to modulating effect of melatonin on sperm functionality via PA/PAI system, the direct role of melatonin in sperm capacitation and AR through binding with melatonin receptors type 2 (MT$_2$; Gonzalez-Arto et al 2014), mediating HCO$_3^-$ secretion (Dragileva et al 1999, Grasa et al 2006), binding with intracellular protein CaM (Colas et al 2009), and by increasing acrosin activity (Kokolis et al 2000) has been elucidated. Although the reason of the seasonal fluctuation of LPC or CI mediated AR in the present study is unclear, however, by keeping in view above explanations, it can be hypothesized that the extra receptivity of ram sperm to undergo AR in fresh, LPC or CI treated groups during winter season observed in the present study, might be due to an expected increase in melatonin during this season. Since seminal plasma proteins have a key modulating role in sperm functionality due to their decapacitating effect (Luna et al 2015), therefore, one another possible explanation of variations in AR induced by LPC and CI in different seasons might be because of seasonal fluctuations in the composition of ram seminal plasma proteins (Dominguez et al 2008).

Regardless of the season both LPC and CI induced AR successfully in ram sperm in present study. However, only 11-13% of sperm underwent AR during first 15min in CI treated group and this induction rate gradually increased up to 180min of incubation. It indicates that some of the sperm reacted rapidly under the influence of CI and others did not react visibly until the incubation time reached to 180min. The exact mechanism of this gradual increase in number of acrosome reacted sperm in CI group is not clear. It seems that AR induction rate was

relatively slow and time dependant in CI treated group. The earlier study in Japanese black bull demonstrated a rapid acrosomal exocytosis (within 1min after stimulation) triggered by ionophore/Ca^{2+} (Murase et al 2001). However, fresh (unfrozen) ram or boar sperm did not initiate acrosomal exocytosis until diacylglycerol was generated (Roldan and Murase 1994, Vazquez and Roldan 1997). Treatment of ram sperm with ionophore/Ca^{2+} resulted in acrosomal exocytosis after 5min and have reached to maximum value after 30min (Roldan and Murase 1994) of incubation. The cryocapacitation triggering by freezing and thawing facilitate the responsiveness of sperm to CI considerably (Almadaly et al 2014). Since the fresh sperm were utilized in the experiments in this study, it can be hypothesized that due to the intactness of sperm coating with seminal plasma remained undisturbed even after centrifugation, which might have prevented the capacitation of some sperm during preincubation period before CI treatment. Hence, all the sperm did not respond at one time and gradually underwent AR depending on their degree of capacitation in CI group. Whereas, in case of LPC, 36-40% of sperm underwent AR within 15min and rate of AR induction remained same over incubation period of 180min. It indicates that AR induction is abrupt, fast and independent of time in LPC treated group. This is in agreement with previous study in human (Byrd and Wolf 1986) where 85-90% of LPC treated sperm underwent rapid acrosomal reaction and motility loss in the absence of extracellular calcium. Similarly, a dose dependant LPC response was reported in the wallaby sperm by undergoing acrosomal loss (>50%) within 10min of incubation period, which laid down the speculation that LPC has detergent like action on plasma and acrosomal membrane in this species (Sistina et al 1993). However, it was demonstrated that rabbit (Kim et al 1989) and hamster (Llanos et al 1993) sperm underwent AR within 5 to 10min in the presence of LPC. Therefore, on basis of abrupt induction of AR by LPC in current study might indicates that LPC bypass some of the signaling transduction pathways which are necessary for *in vitro* induction of AR in ram sperm.

The wider osmotic tolerance limit of sperm has fundamental importance during desiccation or cryopreservation. The human (Gao et al 1995) and mouse (Willoughby et al 1996) sperm appeared more resistant to osmotic stress. In contrast, the ram sperm appeared to have high sensitivity to osmotic stress (Curry and Watson 1994) like equine (Ball and Vo 2001) and boar (Gilmore et al 1998) due to lower cholesterol to phospholipids ratio in their membranes (Bailey et al 2000) and high hydraulic conductivity (Duncan and Watson 1992) and hence

remain difficult to cryopreserve. Since the season has significant influence on ram sperm quality and freezability (Alessandro and Martemucci 2003, Olah et al 2013) and it is evident that the reproductive performance has markedly increased during late summer and autumn seasons in ram (Rekkas et al 1993, Avdi et al 2004). Seasonal variation in osmotic tolerance limit was expected in ram sperm in the present study. The highest membrane integrity rate in terms of live, intact and live-intact sperm proportions after exposure to anisoomotic media were recorded in autumn season whereas, these parameters were lowest during winter season under the same osmotic conditions in this study. The abrupt decrease in intact and live-intact sperm percentages after exposures to >500mOsm/L during all seasons indicates the higher sensitivity of ram sperm towards hyperosmotic stress. It is well known that low temperatures impair the sperm functions by destabilizing the plasma membranes. The destabilizing phenomenon during cold-shock stress is very well marked in ram sperm than those of other species. The addition of seminal plasma proteins to spermatozoa before and ∕ or after cooling is able to minimize cryoinjury effects (reviewed by Muino-Blanco et al 2008). Dominguez et al (2008) reported that the treatment of ram sperm with seminal plasma which was previously collected during autumn season had increased the concentrations of certain proteins on sperm surface. The binding of these specific proteins during autumn season might have stabilizing effect on ram sperm membrane and hence showed best membrane integrity even after exposure to anisoosmotic conditions in the present study.

The previous reports demonstrated that addition of cholesterol to ram sperm increased their osmotic tolerance limits in a wide range (Moce et al 2010, Ahmad et al 2013). It seems that increased cholesterol in the plasma membrane appears to increase the volume changes that sperm can endure. In the present study increased osmotic tolerance in sperm was observed during autumn season which might be due to increased cholesterol contents in sperm membrane during autumn season compared to other seasons (Gondogan 2006). Moreover, the presence of melatonin and testosterone in ram seminal plasma has seasonal variations. It was demonstrated that melatonin is involved in the regulation of semen quality by maintaining the membrane integrity in ram sperm (Casao et al 2010).

The sperm behaves like an ideal osmometer and is very sensitive to changes in extracellular osmolality. Exposing sperm to hypoosmotic condition cause water influx due to concentration gradients and in response the sperm continue to swell until the maximum volume

to its given surface area (Curry and Watson 1994). In contrast, the sperm become shrink upon exposure to hyperosmotic condition and membrane damages occur due to continuous outflux of water until equilibration is achieved (Meyers 2005). Remodeling of actin cytoskeleton in sperm in response to hyperosmotic stress may cause sublethal cell damage (Correa et al 2007). Although, the plasma membrane integrity after exposure to hyperosmotic solutions is relatively innocuous, becomes damaging after returning to isosmotic conditions. The cell shrinkage induces membrane loss, so that when returned to isosmolality, the cells lyse in attempting to return to their normal volume (Willoughby et al. 1996). In the present study the sperm were returned to hypoosmotic condition after their first exposure to iso or hyperosmotic challenges. Since exposures to hyperosmotic condition was damaging and returning back to hypoosmotic condition exaggerate the existed damage might be the reason of abrupt decrease in membrane integrities in 500 or more osmotic challenge groups of present study.

In conclusion the AR induced in the presence of LPC or CI showed different pattern in different seasons. This indicates that both LPC and CI use different signal transduction pathway for AR induction. It can be hypothesized that the extra receptivity of ram sperm to undergo AR in fresh, LPC or CI treated groups during winter season as observed in the present study, might be due to an expected increase in melatonin during this season. Moreover, season has significant influence on osmotic tolerance limit of ram sperm. The best osmotic tolerance during autumn might be the reason of better fertility in breeding season in ram.

5. CONCLUSIONS

Based upon the results of present study it can be concluded that ram sperm sensitivity to different AR inducing agents is varying seasonally. The percentage of acrosome reacted sperm was higher in fresh, LPC and control samples during winter season compared to other seasons at all incubation time intervals. Conversely the percentage of acrosome reacted sperm was higher in winter and summer seasons in CI treated samples. The pattern of AR induction depends upon the type of inducing agent used. It has been observed both LPC and CI can induce AR effectively in ram sperm however; AR induced by LPC is abrupt within 15 min and remained platitude. Whereas, AR induced by CI is time dependant and increased in a linear fashion over incubation time (reacted sperm rate gradually increased). The exact mechanism of seasonal fluctuations in AR induction is not well understood, however, it can be hypothesized that the alterations in receptivity of ram sperm to undergo AR in fresh, LPC or CI treated groups during different seasons might be related with seasonal changes in melatonin and cholesterol.

Season has significant influence on osmotic tolerance limit of ram sperm. During winter season the membrane integrity was recorded lowest at all hypo-, iso- and hyper-osmotic conditions. Although live cell percentages remained high in all osmotic challenge groups, the intact and live-intact percentages abruptly dropped down to 5-10 % (overall) after exposure to > 500 mOsm/L during all the seasons. In contrast these parameters were not decreased to this much extends in hypoosmotic side. Regardless of the season the percentage of live, intact and live-intact sperm decreased under hyperosmotic condition. It can be assumed that cholesterol contents of plasma membrane, seminal plasma proteins, melatonin and testosterone in ram seminal plasma has seasonal variations, which might be involved in regulating the membrane integrity in ram sperm. In sum up this study reveals that ram sperm receptivity to undergo AR and plasma membrane permeability fluctuates under the influence of different seasons.

Seasonal Dynamics in Acrosome Reaction Induction in Ram Spermatozoa

SUMMARY

Two longitudinal studies were conducted to evaluate the seasonal dynamics in acrosome reaction induction and osmotic tolerance limit of ram sperm. Semen was collected using an electroejaculator once a month from 6 mature rams (three ejaculates per each ram per season) throughout study period during winter (Dec-Feb), spring (Mar-May), summer (Jun-Aug) and autumn (Sep-Nov). In study 1 acrosome reaction was induced *in vitro* by using different inducing agents; LPC and CI. The samples were then incubated in water bath at 37°C for 3 hrs. At various intervals (15, 30, 60, 120 and 180 min) after stimulation with LPC or CI, the acrosomal status of the samples was monitored by using fluorescent staining (FITC-PNA). The AR induced in the presence of LPC or CI showed different pattern in different seasons. The percentage of acrosome reacted sperm was higher ($p < 0.05$) in LPC and control during winter season compared to other seasons at all incubation time intervals (15-180 min). However, CI induced the AR in a time dependant manner (reacted sperm rate gradually increased over incubation time of 180 min) and it varied dramatically between seasons. During winter and summer seasons, the CI-induced AR remained higher ($p < 0.05$) at all incubation time points. In study 2 the sperm samples were exposed to fructose-sodium citrate solutions of different osmolalities (50,100, 290, 500 and 1000 mOsm/L). The samples were then incubated at 35°C for 15 min. The isosmotic and hyperosmotic incubation samples (290, 500, 750 and 1000 mOsm/L) were returned to hypo-osmotic condition. After osmotic challenges, the sperm viability and membrane integrity were estimated using a combined hypo-osmotic swelling and eosin test (HE test). The live sperm percentage was recorded lowest ($p < 0.05$) at all hypo, iso and hyperosmotic conditions during winter season. Similarly lowest ($p < 0.05$) intact and live-intact sperm percentages were observed in 50, 100, 290, 500 and 100 mOsm/L conditions during winter season. The highest membrane integrity rate in terms of live, intact and live-intact sperm proportions after exposure to anisoomotic media were recorded in autumn season whereas, these parameters were lowest during winter season under the same osmotic conditions in this studyBest membrane integrity was recorded during autumn season. In conclusion both LPC and CI are use different pathways for AR induction and responsible to provoke signaling

transduction pathway. It can be hypothesized that the extra receptivity of ram sperm to undergo AR in fresh, LPC or CI treated groups during winter season as observed in the present study, might be due to an expected increase in melatonin during this season. Moreover, season has significant influence on osmotic tolerance limit of ram sperm. The best osmotic tolerance during autumn might be the reason of better fertility in breeding season in ram.

Key word: Season, ram sperm, acrosome reaction, osmotic tolerance

Koç Spermatozoonlarinda Akrozom Reaksiyonunun Uyarilabilme Oranlari Üzerine Mevsimsel Etkilerin Araştirilmasi

ÖZET

Koçlarda akrozom reaksiyonunun uyarılması üzerine mevsimsel etkilerin ve ozmotik tolerans limitlerinin incelenmesi amacıyla iki paralel çalışma yürütüldü. Toplam 6 baş ergin koçtan ayda bir kez olmak üzere tüm çalışma süresince kış (Aralık-Şubat), ilkbahar (Mart-Mayıs), yaz (Haziran-Ağustos) ve sonbahar (Eylül-Kasım) mevsimleri süresince (her koç her mevsimde 3 kez ejekülat verdi) sperma örnekleri elektroejekülatör yardımıyla toplandı. Çalışmanın ilk bölümünde akrozom reaksiyonunun uyarılması amacıyla farklı ajanlar, LPC ve CI, kullanıldı. Sperma örnekleri 37°C de 3 saat boyunca inkübe edildiler. LPC veya CI ile uyarıldıktan sonra çeşitli aralıklarla (15, 30, 60, 120 ve 180 dakika) örnekler toplanarak akrozomal bütünlük floresan boyama (FITC-PNA) ile tespit edildi. LPC ve CI ile uyarılan akrozom reaksiyonu oranları farklı mevsimlerde farklı oranlar oluşturdu. Tüm inkübasyon süreleri (15-180 dakika) için akrozom reaksiyonuna giren spermatozoon oranları LPC ve kontrol grupları için kış aylarında maksimum düzeye ulaştı ($P<0.05$). Ancak, CI ile uyarılan akrozom reaksiyonu oranları inkübasyon süresine bağlılık gösterdi (reakte akrozom oranı 180 dakikalık inkübasyon süresince kademeli olarak yükseldi) ve mevsimlere göre önemli ölçüde değişti. Kış ve yaz aylarında CI ile uyarılarak akrozom reaksiyonuna giren spermatozoon oranları tüm inkübasyon süreleri için daha yüksek ($P<0.05$) bulundu. Çalışmanın ikinci bölümünde sperma örnekleri farklı ozmotik basınçlara sahip (50,100, 290, 500 ve 1000 mOsm/L) früktoz-sodyum sitrat solüsyonlarına maruz bırakıldılar. Bu örnekler 35°C de 15 dakika boyunca inkübe edildiler. İzoozmotik ve hiperozmotik solüsyonlarda inkübe edilen örnekler (290, 500, 750 ve 1000 mOsm/L) hipoozmotik koşullara yeniden döndürüldüler. Bu ozmotik şok uygulamalarından sonra spermatozoon canlılık oranları ve membran bütünlükleri eosin boyaması ve hipoozmotik şişme testinin kombine kullanıldığı (HE testi) bir metot yardımıyla değerlendirildi. Hipo, izo ve hiperozmotik koşullarda inkübe edilen spermatozoonlarda en düşük ($P<0.05$) canlı spermatozoon oranları kış mevsiminde elde edildi. Benzer biçimde 50, 100, 290, 500 ve 100 mOsm/L lik solüsyonlarda inkübe edilen örneklerde en düşük ($P<0.05$) membran bütünlüğü tam ve membran bütünlüğü tam-canlı spermatozoon oranları kış aylarında tespit edildi. Anizoozmotik

solüsyonlarda inkübasyon sonrasında canlı, membran bütünlüğü tam ve canlı-membran bütünlüğü tam spermatozoon oranları en yüksek olarak sonbaharda tespit edildi, bununla beraber bu parametrelerin aynı ozmotik inkübasyon koşullarında kış aylarında en düşük olduğu gözlendi. Bu açıdan bakıldığında membran bütünlüğü açısından en iyi mevsimin sonbahar olduğu tespit edildi. Sonuç olarak, LPC be CI un akrozom reaksiyonunun uyarılması için farklı yolakları kullandıkları ve sinyal iletim yolaklarının uyarılmasından sorumlu oldukları belirlendi. Taze, LPC ve CI ile akrozom reaksiyonu uyarılan koç spermatozoonlarında bu çalışmada kış aylarında belirlenen aşırı duyarlılığın bu dönemdeki artan melatonin düzeyleri ile ilişkili olabileceği düşünülebilir. Bununla birlikte, mevsimlerin koç spermatozoonlarının ozmotik toleransları üzerine önemli etki yaptığı görülmüştür. En yüksek fertilite düzeylerine ulaşılan, aşım sezonu olan sonbahar aylarında en geniş ozmotik tolerans sınırlarının belirlenmesinin aslında rastlantısal olmadığı düşünülmektedir.

Anahtar Kelimeler: Mevsim, koç sperması, akrozom reaksiyonu, ozmotik tolerans

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RESUME

I was born on July 15, 1985 in a small village (65/12.L) of Tehsil Chichawatni District Sahiwal, Punjab, Pakistan. I passed matriculation and intermediate exams from the Board of Intermediate and Secondary Education Multan, Pakistan in 2000 and 2002, respectively. Later I joined the University of Veterinary and Animals Sciences (UVAS), Lahore, where I got DVM and M.Phil degrees in 2007 and 2010, respectively. UVAS is one of the oldest veterinary institutes in South Asia and I am proud of being alumni of this prestigious institute.

My earlier studies on "Relationship of age to body weight, scrotal circumference, testicular ultrasonograms, and semen quality in Sahiwal bulls" dealt with the development of physical and reproductive standards for selection of Sahiwal breeding bulls. This pioneer study published in Tropical Animal Health and Production. During the year 2008-2010, I assisted Prof. Dr. Nasim Ahmad of UVAS, Lahore as an integral part of his research team and contributed actively in different research projects.

During the years 2010-2011, I have been engaged in teaching reproductive physiology in two different universities; UAVAS, Lahore and PMAS Arid Agriculture University, Rawalpindi. In 2011, I competitively won the fellowship grant from Scientific and Technological Research Council of Turkey (TUBITAK) and then started my carrier as Ph.D scholar at Adnan Menderes University, Aydin, Turkey. I presented my research work at international conferences like ICAR-2012, Vancouver, Canada and ESDAR-2014, Helsinki, Finland. I also participated in national and international trainings and workshops held in Turkey, Germany and Czech Republic during the course of Ph.D program. It is worth mentioning that my contributions in the field of veterinary sciences have been recognized by the National Academy of Young Scientists (NAYS), Pakistan and I was decorated with best young scientist prize-2013 (2nd position).

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