INFLUENCE OF OSMOLALITY, pH AND UREA ON THE BULL *(BOS TAURUS)* SPERM FUNCTIONS *IN VITRO*

Thesis

Submitted to the
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IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Master of Veterinary Science
(Veterinary Gynaecology and Obstetrics)

2022

Dedicated to....

My

Beloved Parents

&

Dear Guide

भा.कृ.अनु.प.–भारतीय पशु चिकित्सा अनुसंधान संस्थान
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Principal Scientist & Head

Dated: 2020

Certificate

This is to be certified that the research work embodied in this thesis entitled **"Influence of osmolality, pH and urea on the bull (*Bos taurus*) sperm functions *in vitro*"** *submitted by* **Dr. M. Lavanya, Roll No. M-6009,** *for the award of* **Master of Veterinary Science** *Degree in* **Veterinary Gynaecology and Obstetrics** *at ICAR-Indian Veterinary Research Institute, Izatnagar, is the original work carried out by the candidate herself under my supervision and guidance.*

It is further certified that **Dr. M. Lavanya, Roll No. M-6009,** *has worked for more than 21 months in the Institute and has put in more than 150 days attendance under me from the date of registration for the* **Master of Veterinary Science** *Degree in this Deemed University, as required under the relevant ordinance.*

(S. Selvaraju)
Thesis Research Guide

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Chairman
Advisory Committee

Certificate

We the undersigned members of Advisory Committee of ***Dr. M. Lavanya, Roll No. M-6009,*** *a candidate for the degree of* **Master of Veterinary Science** *with the major discipline in* **Veterinary Gynaecology and Obstetrics**, *agree that the thesis entitled* **"Influence of osmolality, pH and urea on the bull (*Bos taurus*) sperm functions *in vitro*"** *may be submitted in partial fulfilment of the requirement for the degree.*

We have gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate, which is being presented for the award of **Master of Veterinary Science** *Degree of this Institute.*

It is further certified that the candidate has completed all the prescribed requirements governing the award of **Master of Veterinary Science** *Degree of the Deemed University, Indian Veterinary Research Institute, Izatnagar.*

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Lavanya

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ABBREVIATIONS

%	:	Percentage
°C	:	Degree Centigrade
μg/mL	:	Microgram per milliliter
μL	:	Microliter (s)
μM	:	Micro molar
μm/s	:	Micrometer/Second
μm^2	:	Micro meter square
μm^3	:	Micro meter cube
ADAM11 B	:	Disintegrin and Metalloproteases 1 B
AI	:	Artificial insemination
ALH	:	Amplitude of lateral head displacement
ANOVA	:	Analysis of variance
AP	:	Acrosome positive
AR	:	Acrosome reacted
BCF	:	Beat cross frequency
BI	:	Before incubation
BO	:	Brackett and Oliphant medium
bp	:	Base pair
BUN	:	Blood urea nitrogen
C	:	Capacitated
cAMP	:	Cyclic Adenosine mono phosphate
CASA	:	Computer assisted sperm analysis
CD	:	Chromatin distribution
CDH1	:	Cadherin 1
cDNA	:	Complementary deoxyribose nucleic acid
CKIT	:	Kit oncogene
CP	:	Crude Protein
CR	:	Conception rate
Ct	:	Cycle threshold
CTC	:	Chlortetracycline
DABCO	:	1,4 diazo bicyclo (2,2,2) octane
DCR	:	Difference in conception rate
DNA	:	Deoxyribonucleic acid
DOI	:	Duration of incubation
DTT	:	Dithiothreitol

EFHD1	:	EF-hand containing 1
EGF	:	Epidermal Growth Factor
EGFR	:	Epidermal Growth Factor Receptor
ENO1	:	Enolase 1
ERK	:	Extracellular signal regulated kinase
ET	:	Embryo Transfer
fg	:	Femtogram
FITC	:	Fluorescein isothiocynate
FITC – PSA	:	Fluorescein isothiocyanate with pisum sativum agglutininin
FMI	:	Functional membrane integrity
g	:	Gravity
g/L	:	Gram/Litre
GAPDH	:	Glyceraldehyde 3 phosphate dehydrogenase
GC	:	Guanine-cytosine content
gDNA	:	Genomic deoxyribo nucleic acid
h	:	Hour (s)
HF	:	Holstein Friesian
HIS	:	High ionic strength medium
HNAN	:	HOS negative and acrosome negative
HNAP	:	HOS negative and acrosome positive
HOS- G	:	Hypo osmotic swelling and Giemsa test
HPAN	:	HOS positive and acrosome negative
HPAP	:	HOS positive and acrosome positive
HSF1	:	Heat shock factor
HSP90AB1	:	Heat shock protein AB1
HYP1	:	Hyperosmotic medium (355 mOsm/kg)
HYP2	:	Hyperosmotic medium (420 mOsm/kg)
Hypo	:	Hyposmotic
IDR	:	Intrinsically disordered region
Iso	:	Isosmotic
ISO	:	Isosmotic media
JC-1	:	5,5‘,6,6‘-tetrachloro-1,1‘,3,3‘-tetraethyl benzimidazolyl carbocyanine iodide
JNK	:	Jun-N Terminal kinases
LIN	:	Linearity index
lncRNA	:	Long noncoding RNA
MAPK	:	Mitogen-activated protein kinases
min	:	Minute (s)

mL	:	Millilitre (s)
mM	:	Millimolar
MMP	:	Mitochondrial membrane potential
mOsm/kg	:	Milli Osmoles / kilogram
mRNA	:	Messenger RNA
MT - ND2	:	Mitochondrial NADH dehydrogenase 2
MT-CO1	:	Cytochrome c oxidase 1
mTEY	:	Modified isotonic tris egg yolk extender
NC	:	Non capacitated
NFAT5	:	Nuclear factor of activated T lymphocytes 5
ORE	:	Osmotic response element sequence
OREBP	:	Osmotic response element sequence binding protein
OVI	:	Oviductal osmo-susceptibility index
OVI 1	:	Oviductal osmo-susceptibility index at 1 h
OVI 2	:	Oviductal osmo-susceptibility index at 4 h
$p < 0.05$	:	Significant at 5 % level
PBS	:	Phosphate buffered saline
pH	:	Hydrogen ion potential
PI	:	Propidium iodide
PM	:	Progressive motility
PRM1	:	Protamine 1
PTPRC	:	Protein tyrosine phosphatase receptor type C
q-PCR	:	Quantitative polymerized chain reaction
r	:	Correlation coefficient
R^2	:	Coefficient of determination
RNA	:	RiboNucleic acid
ROC	:	Receiver Operating characteristic curve
ROS	:	Reactive oxygen species
RPL23	:	Ribosomal protein large subunit
RR	:	Rejection rate
RT	:	Room temperature
RVI	:	Regulatory volume increase
s	:	Second (s)
SEM	:	Standard error of mean
SLC9C1	:	Solute carrier protein 9C1
sncRNA	:	Small non-coding RNA
sNHE	:	Sperm specific sodium proton exchanger
snoRNA	:	Small nucleolar RNA

snRNA	:	Small nuclear RNA
STR	:	Straightness index
SUPER	:	Surface-exposed (during apoptotic cell death), ubiquitously-expressed, protease-sensitive, evolutionarily-conserved, and resident normally
TBE	:	Tris Borate EDTA
TBM	:	Tyrode balanced salt solution
TEY	:	Tris egg yolk extender
T_m	:	Melting temperature
TonEBP	:	Tonicity responsive enhancer binding protein
tRNA	:	Transfer RNA
U	:	Urea treatment media
UT-A	:	Urea transporter A
UT-A	:	Urea transporter B
UTI	:	Uterine osmo-susceptibility index
UTI 1	:	Uterine osmo-susceptibility index at 1 h
UTI 2	:	Uterine osmo-susceptibility index at 4 h
V_{hypo}	:	Volume of sperm in hypoosmotic solution
V_{iso}	:	Volume of sperm in isosmotic solution
VAP	:	Average path velocity
VCL	:	Curvilinear velocity
V_s	:	Volume shift
VSL	:	Straight-line velocity
w/v	:	Weight/ volume
WOB	:	Wobbling index

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Introduction

1 INTRODUCTION

Reproductive success depends on a series of factors including genetics, nutrition and management of parents. Artificial insemination is a successful first generation reproductive technology, wherein, a male influences fertility of a larger population of females; however, the conception rate is lower (35%) in AI than the natural service in cattle. The reasons for lowered fertility could be male, female and breeding managemental techniques. In fact, reproductive problems are the dominant reasons for culling in India (Gupta *et al*., 2015). Though the male and female equally contribute to fertilization in terms of gametes, ovum once ovulated resides at its domicile whereas the sperm take a precarious journey from the testes through the male excurrent ducts and uterus to oviduct, the site of fertilization; hence, sperm are exposed to diverse micro milieu with different pH, osmolality and are prone to more stress than the oocyte. Moreover, in the evolutionary ladder, the sperm reaches the ovum overcoming the barriers in most species, and hence it is imperative to investigate the effect of the osmolality, and pH on sperm. If these physical factors of uterus influence the sperm functions, then, they are likely to be associated with bull fertility.

In mammals, where the internal fertilization takes place, the microenvironment prevailing in the reproductive tract can also substantially influence reproductive success. The uterine and oviductal secretions influencing the sperm function are the result of the combination of selective transudation from blood and endometrial secretions. These reproductive tract fluids also vary in the osmolality, pH and ionic concentration which significantly play a vital role in successful fertilization. In fish, in which external fertilization takes place, the external microenvironment of the media are the stimuli for the sperm to attain fertilization competence and thereby ensuring the reproductive success (Alavi and Cosson, 2005; 2006).

In mammals, after ejaculation or insemination, the sperm have to travel in the female reproductive tract and get exposed to various pH and osmolality gradients. In the bovine, the uterine and oviductal osmolality is hyperosmotic of 350-355 mOsm/kg. The pH of the uterus is 6.8-6.9, whereas the same in the oviduct is 7.4. Interestingly, the osmolality of the epididymis is 350 mOsm/kg and seminal plasma is 280-300 mOsm/kg; hence, the sperm have to travel from hyperosmotic to isosmotic in the male and again to hyperosmotic in the female reproductive tract microenvironment. Besides, the transit at the uterus is longer than the other segments of the travel path after ejaculation. This behooves that the sperm should be capable of withstanding, adapting to the reproductive tract environment and responding to the environmental cues at respective sites and undergoes desirable changes for successful accomplishment of fertilization such as maturation in the epididymis, capacitation in the uterus and oviduct and subsequently the acrosome reaction when they come in contact with zona pellucida. Though the composition (Hugentobler *et al*., 2007; Lees *et al*., 2007), transcriptome (Forde *et al*., 2011) and proteome (Faulkner *et al*., 2012) of the uterine and oviductal fluids of the cow are known, the effect of physical micro milieu on the sperm function is not known *hitherto*.

Differences in the osmoadaptation ability of the bulls may directly influence the sperm functions, for instance, the intricate mechanisms involved in the sperm activation, capacitation, hyperactivation, acrosome reaction in the female reproductive tract can, in part, be explained by osmotic stress and pH of the female reproductive tract. It is known that the feed, metabolic status, and existing uterine and oviductal pathologies status also influence the pH and osmolality. For instance, the uterine pH changes due to high urea content in the histotroph when cows were fed with a high protein diet. Similarly, the metabolic derangements such as ketosis and diseases like mastitis alter the blood urea nitrogen and thereby uterine pH and osmolality. The elevated uterine urea concentration was proposed to affect semen quality and fertility. The perusal of literature revealed the paucity of information on the influence of urea on sperm functional attributes at uterine and oviductal micro-environment.

The present study, hence was conducted with the following objectives:

1. **To study the osmotic and pH adaptation ability of sperm and their influence on functional attributes in HF bulls**

2. **To assess the effect of urea on bovine sperm functional attributes *in vitro***

✍✍✍

Review of Literature

2 REVIEW OF LITERATURE

The evolution selects and supports only better adapters for survival. Similarly, the selection pressure imposed on the sperm over centuries includes phenotypic plasticity and cryptic female choice (Fitzpatrick and Lupold, 2014). The phenotypic plasticity, the ability of an organism/ individual/ cell altering their morphology or behavior in response to the external environment is well studied in fishes, where the sperm in milt/ seminal plasma is immotile, but get activated and become motile when osmotic stress is imposed (Cosson *et al*., 2019). The mammalian sperm exhibits a similar phenomenon. As long as in the epididymis, the sperm remain quiescent, but on ejaculation, it gains motility in the seminal plasma (hypoosmotic stress) where they exhibit progressive motility. Upon reaching the vicinity of an ovum in the oviduct, the sperm changes their motility behavior to hyperactivation kinematics, which is necessary for fertilization. The cryptic female choice is another selection pressure employed by the female over the ejaculated sperm by modifying the physical or chemical environment of the reproductive tract microenvironment to select competent male/sperm for the fertilization. The osmotic stress and pH alterations imposed on the sperm by the female reproductive tract can be either to facilitate phenotypic plasticity of sperm or evolved mechanism of cryptic female choice for successful reproduction.

2.1. Reproductive tract – the privileged compartment

The reproductive success depends on the quality of gametes and the favorable microenvironment of the male and female reproductive tract (Alminana and Bauersachs, 2019). From the production of gametes to maturation and transport, the reproductive tract renders exceptional support to maintain the integrity of gametes and promote consecutive events of the fertilization process.

The female reproductive tract is appreciative of sperm by protecting and preserving the functions of the sperm for a day or two till fertilization. The female allows selective colonization of sperm at privileged sites like cervix, utero tubal junction, and even cumulus cells. At the same time, it acts discriminatory for abnormal sperm by favoring phagocytosis at the uterus and may selectively protect efficient and good quality sperm to assure fertilization by the healthy sperm.

2.2. Spermatogenesis, a well-orchestrated process

The sperm are continuously produced from the progenitor spermatogonial cells in the temperature regulated and immunologically privileged testis (Chakradhar, 2018). The haploid antigenic sperm are well protected from the systemic immune response by blood-testis barrier and sperm are subsequently transported to the epididymis for maturation. The epididymis accounts for the final functional maturation of sperm and also serves as a repository for sperm till ejaculation. The epididymis offers a unique microenvironment with high osmolality, low pH, and low oxygen tension. This preserves the immotile sperm for a longer period of 30-45 days in the bull (De Pauw, 2003). The sperm at ejaculation are distinctive from the somatic cells both structurally and functionally. The sperm are specially structured for travel and designed for the fertilization process by the presence of condensed nucleus with depleted cytoplasm and specialized acrosomal cap at the head, and other cellular organelles with abundant mitochondria in the midpiece and a motility apparatus in the tail. These terminally differentiated cells do not support transcription and translation (Ren *et al*., 2017). Hence, they are packed with all the specialized biomolecules (transcriptome, proteome, and metabolome) in a self-sufficient manner to achieve fertilization. These sperm acquire motility and becomes metabolically active for the first time when they are dispensed with the seminal plasma during ejaculation (Juyena and Stellata, 2012). The knowledge on motility acquisition in mammals, however, is still in infancy. Altogether the sperm are loaded with the self-sustained biomolecules and dispatched in multitudes into the female reproductive tract for the successful execution of fertilization process.

2.3. The evolvement of fertilizing capacity of sperm

The ejaculation takes place at the anterior vagina in case of natural service in cattle (Suarez & Pacey, 2006) or deposited at the body of the uterus during artificial insemination (Lopez – Gatius, 2000). Sperm are a heterogeneous group of cells differing in their functional attributes even within the ejaculate (Holt and Van look, 2004). The female hence adopts various strategies right from the cervix to hand-pick sperm subpopulation of supreme quality for successful fertilization. These include the cervical mucus where the sperm take the less resistance pathway deeper in the crypts rather than the center of the cervical lumen (Katz, 1981). The poor quality sperm lacking this ability are discarded thereof. The myometrial contraction helps the sperm rapidly to reach the utero-tubal junction as the sperm in the uterus elicit leucocytic infiltration (Suarez and Pacey, 2006). In the oviduct, the sperm are attached to the ciliated epithelium and reside inside the folds until ovulation (Lefebvre *et al*., 1997). During this period, the sperm undergo capacitation, a necessary preliminary step for sperm-zona binding. From here, the sperm with hyperactivated motility are released in waves. However, the signal for capacitation, hyperactivated motility, and programmed release from the oviductal epithelium are not decoded yet (Tulsiani *et al*., 2012). The hyperactivation with increased flagellar beating helps the sperm to pass through the viscid mucus and the altered motility pattern holds the sperm in the vicinity of the ovum (Ho *et al*., 2002). After the acrosomal reaction, a sperm successfully penetrates the ovum and discharges its long-carried possessions including the nucleus, and various biomolecules for successful fertilization and embryo development. The osmolality, pH, and temperature excursions are physical signals regulating this complex array of sperm functions at the right time and the right place to culminate reproductive success in mammals.

2.4. A strenuous path to fertilization- a sperm perspective

Appreciating the fertilization process from a sperm point of view is highly challenging and demanding. Sperm progressing from epididymis to the site of fertilization are confronted with various stresses via gradients of pH, osmolality, and diversified metabolites in the female reproductive tract in differing concentrations between the segments (Aguillar and Reyley, 2018).

The different segments of the reproductive tract though unique, are tightly regulated and maintain homeostasis in the microenvironment they offer. For example, dynamic changes were observed between the ampullary and isthmus fluid the adjacent oviductal segments in bovine (Way *et al*., 1997). The sperm, hence, should be capable of withstanding the imposed stresses and respond positively to the various cues from the female reproductive tract at respective stages to accomplish the fertilization successfully. Understanding such mechanisms help us in improving sperm handling procedures in assisted reproductive techniques and for better fertility prediction of males.

2.5. Osmotic stress and adaptive ability

Among various abiotic stresses imposed on the organisms, osmotic stress is one of those well-characterized adaptation features from single cellular metazoan to multicellular eukaryotes. Both the hyperosmotic and hypoosmotic stress affect the eukaryotic cells considerably ranging from reactive oxygen species (ROS) attack, DNA damage to cell death. These stresses also influence DNA condensation, increase nuclear lacunarity, and facilitate nucleocytoplasmic transport (Finan and Guilak, 2010). But the response and ability to overcome the osmotic stress differ among various cell types. The choice of either regulating the cell volume back to the native state or apoptotic cell death depends on the ability of the cell to orchestrate the events including sensing and recruitment of various signaling cascades, and effectors to overcome the imposed stress. The cells which are consistently exposed to osmotic stress like nephrons and hepatocytes as a part of their destined function are endowed with sufficient osmoregulatory mechanisms (Zhang *et al*., 2003). The sperm are also exposed to such demanding environments during their journey to fertilization and to achieve nuclear modulation in preparation of syngamy (Figure 1). Hence, exploring the osmoadaptation ability of the sperm receives significance.

2.6. Osmotic difference between semen and uterus across species

An osmotic difference exists between epididymis, seminal plasma, and the uterine fluid of most mammals. In monogastrics like human, mouse, rat, and pig the seminal plasma is comparatively hyperosmotic to uterus whereas in ruminants, the uterus is relatively hyperosmotic to seminal plasma (Table 1).

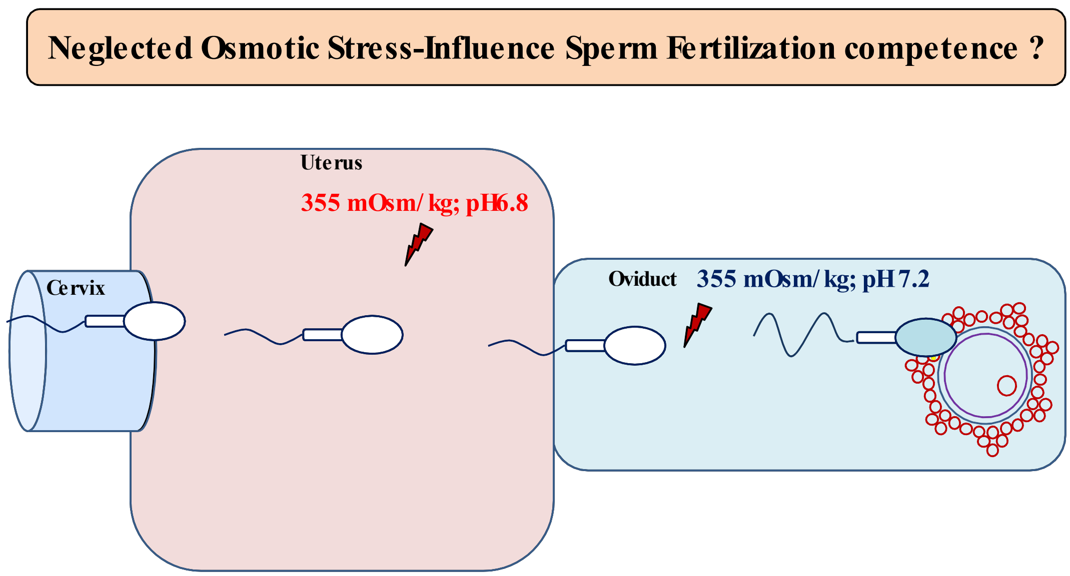

Fig. 1: Exploring the osmoadaptation ability of the sperm in female reproductive tract. The sperm are exposed to demanding micro-environments during their journey to fertilization to achieve successful syngamy

Table 1: Osmotic difference between seminal plasma and uterus of mammals

Species	Seminal plasma (mOsm/kg)		Uterus (mOsm/kg)	
Human	360-380	(Polak *et al*., 1984)	280	(Casslen *et al*., 1984)
Mouse	415	(Miyamato, 1973)	330	(Miyamato, 1973)
Rat	365	(Dam and Copper, 2010)	287	(Dam and Copper, 2010)
Pig	280- 345	(Davies *et al*.,1975)	290-320	(Li *et al*., 2007)
Bovine	280- 300	(Drevius *et al*., 1972)	350 355	(Olds *et al*., 1957)
Ewe	300-325	(Matsuoka *et al*., 2006)	349-370	(Wales, 1973)

2.7. The osmotic difference among various segments of the reproductive tract in bovine

In the bovine, the osmolality of the reproductive tract secretions differs greatly (Figure 2). The epididymis, uterine and oviductal secretions are hyperosmotic as compared to seminal plasma which is isosmotic to plasma (Table 2). As osmotic difference exists, it is mandatory for a sperm to adapt to osmotic stress and regulate its volume for further progress in the reproductive tract to reach the site of ovum for fertilization.

Table 2: The osmotic difference across the bovine reproductive tract fluids

Reproductive tract	Osmolality (mOsm/kg)	References
Epididymis	350-355	(Drevius *et al*., 1972;Verberckmoes *et al*., 2003)
Seminal plasma	280- 300	(Drevius *et al*., 1972)
	292	(Present study)
Uterus	350- 355	(Olds and Vandemark, 1957)
Oviduct	350- 355	(Olds and Vandemark, 1957)

When the sperm are exposed to the hyperosmotic environment in the uterus, they are expected to lose their water molecules and, eventually, the cell volume shrink. Subsequently, the osmoregulatory pathways are activated and the cells regain their original cell volume called regulatory volume increase (RVI) due to the retained evolutionary memory (Hoffman *et al.*, 2009). Such an osmoadaptation process is expected to happen in the female reproductive tract. During this osmoadaption process, the motility of the sperm may be hampered for a short period, but at the end of RVI the motility is regained, which is highly necessary for penetrating the ovum.

Though the motility acquisition and sperm activation are not deciphered adequately in mammals, it has been documented in fishes. The sperm in milt acquires motility in milliseconds on exposure to the external environment. The key factors involved in this signaling cascade are enumerated as osmotic pressure, ionic and gaseous components of external media. The seawater of approximate osmolality 1100 mOsm/kg and freshwater osmolality 50 mOsm/kg serve as hyperosmotic and hypoosmotic medium, respectively to the seminal plasma of fish milt, 300 mOsm/kg. Such osmotic difference plays a significant role in preparing the fish sperm for fertilization. The subcellular changes associated with this signaling cascade are tyrosine phosphorylation, calcium influx, potassium influx, hyperpolarization of the plasma membrane, and elevation of intracellular pH, cAMP, and ROS production (reviewed in Cosson *et al.,* 2019) which are also the established events as capacitation associated changes. But the observed osmotic difference and their possible role in fertility have not been foreseen in mammals.

As sperm of different bulls vary in their different functional attributes they may also differ in their osmoadaptation ability and may influence the bull fertility. Though the osmo-tolerance ability of the sperm was studied in isosmotic and hypoosmotic conditions (Liu and Foote, 1998), the sperm never experience a hypoosmotic condition in the female reproductive tract. Hence, it is essential to understand the osmoadaptation ability of the sperm in the hyperosmotic condition prevailing in the uterus. Understanding such osmoadaptation ability of the sperm will provide knowledge on the fertilizing ability of the bull and eventually may aid in developing bull fertility prediction tests.

2.8. Effect of osmotic stress on sperm

2.8.1. Cell volume perturbations

Initial studies on the osmotolerance ability of the sperm revealed no detectable difference in the volume of sperm in anisosmotic solutions (Pursley *et al*., 1950; Rothschild *et al*., 1959). This may be attributed to the less precision instruments used and minor volume perturbations

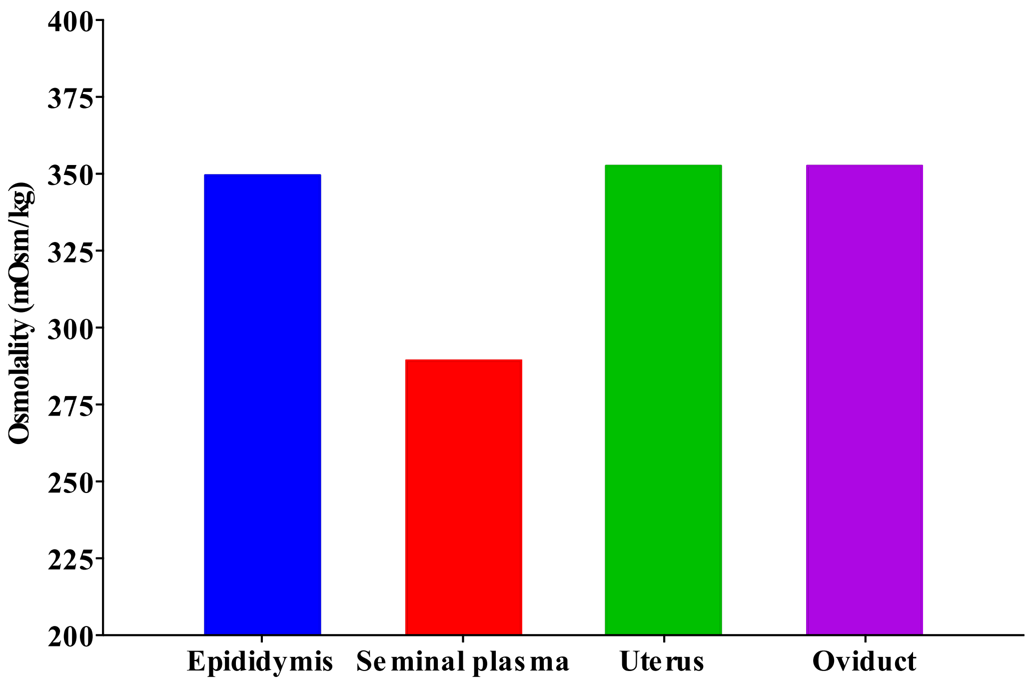

Fig. 2: The osmotic gradient changes experienced by the sperm across the bovine reproductive tract fluids

due to the relative absence of cytoplasm in sperm compared to other cells. The volume of ejaculated bull sperm was reported as 25 μm^3 (Drevius *et al*., 1966). The critical medium osmolality (the osmolality at which the 50% of the cells swell and burst) of bull sperm was established as 36 mOsm/kg (Watson *et al*., 1992). The mean volume and area of swollen sperm at critical medium osmolality were determined as 126 μm^3 and 145 μm^2, respectively. Hence, bull sperm were found to increase in volume by 3.78 to 3.94 times without apparent increase in area (Drevius, 1972). A study on the effect of anisosmotic solutions on the bull sperm revealed that bull sperm behaved as linear osmometers from 150 to 400mOsm/kg but the cell volume did not decrease beyond 500 to 1000 mOsm/kg (Liu *et al*., 1998). These findings revealed that the bull sperm respond to anisosmotic solutions ideally and behave as linear osmometers.

2.8.2. Cell kinematics and viability

Motility and plasma membrane integrity of the bull sperm were observed to be optimally maintained within physiological (200-300 mOsm/kg) osmolalities (Liu *et al*., 1998). The motility was highly compromised in hypoosmotic (below 150 mOsm/kg) and hyperosmotic (500 mOsm/kg/L and above) solutions whereas plasma membrane integrity was maintained in hyperosmotic solutions (up to 732 mOsm/kg). The partial recovery of sperm motility was observed when sperm were returned from hyperosmotic to isosmotic solutions (Liu *et al*., 1998). In another study, the bull sperm progressive motility was reported to be maximum in isosmotic Tyrode solution (300 mOsm/kg), low at hyperosmotic modified Tyrode solution (400 mOsm/kg) and very low at hypoosmotic modified Tyrode solution (200 mOsm/kg) (Barati *et al*, 2011). These findings revealed that the hypoosmotic solution was comparatively more detrimental to bull sperm than a hyperosmotic solution.

These studies were also conducted in the buffalo semen for establishing optimal extender osmolality and pH for cryopreservation. The effect of different osmotic pressures of Tris extender on post-thaw quality of cryopreserved buffalo bull sperm revealed significantly higher motility, acrosome integrity, and DNA integrity in 275 and 295 mOsm/kg compared to 255 mOsm/kg (Mughal *et al*., 2013). The viability, acrosome integrity, plasma membrane integrity, and motility of buffalo bull sperm were significantly higher in 260 mOsm/kg tris extender at 5°C than that of tris extender at 240, 280 and 300 mOsm/kg (Bishist *et al.*, 2018). These studies provided inconclusive results about the role of osmolality on the cryopreservability of buffalo semen.

2.8.3. Capacitation status and acrosome integrity

The osmolality of capacitation medium in most species is isosmotic ranging between 290-300 mOsm/kg. But *in vitro* fertilization is achievable over a wide range of osmolality between 308 to 372 mOsm/kg in the mouse and 292 to 392 mOsm/kg in the hamster (Miyamato and Chang, 1973). The high ionic strength (HIS) medium has been used as the capacitation medium in rabbit (Brakett *et al.*, 1978) and bovine (Brackett *et al.*, 1982) sperm resulting in successful *in vitro* fertilization. The capacitating effect of HIS media was, however, attributed to the better removal of inhibitory or sperm stabilizing factors coated on sperm. The hyperosmotic condition was also associated with increased tyrosine phosphorylation and zona pellucida binding in human sperm (Liu *et al.*, 2006). The sperm undergoing cryopreservation experience osmotic stress and they exhibit premature capacitation like changes known as cryocapacitation (Cormier *et al.,* 1997). Similarly, the bovine sperm stored in tris extender (320 - 325 mOsm/kg) at 4°C for 4-8 hours resulted in higher zona-free egg penetration without obvious capacitation agents (Ijaz *et al.*, 1987). The osmotic stress is well known to induce mitogen-activated kinases (MAPK) which are intimately associated with the sperm capacitation process (Sun *et al*, 1999). The kinases mediate post-translational modification of proteins in sperm which are involved in the capacitation, acrosome reaction, and fertilization process (Homer *et al.*, 1997). The growing evidence suggests the possible involvement of evolutionarily conserved osmotic stress in mediating the capacitation process.

2.8.4. Chromatin distribution

The chromatin in the sperm nucleus is highly condensed with protamines replacing conventional histones, although not complete (Hammoud *et al.*, 2009). The chromatin decondensation takes place after fertilization. The sperm undergoing cryopreservation experience hyperosmotic stress which affects chromatin condensation and favors chromatin relaxation (Le *et al*., 2019, Jee *et al*., 2008). The sperm subjected to hypoosmotic swelling test as a selection for intracytoplasmic sperm injection was also said to cause chromatin decondensation and precondition the sperm for nuclear reprogramming post-fertilization (Lima *et al*., 2018). Thus osmotic stress is known to cause chromatin loosening and recruitment of RNA polymerase ii, which further decides the cellular metabolism and thereby its fate (Lima *et al*., 2018)

2.8.5. Mitochondrial membrane potential

ATP synthesis via oxidative phosphorylation is the most efficient energy deriving pathway in bovine sperm (Storey, 2008). The mitochondrial membrane potential is thus adopted as an efficient test for predicting the energy available for downstream sperm motility and metabolism as mitochondria play a key role in motility, capacitation, hyperactivation, acrosome reaction, and fertilization (Moraes *et al*., 2018). The mitochondrial membrane potentialis further highly correlated with sperm motility, *in vitro* fertilization outcome and fertility (Kasai *et al*., 2002; Sousa *et al*., 2011)

Stallion sperm exposed to osmotic stress does not affect mitochondrial membrane potential over a wide range (150 to 600 mOsm/kg) whereas the osmotic stress below 150 and above 600 – 1500 mOsm/kg considerably reduced the mitochondrial membrane potential (Garcia *et al*., 2012). Similar findings have been reported in rat sperm that anisosmotic conditions severely hamper mitochondrial membrane potential (Kim *et al*., 2013).

Apart from energy synthesis for motility, the mitochondrial membrane potential is also necessary for ion transport between mitochondria and cytoplasm. Similarly, the translation of nuclear encoded genes in the mitochondria is an energy-dependent process that has been proven in bovine sperm (Gur and Breitbart, 2006).

Those studies assessed the osmotic stress response of bull sperm using anisosmotic solutions ranging from 50 to 1700 mOsm/kg by modifying the medium with sodium chloride did not account for the pH change that would have resulted. The functional attributes of sperm like motility and membrane integrity were assessed after returning the samples from anisosmotic solution to isosmotic solution. This method may not be appropriate for assessing the osmotolerance effect of sperm functional attributes and eventually cannot predict the osmotolerance or osmoadaptation ability of sperm. Further, these studies evaluated the sperm function at a single time point after incubation in the anisosmotic solution. Studying any adaptation mechanism needs monitoring over some time continuously for better understanding. The effects of osmotic stress on the functional parameters like capacitation, acrosome reaction, chromatin distribution, mitochondrial membrane potential also need to be studied for elucidating the osmoadaptation mechanism in sperm.

2.9. Tests evolved to exploit osmotolerance of sperm

2.9.1. Milavanov's resistance test

This test explored the resistance of bull sperm to 1 % sodium chloride solution on several steps of dilution. In this test, 10 mL of 1% sodium chloride solution is added to 0.02 mL of semen, until the progressive motility of all the sperm is ceased (Milavanov, 1934).

$$\text{Value} = \frac{\text{Volume of NaCl solution required (mL) to cease the progressive motility}}{0.02} \times 100$$

Though the author conferred the observed effect as resistance to sodium chloride, he did not attribute it to the osmotic resistance of sperm. Further, the observed effect was attributed to dilution rather than sodium chloride by Blackshaw and Emmens (1951). However, the test was not presently employed in routine semen analysis.

2.9.2. Hypoosmotic swelling test

This test was developed to explore the functional plasma membrane integrity of sperm by subjecting to 150 mOsm solution at 37 °C for 30 min (Jeyendran *et al.*,1984; Revell and Mrode, 1994). A significant correlation was observed between the percentage of functional membrane intact sperm with in vitro zona penetration ability. This test has found a place in the basic semen evaluation tests to access the functional membrane integrity of bull sperm (Minimum standards for bovine frozen semen production, GOI). Further, this cost-effective test has taken an irreplaceable position in selecting a single effective sperm for intracytoplasmic sperm injection (Sallam *et al.*, 2001).

2.9.3. Short-osmotic resistance test

The hypoosmotic swelling test was further modified to access the plasma membrane integrity of boar sperm by subjecting to 75 mOsm solution at 37°C for 5 min (Perez-Llano *et al*., 2000). A significant correlation was observed between functional membrane integrity and *in vivo* fertility in boar and claimed it as a sensitive boar semen fertility prediction test. Though modified with short reaction time it is not widely adopted for routine semen evaluation.

2.9.4. Osmotic tolerance test

In this test, the boar sperm were subjected to different osmotic conditions (100 to 4000 mOsm) and isosmotic medium (300 mOsm) for a fixed point of time ie, 15 mins. At the end of the incubation, the progressive motility, morphology, viability, and acrosomal integrity were evaluated in both anisosmotic and isosmotic treatments. The osmotic tolerance was calculated as follows,

$$\text{Osmotic resistance} = \frac{\text{Value in osmotic treatment}}{\text{Value in isosmotic treatment}} \times 100$$

The observed osmotic resistance values were correlated with 60 days non-return rate (NRR) and litter size in pigs (Marc Yeste *et al*., 2010). This study associated the motility, morphology, viability, and acrosomal integrity of the osmotolerant subpopulation to porcine fertility. The precise osmotic stress to be provided was not emphasized and moreover, the study used a wide range of osmotic conditions (100 to 4000 mOsm/kg) on the account of cryopreservation.

2.9.5. Volume shift

Sperm were incubated in hypoosmotic media of 150 mOsm for 15 mins and a shift in volume was assessed using coulter counter.

$$\text{Vs} = \frac{\text{V hypc}}{\text{V iso}}$$

(V_s – Volume shift, V_{hypo} – Volume of sperm in hypoosmotic solution, V_{iso} – Volume of sperm in isosmotic solution). The volume shift observed was correlated with non-return rate

(Petrunkina, 2001). The study though established a correlation between the cell volume shift and non-return rate, the involvement of relatively costly and exiguous instruments in the semen analysis, the test did not find a practical place in the routine semen analysis.

All the above mentioned tests explored the osmotic tolerance ability of the sperm by assessing the functional membrane integrity and hence the semen quality. None of the tests accounted for the osmoadaptation process of sperm by continued monitoring and characterization of recovery of sperm subpopulation capable of withstanding the osmotic stress.

2.10. Hydrogen ion potential of reproductive tract secretions in bovine

The pH of the biological fluid is of paramount importance as it can influence nutrient availability, rate of biochemical reactions, behavior of different substrates in the medium, and microbial activity. The reproductive tract secretions also serve as a transport medium and nutrient bowl to the gametes and embryo, the hydrogen ion potential existing in different segments might influence the functions of the gametes. The sperm traveling from the testis through the excurrent ducts with the seminal plasma to the female reproductive tract experience a wide range of hydrogen ion potential (Table 3)

Table 3: Hydrogen ion potential across the bovine reproductive tract fluids

Reproductive tract	pH	
Epididymis	5.77- 5.86	(Acott and Carr, 1984)
Ejaculated semen	6.7- 6.99	(Berger, 1951)
	6.4 – 6.5	(Hossain *et al.*, 2012)
Vagina	7.2 – 7.3	(Swartz *et al.*, 2014)
	7.3 – 7.5	(Cohen *et al.*, 2012)
Cervix	6.5 – 6.7	(Tsiligianni *et al.*, 2001
	6.2 – 6.3	(Wehrend *et al.*, 2003)
Uterus	6.96- 6.97	(Hugentobler *et al.*, 2004)
	6.81- 6.84	(Elrod and butler, 1993)
Oviduct	7.41 – 7.43	(Hugentobler *et al.*, 2004)

The optimal pH of the fluid in the reproductive tract is essentially important for the metabolically active sperm to retain their functional attributes and fertilizing potential. The change

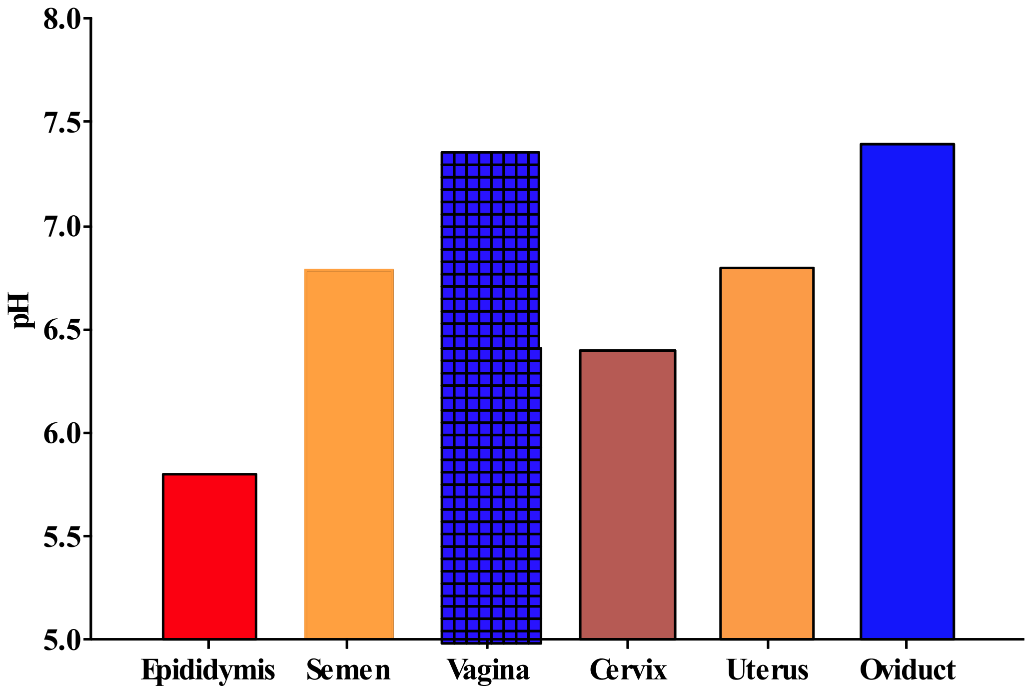

Fig. 3: The hydrogen ion potential changes experienced by sperm across the bovine reproductive tract

in pH among the segments may influence the sperm functions mainly the hyperactivation and capacitation by regulating the various molecular events such as tyrosine phosphorylation, cholesterol efflux, calcium influx, alkalization of cytoplasmic pH, etc., (Vredenburgh Wilberg and Parrish, 1995). From a sperm point of view, it should adapt and respond positively to the changing hydrogen ion potential across the different regions of the reproductive tracts (Figure 3) to become a potential candidate of fertilization.

2.10.1. Effect of pH on sperm functions

At physiological pH (6.9 and 7.4), the maximum metabolism of bull sperm was observed. However, while reducing the pH to 6.4 and below imposed a significant decrease in sperm metabolic activity (Rothschild *et al*., 1959). In accordance with these findings, the motility of bull sperm was found maximum at pH 6.5 and 7.5 (Tampion & Gibbons, 1962). The alkaline pH was hypothesized and proved as a trigger for the vigorous motility of bull sperm in the oviduct (Lindemann and Kanous, 1989). These findings led to the school of thought that subjecting the sperm from acidic epididymal plasma to comparatively alkaline seminal plasma triggered motility in the quiescent sperm. The high pH could be the reason for the hyperactivation of sperm in the alkaline oviduct.The motility, viability and mitochondrial activity of bull sperm were significantly higher at pH 7 and 7.5. At pH below 6.5 and 8, these attributes were significantly affected **(**Contri *et al*., 2013). In the same way, the motility, viability, penetration ability of human sperm was significantly decreased in acidic (5.2 and 6.2) compared to alkaline (7.2 and 8.2) environment (Ji Zhou *et al*., 2015). In contrast, the progressive rheotaxis of bull sperm to chemoattractants was found maximum at pH 6.4 and 6.6 compared to 6.0, 6.2, 6.8, and 7.0 (El- sherry *et al*., 2017). The variations between the species and biological significance are not clear.

These studies accounted for the effect of pH on sperm at isotonic solutions. But the hyperosmotic environment prevailing in the uterus and oviduct of the cow was not considered. Further, the effect of pH on various other functional attributes like capacitation and acrosome reactions, which are happening in the uterus and oviduct were not studied.

2.11. Sperm transcriptomics – A tool for understanding the molecular process and fertility prediction

The sperm are endowed with the necessary transcripts, proteins and metabolites for accomplishing the downstream processes as they are believed to be transcriptionally and translationally silent. The sperm retain RNA which may be remnants of spermatogenesis as well as potential RNAs to be delivered to oocyte upon penetration. This includes a complex population of transcripts including coding messenger RNA (mRNA) and non-coding RNAs like transfer RNA (tRNA), ribosomal RNA (rRNA), small non-coding RNA(sncRNA), long noncoding RNA(lncRNA), small nucleolar RNA (snoRNA) and small nuclear RNA (snRNA) (Selvaraju *et al*., 2018). Bovine sperm carry transcripts for 13,833 genes involved in the pre-fertilization, fertilization, and post-fertilization process (Selvaraju *et al*., 2017). Thus transcriptome profiling of the sperm RNA and identification of the variations in their levels of expression aids in differentiating bulls varying in fertility traits (Parthipan *et al*., 2017).

2.12. Genes associated with osmoregulation

Stress causes perturbations in cellular function which is reflected in the gene expression. The gene expression alteration depends on the degree and duration of stress. The sperm from the testes are subjected to different osmotic gradients at epididymis, seminal plasma, and female reproductive tract. As sperm respond to the external stimuli with the limited transcriptional activities, assessing the osmotic stress associated genes may shed light on the sperm osmotic adaptation ability and thus the fertilizing ability of the bulls. The sperm though believed to be transcriptionally silent, presence of molecular features such as post-translational modification of proteins, mitochondrial translation of nuclear-encoded proteins (Gur and Braiebart, 2006), nuclear shuttling of transcription factors and their localization on histone retained regions (Dadoune *et al*., 2005) suggest obscure molecular events in sperm.

The genes differentially expressed under osmotic stress and are the relevance of sperm function were selected for the study by a literature survey (Figure 4). The osmoresponsive genes *NFAT5* (Woo *et al*., 2000), *ADAM1B* (Fischer *et al*., 2004),*SLC9C1* (Grienstein *et al*., 1992), *HSP90AB1* (Wang *et al*., 2014), *ENO1, EFHD, MT-ND2* and *GAPDH* (Gabert

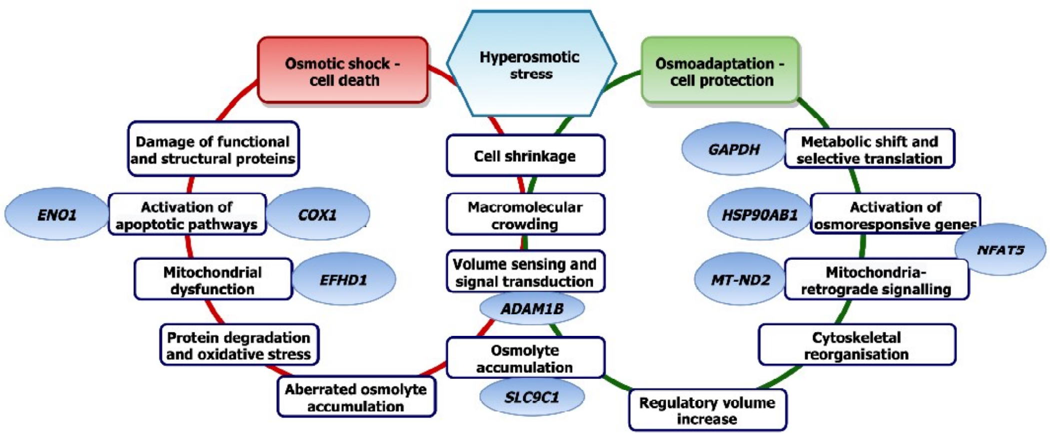

Fig. 4: The osmoresponsive genes and their function pertaining to osmotic stress

and Kultz, 2010), *MT-CO1* (Fontanisi *et al*., 2006) were found to be involved in various functions like oxidative phosphorylation (*MT- ND2, COX 1*), glycolysis (*ENO1, GAPDH*), mitochondrial calcium sensor (*EFHD1* / mitocalcin), chaperone (*HSP90AB1*), a transcription factor that acts as the major controller of osmoresponsive genes (*NFAT5*), an osmotic stress effector ion channel (*SLC9C1*), and a disintegrin and metalloprotease (*ADAM1B*). Since these genes were altered under stress conditions the relative abundance of these genes in the sperm were expected to guide us on the osmoadaptation ability of sperm.

2.12.1. Nuclear factor of activated T lymphocytes 5 (*NFAT5*)

*NFAT5*is also known as tonicity responsive enhancer binding protein (TonEBP) (Ferraris *et al*., 2002). It is a protein encoding gene and a DNA binding transcription factor that induces expression of osmo-responsive genes including aquaporins, aldolase reductase, sodium myoinositol transporter (Lopez Rodriguez *et al*., 2014), taurine transporter, betaine transporter (Miyakawa *et al*., 1998), heat shock proteins (Navarro *et al*., 2008) and urea transporter (Nakayama *et al*., 2000) involved in osmolyte accumulation for regulating the osmo-adaptation ability. The NFAT5 has an intrinsically disordered region (IDR) (Dumond *et al*., 2014) which involve in the direct sensing and activation of genes responsible for cellular protection from osmotic stress and heat stress. It is a target gene product of mitochondrial retrograde signaling (De Cunha *et al*., 2015) an established mode of protein synthesis in bovine sperm (Gur and Breitbart, 2006). Further, it is closely associated with the MAPK pathway and protein kinase A (Zhou *et al*., 2016) which are involved in sperm capacitation and post-translational modifications. Gene-specific knock out studies in mice revealed severe embryonic mortality (Mak *et al*., 2011). The significance of the NFAT5 gene expression on sperm function has not been reported.

2.12.2. Disintegrin and Metalloproteases 1 B (*ADAM1B*)

ADAM1 is also known as fertilin alpha, in view of its role in sperm-egg fusion characterized by sperm (Bronson *et al*., 1999). It encodes for a membrane-anchored protease that belongs to the class of disintegrin and metalloproteases. They are typical glycosylphosphatidylinositol-anchored proteins with a cytoplasmic domain, transmembrane domain, and an extracellular proheparin binding EGF ligand (Weber and Paul, 2012). The osmotic stress is mediated by the EGFR activation of ADAM and downstream activation of signaling cascades namely MAPK, and JUNK as evidenced in cell lines (Fischer *et al*., 2004). The MAPK, ERK, and JUNK are the vital kinases involved in mediating post-translational modification and capacitation of the sperm (Silva *et al*., 2015). They also play an inevitable role in the early embryo development as it regulates the notch pathway (Groot and Vooijs, 2012). Knock out studies in mice further confirmed its involvement in embryo development (Weber *et al*., 2011). In sperm, ADAM1 is localized in the plasma membrane of the head region and are involved in various functions like sperm maturation and transport in uterus, sperm aggregation, and egg interaction (Yamaguchi *et al*., 2009).

2.12.3. Solute carrier protein 9C1 (*SLC9C1*)

SLC9C1 is also known as sperm specific sodium proton exchanger (sNHE). These class of plasma membrane ion transporters is well characterized in both sperm function and regulatory volume increase in hyperosmotic stress. The cell shrinkage caused by hyperosmotic medium is counterbalanced by the activation of sodium proton exchanger (NHE) ion transporters, which exchange one intracellular proton to extracellular sodium thereby facilitating the influx of water through aquaporins and regaining the cellular volume (Grienstein *et al*., 1992;Ruiz-Martínez *et al*., 2011). They are also well recognized for their role in the alkalization of intracellular pH as they transport the weak acids having the functional group of hydrogen outside the cell. Hence it plays an important role in intracellular alkalization of sperm mediating capacitation and hyperactivation aiding in the fertilization process. It is principally localized in the sperm flagellum, and their irreplicable role in sperm motility and in vitro fertilization have been established as the sNHE null mice experience severely hampered sperm motility and protein tyrosine phosphorylation under capacitation conditions (Wang *et al*., 2006).

2.12.4. Heat shock protein*AB1* (*HSP90AB1*)

Heat shock proteins belong to the molecular class of chaperones and are upregulated under osmotic stress in sperm (Wang *et al*., 2014). They are involved in the stabilization of various proteins under osmotic stress and also in the degradation of misfolded proteins to ease the cell function (Nathan *et al*., 1997).In sperm HSP90 expression is increased during sperm capacitation and are known to be involved in key functions like intracellular calcium homeostasis, protein tyrosine phosphorylation and progesterone mediated signaling events (Li *et al*., 2014). Further, their expression levels are positively correlated with the cryotolerant capacity of the bull sperm (Wang *et al*., 2014). Under demanding stress conditions including altered pH and osmolality, the heat shock protein (HSP90) is associated with the HSF1 (Heat shock factor), a transcription factor and regulate the expression of other genes of the chaperone complex including histone deacetylases and DNA methyltransferases (Mazaira *et al*., 2018). Recently, the heat shock protein is also reported as chromatin remodelers in human sperm (Beeram *et al*., 2019). Thus, the role of *HSP90* in protein stabilization and nuclear remodeling in sperm under osmotic stress can be of immense importance in easing the fertilization process.

2.12.5 Protamine 1*(PRM1)*

PRM1 is one of the most abundant transcripts in the sperm owing to their role in the sperm chromatin condensation and stabilization. And hence the protamine transcripts are mostly believed as the remnants of spermatogenesis and their abundance are even considered as fertility biomarkers for efficient spermatogenesis (Kempisty *et al*., 2007). At the same time, another school of thought is that their mRNA abundance has a functional role of undergoing translation during the sperm transit in the female reproductive tract to replace damaged protamines (Feugang *et al*., 2010). Contradicting these findings, the protamine transcripts abundance is also linked with poor semen quality and sperm motility (Lambard *et al*., 2014) which is conceptualized due to poor translational machinery in the spermatogenesis.

2.12.6. Enolase 1 *(ENO1)*

This gene encodes for a protein coding gene namely Enolase 1. It is a glycolytic enzyme and responsible for glycerol synthesis. Though it is a member of glycolysis pathway it is not a constitutive enzyme, and is abundantly expressed during cellular stress like hypoxia, heat, and osmotic stress (Didiasova *et al*., 2019). It is also known as HSP48 due to its increased expression levels during stress. The ENO1 is also termed as “SUPER” protein ie, surface-exposed (during apoptotic cell death), ubiquitously-expressed, protease-sensitive, evolutionarily-conserved, and resident normally in viable cells. Hence the surface-expressed enolase is considered as the early cell marker of apoptosis (Ucker *et al*., 2012). The enzyme is evidenced in sperm and the less abundant expression of enolase 1 protein is linked with low fertility (Park *et al*., 2012 ;Soggiu *et al*., 2013). In contrast, abundant expression of ENO protein in human sperm to poor quality embryo development in idiopathic infertile couples (Jodar *et al*., 2020).

2.12.7. EF-hand containing 1 (*EFHD1*)

It is also known as mitocalcin. It is a mitochondrial calcium binding protein that is upregulated in stress conditions like oxidative and osmotic stress (Li *et al*., 2016). It is involved in the mitochondrial flash activation process (Hou *et al*., 2016). The mitochondrial calcium signal is an intrinsic regulation mechanism when the cell is under stress conditions characterized by the production of reactive oxygen species. When the stress is pathological and the cell is unable to maintain the homeostasis, there is a burst of calcium and reactive oxygen species in mitochondria which is known as mitochondrial flash activation. EFHD 1 is recently found as the calcium signal sensing protein of the mitochondrial flash activation process and involved in the pro-apoptotic mechanisms leading to cell death (Hou *et al*., 2016).

2.12.8. Cytochrome c oxidase1 (*MT-CO1)*

This mitochondrial gene encodes for the cytochrome c oxidase, the terminal enzyme complex (Complex VI) of the mitochondrial electron transport chain. This transmembrane complex transmits the electrons and translocates protons to the oxygen molecules and thereby increasing the proton electrochemical gradient contributing to the mitochondrial membrane potential. The mitochondrial function is highly necessary and regulated during osmotic stress. The transcript data under osmotic stress revealed downregulation of cytochrome c oxidase and upregulation of nuclear coded RNA processing and assembly factors which may be due to the translational activation under osmotic stress (Mansilla *et al.,* 2018). Also, cytochrome c oxidase is released to the cytoplasm and activates caspase signaling cascade which causes apoptosis. The same intrinsic apoptotic regulation is also reported in testes (Liu *et al*., 2006).

2.12.9. Mitochondrial NADH dehydrogenase 2 (*MT - ND2*)

It encodes for mitochondrial NADH dehydrogenase enzyme of the largest NADH ubiquinone oxidoreductase complex (Complex I) in the mitochondrial electron transport chain. It transports electron from the NADH to ubiquinone and the released energy is used for the ATP synthesis. The energy production by the mitochondria is the dominant pathway of ATP production over glycolysis in sperm (Storey *et al*., 2008) and in this regard, expression of ND2 receives significance. Mutation in the ND2 gene has been reported to cause disruption of energy production, motility, and cause complete failure of fertilization (Zhang *et al*., 2018). Though the osmoregulatory functions of ND2 have been highlighted in fishes (Cabellaro *et al*., 2015), but the role of ND2 need to be explored in mammals.

2.13. Factors affecting female reproductive tract microenvironment

The survivability of the gametes and the appropriate development of an early embryo depend on histotrophic mode of nutrition and hence the maternal microenvironment is of vital importance for their survival and optimal function. Evidence also suggests that mouse embryos cultured in vitro are extremely sensitive to medium and even slight acidity or alkalinity (0.2 – 0.4 units) on either side of the normal pH (7.2) disrupts mitochondrial localization and actin cytoskeleton (Swain, 2010). Similarly, the embryo development (8-16 cell stage) was arrested at alkaline pH above 7.2 (Lane and Bavister,1999)

Various conditions such as nutrition and diseases influence the maternal uterine environment. The quantity and quality of the maternal diet is found to influence the reproductive success by causing perturbations in the composition of reproductive secretions or the circulating metabolites, growth factors and hormones (Ashworth *et al*., 2009). Recent studies provide significant evidence on the positive and negative effects of focus feeding on pregnancy and embryo development in livestock around the peri-conception period. Some of them include skewing of sex by maternal feeding (Arangasamy *et al*., 2015), feeding fiber rich diet resulting in increased litter size in pigs (Ferguson *et al*., 2004), feeding urea in ewes before mating leading to developmentally retarded and abnormal embryos on day 3 of recovery (McEvoy *et al*., 1997). These studies strongly suggest that the success of the complex fertilization process and embryo development is mainly influenced by the maternal microenvironment.

2.14. Blood urea nitrogen and fertility

Blood urea nitrogen (BUN) concentration has been cited as a cause for reduced fertility in dairy cattle. BUN being an end product of protein metabolism can be derived from excessively fed protein diet or increased protein degradation in the body. Elevated blood urea nitrogen has been reported as one of the reasons for the repeat breeding in cattle (Gupta *et al.*, 2008; Selvaraju *et al.*, 2017; Widayati *et al.*, 2018). The postpartum cows in their transition period become more prone to metabolic diseases. Such cows with clinical and subclinical ketosis were also reported with elevated blood urea nitrogen (Issi *et al.*, 2015; Sudhakara Reddy *et al.*, 2014). It is established that postpartum cows at their peak lactation exhibit poor fertility characterized by increased calving to conception interval, increased number of services per conception, etc. The animals with mastitis were also reported to have elevated BUN levels and such animals exhibit declined fertility (Nozad *et al.*, 2012; Roy *et al.*, 2011). Though the elevated BUN levels were reported in the postpartum animals with metabolic diseases and mastitis, no studies reported that the elevated BUN levels were the cause for low fertility in these diseased animals. However, the research evidences suggest a negative influence of elevated blood urea nitrogen on cattle fertility (Ferguson *et al.*, 1993, Raboisson *et al.*, 2017)

2.15. Impact of high protein diet on fertility

The dairy cattle fed with high protein or rumen degradable protein diet have always been accused of low fertility over decades (Table 4). The conception rate is reported to vary from 10 to 20% between animals fed with isocaloric feed differing in protein levels. Though this problem is age-old and sufficient research evidence exists on various hypotheses behind the scenario, no definite cause and effect was established.

Table 4: The difference in conception rate between high and low crude protein fed cattle

Low protein diet			High protein diet group			DCR	Reference
CP %	BUN (mg/dL)	CR (%)	CP %	BUN (mg/dL)	CR (%)	(%)	
16	8.8	56	20	15.4	44	**12**	Folman *et al*. (1981)
15	15	87	20	26	85	**2**	Howard *et al*. (1987)
12	11	64	20	24	56	**8**	Carroll *et al*. (1988)
16.5	12	48	19.2	19	31	**17**	Canfield *et al*. (1990)
15.45	10.2	82	21.8	14.8	61	**21**	Elrod & Butler, (1993)
13	8	87	20	21	75	**12**	Barton *et al*. (1996)
16	16.5	68	21	21	50	**18**	Chapa *et al*. (2001)

CP% - Crude Protein, BUN – Blood Urea Nitrogen, CR % - Conception rate,DCR – Difference in conception rate between high and low crude protein fed group.

The decline in conception rate was attributed to both systemic and local effects of urea nitrogen. The BUN urea nitrogen varies from 2 to 25 mg/dL depending on the diet and metabolic status of the animal (Table 7). The increased urea and ammonia concentration in the blood affect the energy metabolism and hypophyseal-pituitary–ovarian axis and thereby hormonal homeostasis of the animal also. Through local effect, the toxic metabolites may have a direct detrimental effect on the gametes and embryo or they may alter the microenvironment of the reproductive tract, for example, decrease the uterine pH which may not support gamete and embryo survival (Westwood *et al*., 2000; Tamminga *et al*., 2006)

The toxic metabolites of protein degradation and negative energy balance were reported to operate together in the disruption of luteinizing hormone release causing poor maturation and the ovulatory response of ovum (Jordan and Swanson, 1979a). But this research evidence was not found consistent in other studies (Canfield *et al*. 1990). The systemic effect was not widely accepted as the increased BUN level caused conception failure as it did not limit the milk production or cause metabolic derangements of energy metabolism. Various hypotheses were also proposed and tested to assess the local effect of toxic metabolites within the reproductive system. The toxic metabolites were proposed to cause luteal insufficiency, but the results in this direction were not consistent as some authors agree to this effect (Garverick *et al.*1972; Jordan and Swanson, 1979b; Sonderman *et al.,* 1987) whereas findings from other studies did not accept the proposed effect later (Carroll *et al,* 1988; Eldon *et al,* 1988). *In vitro* studies reported that *in vitro*maturation of bovine oocytes and fertilization was affected by the presence of urea (De Wit, 2001) but no effect was observed in the cleavage of the zygote (De Wit, 2001; Ocon and Hansen, 2003). A decrease in the uterine pH was proposed to be the cause for low fertility in animals having high uterine urea concentration, but in cows fed with high protein diet had no change in uterine pH (Table 5) even though the BUN and uterine urea concentration was considerably high (Elrod and Butler, 1993; Amundson *et al.* 2016). But the increase in uterine pH from 6.8 to 7.0 a week (7 days) after estrus was prevented by the high level of urea in the uterus (Elrod and Butler, 1993). A significant correlation exists between blood urea nitrogen and uterine urea concentration in cows (Hammon *et al.*, 2005; Kenny *et al.*, 2002). The adverse effect of urea nitrogen levels in the female reproductive tract on embryonic development is well studied, but on gamete quality, especially on sperm function has not been established.

Table 5: Relationship between blood urea nitrogen and uterine pH in cattle

Phase of estrous cycle	Control		High protein group		Reference
	BUN (mg/dL)	Uterine pH	BUN (mg/dL)	Uterine pH	
Estrus	10.03-10.37	6.83- 6.85	14.61- 14.99	6.81- 6.84	(Elrod & Butler, 1993)
Diestrus (day 7)	10.03-10.37	7.09- 7.10	14.61- 14.99	6.79 – 6.8	(Elrod & Butler, 1993)
Estrus	6.59 – 6.74	6.85- 6.86	11.56 – 11.92	6.81- 6.85	(Amundson *et al.*, 2016)

2.16. Urea in uterine tract secretions

There was a strong correlation observed between the blood urea concentration and uterine urea concentration (Table 6).

Table 6: Relationship between BUN and uterine urea concentrations in high and low crude protein fed cattle

Low protein diet		High protein diet		Reference
BUN (mg/dL)	Uterine urea conc. (mg/dL)	BUN (mg/dL)	Uterine urea conc. (mg/dL)	
16-20	19.7 – 21.1	21-25	25.6 – 28.2	(Hammon *et al*., 2005)
4.03	5.92 – 6.20	9.15	14.36 – 14.66	(Kenny *et al*., 2002)

The research though carried out on various existing hypotheses of the possible detrimental effect of urea on oocyte, corpus luteum function, embryo development, and uterine microenvironment, the results were not consistent as few studies support and other studies reject leaving an indefinite conclusion for the existing long-lived problem. However, a strong correlation between the higher urea concentration and higher uterine urea concentration subsequently with low fertility has been accepted largely. This revealed a significant lacuna in understanding the urea mediated fertility compromise in bovine. Thereby insufficient studies on the influence of urea on sperm function need much attention.

2.17. The possible effect of urea on sperm

Repeat breeders with significantly high plasma urea nitrogen levels (>18mg/dL) had low fertility (Gupta *et al*., 2008; Widayati *et al*., 2018). These findings suggested that either fertilization failure or early embryonic mortality could be a reason for decreased conception rate since the length of the estrous cycle was not altered (Figure 5). The superovulatory response and embryo quality were significantly affected in embryo transfer donors fed with a high protein diet, whereas high protein levels in the diet of ET recipients did not affect conception rate suggested that the effect of urea could be within day 7 after AI (Rhoads *et al*., 2006). The embryo quality was poor in cows fed with a high protein diet at the time of insemination rather than from the previous mid-luteal phase (Dawuda *et al*., 2002). Another study also reported that the embryo quality was not deteriorated when animals were continuously fed with a high protein diet and given a washout period at the time of artificial insemination (Westwood, 1998). These findings suggest that the effect of urea leading to fertilization failure or poor embryo quality might be mediated via sperm. Though the possible effect of urea on sperm function could be one of the reasons for low fertility in animals with high urea content in the uterus, very few studies were conducted on these aspects.

Though urea is hypothesized to affect sperm functions in utero, urea is not a strange chemical to the sperm. Urea is one of the most abundant organic metabolites found in the seminal plasma of bulls (Menezes *et al*., 2019; Velho *et al*., 2017) even though its role in the seminal plasma is not known. Bull sperm also contain urea cycle enzymes like carbamyl phosphatase synthetase, ornithine carbamyl transferase, and arginase, but were less active than rabbit sperm (Dietz and Flipse, 1969).

2.18. Urea concentration in seminal plasma

The urea concentration in the seminal plasma is found to vary between species and among individuals of the same species. The urea is found to be one of the most abundant metabolites in bull seminal plasma (Velho *et al.,* 2017). But the functional significance is not elucidated yet. But the pathological increase in the urea concentration may bound to have harmful effects on the semen quality.

Table 7: Urea nitrogen concentration in plasma and seminal plasma

Species	Plasma urea	(mg/dL)	Seminal plasma urea(mg/dL)	
Bull	2.10 – 2.50	(Hennessey and Williamson, 1990)	29.85 – 45.00	(Cevik *et al*., 2008)
	6.20 – 15.5	(Hammond, 1992)		
	4.90 – 16.5	(Lumsdon *et al*., 1980)		
	17.0 – 21.7	(Roseler *et al*., 1993)		
Stallion	33.3 – 39.6	(Gough *et al*., 2002)	27.62 – 35.43	(Atroschenko *et al.,* 2019)
Ram	18.3 – 51.9	(Cortoda *et al.,* 2000)	28.80 – 63.50	(Cortoda *et al.,* 2000)
Human	20.0- 34.0	(Musch *et al.,* 2006)	22.46 - 70.20	(Allahkarni *et al.,* 2017)

2.19. Studies on the effect of urea on sperm

The sperm motility was significantly affected when the sperm were incubated in uterine flushings of post-insemination in cows fed with high protein diet (Hossain, 1993). In the same study, a decrease in the motility was observed in the sperm incubated with urea containing

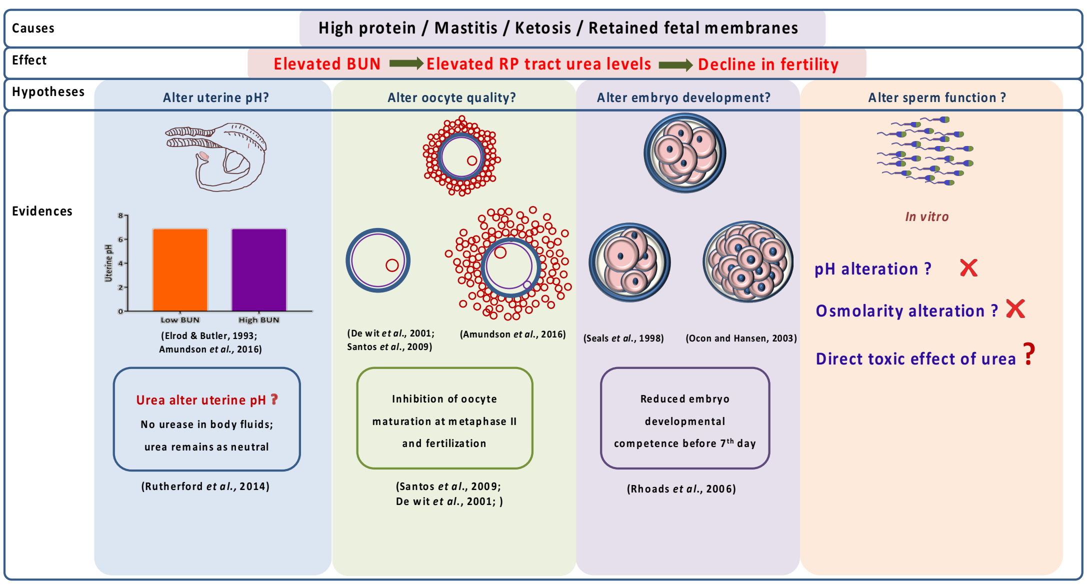

Fig. 5: **The causes, effect, hypotheses and evidences available for urea mediated decline in fertility in bovine**

medium. Similarly, the cervical mucus penetration ability of bull sperm was hampered in urea containing medium in vitro (Breau *et al*., 1985). The epididymal rat sperm experienced a severe decline in motility in isotonic urea solution (Turner and Howard, 1978). But the human sperm incubated in urea containing medium did not experience decreased motility (Kim *et al*., 1998). As the urea cycle enzyme activity differs among species (Dietz and Flipse, 1969), the urea tolerating ability of sperm may also differ. Hence, there is a need to understand the role of urea on sperm function in order to establish the effect of high urea levels on bull sperm function and fertility in cattle.

2.20. Research gap

- Osmo-adaptation ability of bull sperm in terms of motility and its influence on other sperm functional attributes at physiological uterine and oviductal osmolality has not been studied.
- Inconclusive hypotheses and observations exist on the probable effect of elevated uterine fluid urea concentration on sperm function. Controlled *in vitro* studies on the effect of urea on sperm functional attributes are lacking.

Materials and Methods

3 MATERIALS AND METHODS

The present work was carried out at Reproductive Physiology Lab, ICAR - National Institute of Animal Nutrition and Physiology, Bengaluru. The experiment was carried out as per the IAEC approval vide: NIANP/IAEC/1/2020/10

The experiment was designed to assess the osmo adaptive behaviors of sperm in the uterine and oviductal environment and for predicting bull fertility (Figure 6). The first part of the study includes selection and standardization of a suitable isotonic sperm transport media, washing media, and fixing the sampling time points for osmoadaptation experiments. The second part includes two experiments consisting of (i) incubation of sperm in media simulating uterine and oviductal osmolality and pH, and (ii)incubation of sperm in media with oviductal osmolality and pH containing different concentrations of urea. In these two experiments, various sperm functional parameters like sperm kinematics, sperm viability, functional membrane integrity, acrosome integrity, sperm subpopulation positive for both functional membrane and acrosomal integrities, mitochondrial membrane potential, chromatin distribution, capacitation reaction, and acrosome reaction were studied.In the third part of the study, the relationship between osmoadaptation ability and the expression levels of osmo-responsive genes in sperm were conducted. Finally, statistical analyses were carried out to assess the effect of physiological osmolality, pH, and urea concentration on sperm functional attributes. The possibilities of predicting bull fertility based on osmoadaptation ability and gene expression levels were also assessed.

3.1. Sample source

The neat semen samples of two to five year-old HolsteinFriesian bulls maintained under standard managemental conditions were procured from Nandini Sperm Station, Hessarghatta, Bengaluru. Semen samples were collected using artificial vagina method. Two ejaculates from each bull (12) was collected (0.5 mL) and pooled to minimize the biological variation (12×2= 24). The ejaculates having at least 500 million sperm/ mL and 70% progressive motility were selected for the study. The semen samples (12) were diluted (1:1) immediately in the transport medium and transported to the laboratory at 25-28°C within 4 h.

3.2. Standardization of sperm transport medium

Semen samples were initially transported in tris egg yolk (TEY) extender (1:1 dilution) with and without glycerol at 25-28°C within 4 h. Sperm showed altered motility pattern characteristic of sperm activation. As the TEY extender with and without glycerol were hyperosmotic (Table 8), and the present study aimed to explore the effect of osmolality and pH, standardization of the suitable transportation and washing media which are isosmotic and of same hydrogen ion potential to that of seminal plasma was imperative (Figure 7).

Table 8: The osmolality of the conventional TEY used for bovine semen cryopreservation

Solution	Osmolality (mOsm/kg)
Tris buffer (n=6)	451± 6.9
Tris with only 20 % egg yolk (n=6)	437± 7.5
Tris with only glycerol (n=6)	1762± 43.3
Tris with glycerol and 20 % egg yolk (n=6)	1735±54.7

Accordingly, the following threetransport media were evaluated for transporting the bovine semen samples

a) Modified isotonic trisegg yolk extender (mTEY)

b) Modified isotonic tris extender

c) Tyrode balanced salt solution (TBM)

Overall workflow of the study

1. Selection and standardization of semen transport medium
2. Selection and standardization of sperm washing medium
3. Fixing sampling time points for osmoadaptation study
4. Influence of uterine and oviductal osmolality and pH on sperm functional attributes
5. Influence of different urea concentrations on sperm functional attributes at oviductal osmolality and pH
6. Differential gene expression studies of osmo-responsive genes
7. Bull fertility prediction based on sperm osmo-adaptation ability

Fig. 6: The overview of the experiments carried out in the present study

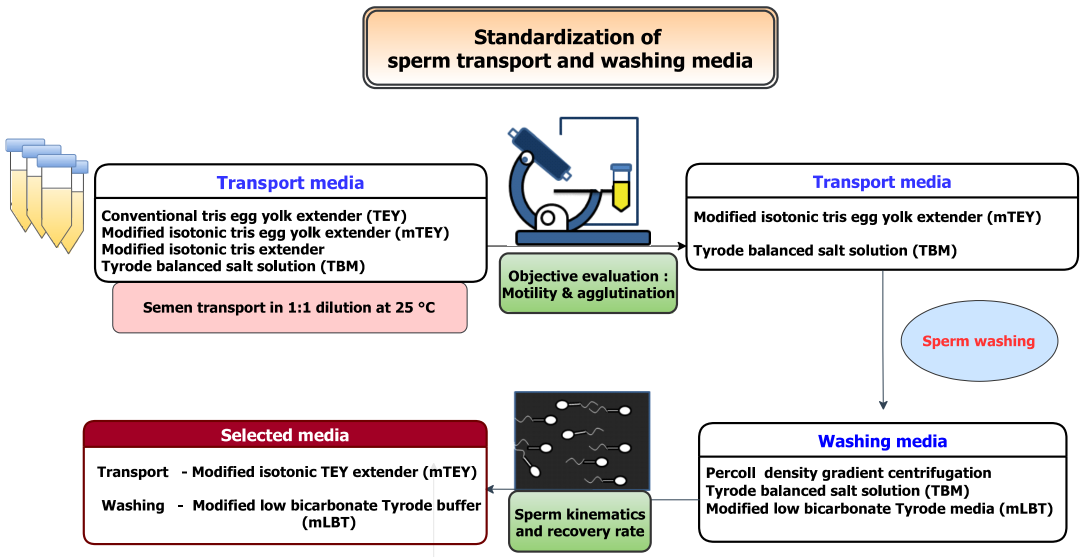

Fig. 7: The overview of the methodology adopted for standardization of isosmotic sperm transportation and washing media

Semen samples were diluted (1:1) in the transport medium and transported at 25-28°C within 4 h of semen collection. After reaching the laboratory, the semen samples were incubated at 37°C for 10 min and subjected to sperm kinematics evaluation as well as for the presence of agglutinated sperm (Table 9). The modified isotonic tris egg yolk extender and Tyrode balanced salt solution were found to retain significantly high progressive motility and very little/no agglutination (WHO laboratory manual for examination and processing of semen, fifth edition) and hence selected as transport media for further studies.

Table 9: Evaluation of sperm motility and agglutination reaction in various transport media

Medium	Individual progressive motility (%)	Agglutination
Modified isotonic TEY extender	80	-
Modified isotonic tris extender	70	Grade 1
Tyrode balanced salt solution	80	Grade 2

3.3. Standardization of sperm washing medium

The samples were washed in three different media,namely

a) 45% Percoll density gradient

b) Tyrode balanced salt solution (TBM)

c) Modified low-bicarbonate Tyrode solution (mLBT)

An aliquot (100 µL) of the semen sample was layered over 1 mL of washing medium in a microcentrifuge tube and centrifuged at 200 g for 5 min at room temperature. The supernatant was discarded and the loose pellet was resuspended in the respective media. The washing efficiency was assessed based on the sperm recovery rate.

The post-washing relative yield of sperm was assessed based on sperm recovery rate (concentration, %) and progressive motile sperm (%) as compared to prewash (Mortier *et al.,* 2000). The washing efficiency was also checked for the efficient removal of egg-yolk particles.

$$Relative\ yield = \frac{\text{Output } (\text{V} \times \text{C} \times \text{PM } (\%))}{\text{Input } (\text{V} \times \text{C} \times \text{PM } (\%))}$$

where, V- volume (mL), C- concentration (number of cells in million/mL), PM – progressive motility.

Modified low-bicarbonate Tyrode (mLBT) media had the maximal sperm recovery rate and also minimal influence on sperm motility parameters. Hence mLBT was chosen as washing media for the subsequent studies.Based on the results, modified tris egg yolk extender (mTEY) and modified low-bicarbonate Tyrode (mLBT) media were selected as transport and washing media, respectively. The subsequent studies were conducted using these media.

3.4. Optimization of sampling time points for osmoadaptation study

A pilot study was performed to optimize the sampling time points from the incubation media for osmoadaptation study (Figure 8). Six ejaculates (1 ejaculate/bull) were transported in mTEY extender and washed inmLBT buffer. After washing, an aliquot of the semen sample (100 µL) containing about 100 million sperm was added to each of the incubation media (900 µL) having different osmolalities (290, 355 and 420 mOsm/kg) and incubated at 37°C for 4 h.

Sperm kinematics were analyzed for every 30 min (0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4 h) using a computer-assisted sperm analyzer (CASA; Sperm Class Analyser, Microptic, Spain). The samples from every time point werealso analyzed for function membrane integrity by hypo-osmotic swelling test.

The result of this study indicated that at 0 min the motility dropped and in the majority of the samples motility regained between 1 to 2 h.

3.5. Experiment 1: Effect of osmolality and pH on sperm functional attributes

The semen samples from 12 bulls (24 ejaculates, 2 ejaculates per bull) were transported in TEY extender and washed in mLBT (Figure 9). Tyrode basal medium (290, 6.8 pH) was considered as the isosmotic medium. The desired hyperosmolality (355 and 420 mOsm) was obtained by adding a calculated amount of fructose (Table 3). The fructose was preferred to increase the osmolality as it belongs to the class of organic and non-ionic osmolyte. Osmolality was measured using a freezing point osmometer (OSMOMAT 3000 – Gonotec, Germany).

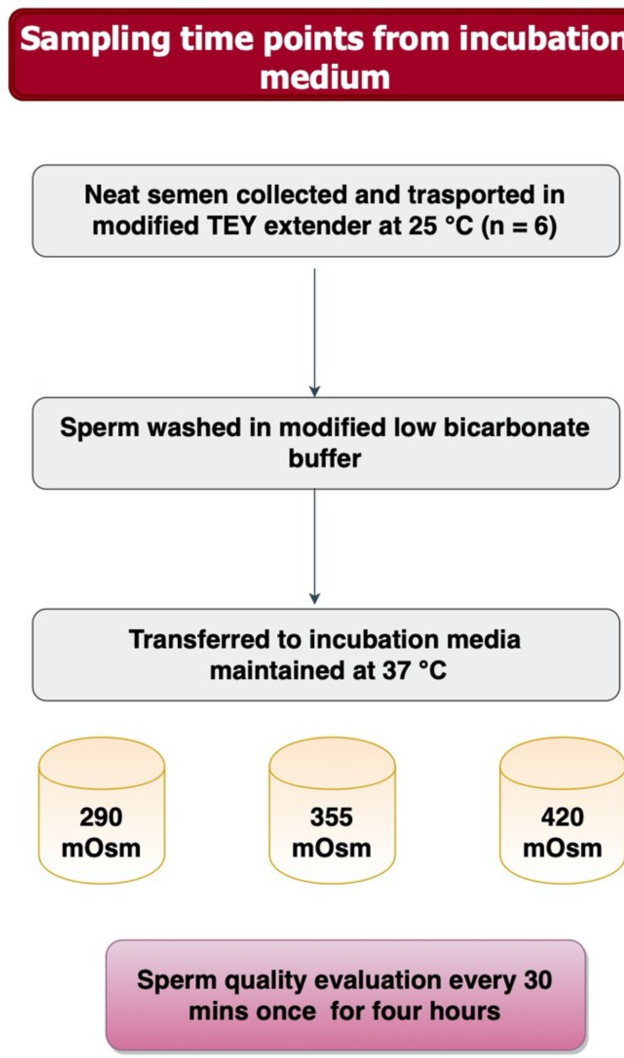

Fig. 8: The methodology followed for optimization of sampling time points for osmoadaptation study

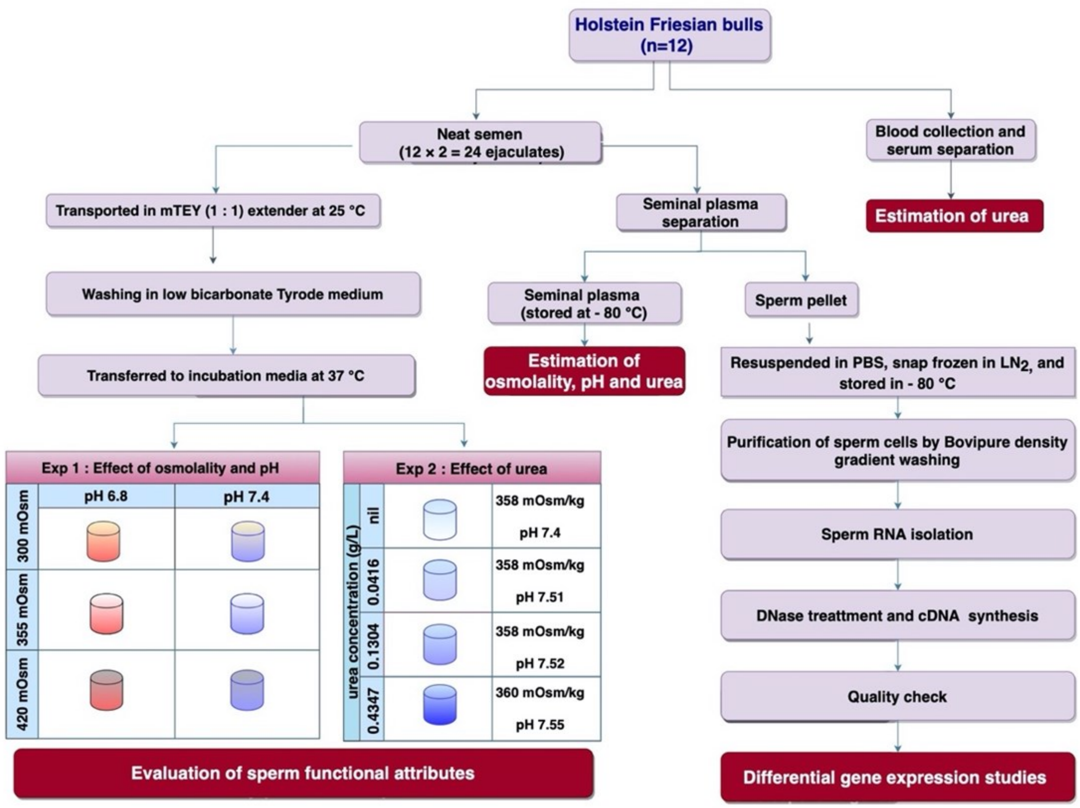

Fig. 9: The methodology followed for evaluation of the effect of osmolality, pH and urea on sperm functional attributes

The pH was adjusted to 7.4 using 5N sodium hydroxide and measured with digital pH meter (EuTech PH 700 benchtop pH meter, Qtech, India). Osmolality after pH adjustment was also measured and confirmed (355 and 420 mOsm).

Table 10: The incubation media of different osmolality used for the study. Preparation of desired hyperosomotic media for the study by adding calculated amount of fructose to isosmotic TBM media

Medium	Tyrode basal medium (mL)	Fructose (g/L)	Calculated osmolality (mOsm/kg)	Measured osmolality (mOsm/kg)
Isosmotic Tyrode basal medium 290 mOsm/kg	1000	nil	290	296
Hyperosmotic medium 355mOsm/kg	1000	11.89	355	358
Hyperosmotic medium 420mOsm/kg	1000	22.7	420	429

After washing, an aliquot of the semen sample (100 µL) containing about 100 million cells was transferred to the respective incubation media (900 µL) maintained at 37°C in a water bath (TC 202, AMETEK Brookfield, USA). The sperm samples were tested at 0, 1, 2 and 4 h intervals for various sperm functional parameters (Table 11) as per the established procedure (Table 12).

Table 11: The functional parameters assessed in sperm during the course of incubation in control and treatment media

0 h	1 h	2 h	4 h
Sperm Kinematics	CASA	CASA	CASA
Viability		Viability	Viability
Morphology		Morphology	Morphology
Functional membrane integrity		Functional membrane integrity	Functional membrane integrity
Chromatin distribution		Chromatin distribution	Chromatin distribution
MMP		MMP	MMP
Acrosome reaction	Acrosome reaction	Acrosome reaction	Acrosome reaction
Capacitation status	Capacitation status	Capacitation status	Capacitation status

Table 12:The tests and methods employed to assess various sperm functional attributes

Sl No	Sperm functional parameters	Method followed	Reference
1	Sperm kinematics	Computer-assisted sperm analyzer	(Perumal *et al*., 2011).
2	Structural membrane integrity (%)	Eosin – Nigrosin staining	(Bloom *et al*., 1950)
3	Functional membrane integrity and acrosomal integrity (%)	Hypo osmotic swelling and Giemsa (HOS- G) test	(Jeyendran *et al*., 1984)
4	Abnormal sperm	Giemsa staining	(Selvaraju *et al*., 2008)
5	Mitochondrial membrane potential	JC-1 stain	(Selvaraju *et al*., 2012)
6	Chromatin distribution	Feulgen's staining	(Selvaraju *et al*., 2008)
7	Capacitation status	CTC staining	(Ward and Storey *et al*., 1984)
8	Acrosome integrity	FITC staining	(Selvaraju *et al*., 2015)

3.6. Experiment 2: Effect of urea on sperm functional parameters

The hyperosmotic Tyrode medium (355 mOsm and 7.4 pH) was selected as basal media for the present experiment and urea was added at the concentration of 4.16, 13.04 and 43.47 mg/mL in order to mimic *in vivo* BUN levels of 0.2, 0.66 and 2.0 mg/mL, respectively (Table 6). The changes in pH and osmolality were noted. The urea treatment media (U) were prepared freshly on the same day of the experiment to avoid the pH fluctuations.The sperm functional parameters were assessed at 0,1, 2 and 4 h of incubation as described under experiment 1 (Table 11 and 12)

Table 13: Influence of urea on the changes in osmolality and pH of the test media. Three different concentrations of urea were added to the treatment media

Media	Urea (g/L)	Predicted invivo urea concentration (mg/mL)	Changes in Osmolality (mOsm/kg)		Changes in pH	
			Before urea addition	After urea addition	Before urea addition	After urea addition
Control (355 mOsm/kg)	-	-	355	358	7.4	7.4
U1	0.0416	0.2	355	358	7.4	7.51
U2	0.1304	0.66	355	358	7.4	7.52
U3	0.4347	2.0	355	360	7.4	7.55

3.7. Assessment of sperm functional parameters

3.7.1. Sperm kinematics

The sperm kinematic parameters were analyzed using a computer-aided sperm analyzer (CASA; Sperm Class Analyser, Version 6.4,Microptic SL, Spain) (Perumal *et al.*, 2011). On a clean prewarmed microscopic slide, 10 µL of sample (concentration adjusted to 2×10^6) cells/mL (to avoid collision) was placed and covered by a coverslip on the microscopic stage maintained at 37 °C with a stage warmer. The analysis was performed by capturing minimum of 10 random homogenous fields using a camera (Nikon eclipse 50Nikon, Japan) with a capturing efficiency of 25 frames/s at 10 x magnification in the negative phase-contrast microscope (Nikon eclipse 50, Nikon, Japan).Egg yolk particles and debris present in the captured fields were manually removed to eliminate them from the analysis. The following CASA settings were adopted for sperm tracking (Table 14).

Table 14: The settings followed in computer assisted sperm analysis for the assessment of sperm kinematics

CASA parameters	Criteria set for analysis		
Motility and Progressivity (%)	Rapid	:	VCL > 50 µm/s
	Medium	:	VCL - 25 – 50 µm/s
	Slow	:	VCL - 10 – 25 µm/s
	Static	:	VCL > 10 µm/s
	Progressive	:	STR >70
Mucus penetration (%)	ALH	:	1.25 - 100 µm
	STR	:	80 - 100%
	VAP	:	>25 µm/s
Hyperactivation (%)	LIN	:	0 - 50%
	VCL	:	>150 µm/s
	ALH	:	> 3.5 µm

VSL (Straight-line velocity) is the distance between the first and last position points in the track segment of the sperm trajectory divided by the total duration of the track segment (Figure 10) and expressed as µm/s. VCL (Curvilinear velocity) is the sum of distances between every two consecutive track position points in a track segment divided by the total duration of the track segment and expressed in µm/s. VAP measures the distance traveled by the sperm along the average trajectory divided by the total duration of the track segment and expressed as µm/s. Linearity (LIN) is the ratio of VSL and VCL and straightness (STR) is the ratio of VSL and VAP and both are expressed in percentage.

The amplitude of lateral head displacement (ALH) is twice the maximum displacement of sperm head from its fitted moving axis in a track segment expressed as µm. Beat cross frequency (BCF) is the frequency that the sperm head moves across the middle plane of the straightened trajectory expressed as hertz. The mean sperm head area of the captured sperm was obtained from the software computed head length and width and expressed as μm^2. The head size in our study was used as the representative measure of cell volume to predict osmoadaptation status.

Thus obtained progressivity (progressive, non-progressive and immotile), velocity (Curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP)) and other motility characteristics (linearity index, straightness index, wobbling, the amplitude of lateral head displacement, beat cross frequency, hyperactivation, mucous penetration) were considered for analysis.

3.7.2. Sperm concentration

The resuspended sperm pellet was diluted with PBS in a microcentrifuge tube and 5 µL of glutaraldehyde (0.3 %) was added to kill the cells. The haemocytometer was charged with 10 µL of the sperm suspension. Once the cells settled down, the hemocytometer was placed under 40x objective in a light microscope and the cells in the 5 large squares bounded by three lines in all four sides (RBC counting squares) were counted. The sperm head only on the top and right border of all the squares were considered. The concentration was calculated using the following formula:

Sperm concentration = Number of cells in 5 large squares x dilution factor x 0.05

3.7.3 Sperm viability

The sperm viability was assessed based on the dye exclusion principle using Eosin and Nigrosin stain (Bloom *et al.*, 1950). An equal volume (10µL) of semen sample from each

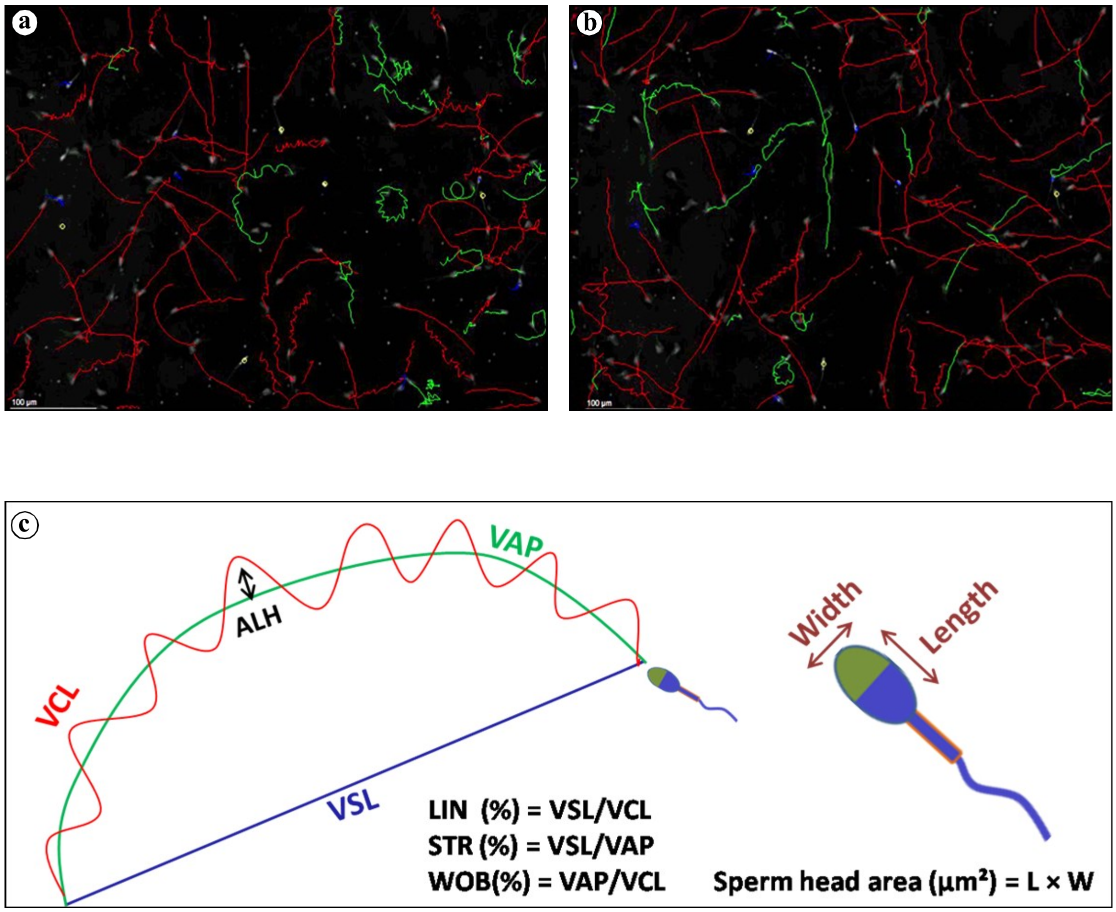

Fig. 10: **Sperm kinematic analysis by computer assisted sperm analysis. Figure a and b show the sperm analyzed based on their curvilinear velocity as rapid progressive (red), medium progressive (green), non progressive (blue) and immotile (yellow). Figure c represents an individual sperm track. The paths shown include curvilinear path (red), average path (green) and straight line path (blue) of a sperm. The mean head size of sperm was obtained from the CASA computed head area by above formula**

incubation medium and Eosin (5%) and Nigrosin (10%) were mixed and smeared onto a clean grease-free glass slide maintained at 37°C. The slides were air-dried and counted using the 100x objective under the phase-contrast microscope on the same day. As the test works on the principle of dye exclusion, those sperm that appear white or unstained were considered as live and those that appear pink were considered as dead.

3.7.4. Functional membrane integrity

Hypo osmotic swelling test was carried out to assess the functional membrane integrity of sperm as described earlier (Jeyendran *et al*., 1984). Semen sample (50µL) from each incubation medium was added into 450µL of 150 mOsm (hypo-osmotic) and 300 mOsm (isosmotic control medium) medium maintained at 37°C and incubated for 30 min at 37°C. At the end of incubation, 5 µL of glutaraldehyde (0.3%) was added to both tubes for fixing the cells. A drop was then kept on a grease-free glass slide with a coverslip and examined under10x objective of negative phase-contrast microscope. The sperm with a hairpin bent at the principal piece of sperm tail were considered as functional membrane intact positive. A minimum of 200 cells was counted for each sample. The percentage of sperm with functional membrane integrity (%) was calculated by subtracting the functional membrane intact in 150 mOsmwith thefunctional membrane intact in 300 mOsm.

3.7.5. Functional membrane and acrosome integrity

Hypo osmotic swelling – Giemsa test (HOS-G) was carried out to assess the subpopulation of sperm positive for both functional membrane and acrosome integrities (Selvaraju *et al*., 2008).From each incubation medium, 50µL of semen sample was added to 500 µL of 150 mOsm (hypoosmotic) and 300 mOsm (isosmotic control medium) medium maintained at 37°C and incubated for 30 min at 37°C. At the end of incubation, a drop of the sample (volume) was smeared on a clean grease-free glass slide and air-dried. The air-dried slides were then fixed in buffered formal saline for 30 min and washed in running tap water for 2 min. The slides were dried and immersed in Giemsa (Himedia, India) working solution overnight. The slides were then washed under tap water for a few seconds and air-dried.A minimum of 200 cells was counted in each slide under oil immersion using the 40x objective of the phase-contrast microscope.The sperm with hairpin bent in the principal piece of the tail was considered as functional membrane intact (HOS positive). The sperm with uniformly pink stained acrosome were considered as acrosome positive. Based on this test, the following four subpopulations were observed:

HPAP	HOS positive and acrosome positive
HPAN	HOS positive and acrosome negative
HNAP	HOS negative and acrosome positive
HNAN	HOS negative and acrosome negative

The results were computed as follows:

$$HPAP(\%) = \text{HPAP at 150 mOsm } (\%) - \text{ HPAP at 300 mOsm } (\%)$$

$$\text{Acrosome positive (AP)}(\%) = \text{HPAP } + \text{HNAP at 300 mOsm } (\%)$$

$$\text{HP (HOS positive)}(\%) = \text{HPAP} + \text{HPAN at 150 mOsm} - \text{ HPAP} + \text{HPAN at 300 mOsm } (\%)$$

3.7.6. Morphology

The percentage of abnormal sperm wascounted from the Hypo osmotic swelling – Giemsa test smears (300 mOsm/kg). A minimum of 200 sperm was counted in each slide under a microscope at 100x (oil immersion) phase-contrast microscope (Nikon Eclipse 80i, Nikon, Japan) and expressed as percent abnormal sperm.

3.7.7. Chromatin distribution

Sperm chromatin distribution was evaluated by Feulgen's staining (Selvaraju *et al.*, 2008). Semen sample (10 µl) from each incubation medium was placed on a clean grease-free microscopic slide and smeared. The prepared smears were fixed in 10% neutral buffered formalin for 30 min and washed in running water and airdried. The slides were then immersed in 5N HCl for 30 minto hydrolyze the purine and deoxyribose bond and to expose the aldehyde group for subsequent reaction with Schiff reagent. The smears were then washed in running water for 5 min and air-dried completely. The smears were incubated in Schiff reagent for 30 min in an amber staining box (since the Schiff reagent is light sensitive) and rinsed in freshly prepared sulfite water thrice. Finally, the slides were washed in running water for 10 min and examined under the microscope at 100x (oil immersion) phase-contrast microscope (Nikon Eclipse 80i, Nikon, Japan) for chromatin distribution. The sperm with evenly distributed chromatin were considered as normal chromatin distribution. The sperm with condensed nuclei, vacuole, abnormal chromatin patterns were considered as abnormal chromatin distribution.

3.7.8. Mitochondrial membrane potential

Sperm mitochondrial membrane potential was evaluated using mitochondria specific cationic fluorophore JC-1(5,5‘,6,6‘-tetrachloro-1,1‘,3,3‘-tetraethyl benzimidazolyl carbocyanine iodide) (Calbiochem, USA). An aliquot of 10 µL of the sample from each of the incubation medium was added to 50 µL of 1x PBS (pH 7.4) containing1 µL of JC- 1 (1 µg/mL) and incubated for 30 min at 37°C. A drop was then smeared on the clean glass slide and counted under 40 x epifluorescence microscopes (Nikon Eclipse 80i, Nikon, Japan) with an excitation filter of 510-560 nm and an emission filter of 505 nm. If JC- 1 enters the mitochondria, form JC- 1 aggregate and the midpiece appearas yellow to orange in sperm indicating high membrane potential. If the JC-1 exists as monomers, the midpiece appears green in the sperm indicating low membrane potential. Those sperm subpopulation without both JC-1 monomers and aggregate were considered dead and included in the low membrane potential. Thus the ratio of high and low membrane potential bearing sperm was calculated from counting 100 cells per sample.

3.7.9. Capacitation status

Chlortetracycline assay was performed for assessing the capacitation status of sperm (Ward and Storey *et al*., 1984) with minor modification. On/over a clean grease-free glass slide, 5µL of the semen sample from each of the incubation medium and 5µL of chlortetracycline (concentration) (Sigma, USA) were added and mixed well. The samples were left for a few seconds to react, and then 0.5 µL of glutaraldehyde (0.6 %) in tris was added and smeared. In order to retain the fluorescence for a longer time a drop of an antifade agent (DABCO- 1,4 diazo bicyclo (2,2,2) octane), was added and stored at 4°C till counting (maximum of 3 days). A minimum of 100 cells was counted under a 100x epifluorescence microscope (Nikon Eclipse

80i, Nikon, Japan) with an excitation filter of 510-560 nm and an emission filter of 505 nm and classified as given in table 15.

Table 15: The description of the patterns observed in the CTC assay for classification of the capacitation status

Capacitation status	Pattern classification	Description of the pattern
Non-capacitated	Pattern F	The sperm showing uniform green fluorescence over the entire head and midpiece
Capacitated	Pattern B	The sperm with bright green fluorescence over the acrosome region of head and midpiece with a dark band on the post-equatorial region
Acrosome	Pattern AR	The sperm with bright green fluorescence on the post-equatorial region and midpiece with the non-fluorescent acrosomal region or the sperm with the dark non-fluorescent head showing the green equatorial ring and fluorescent midpiece

3.7.10. Acrosome integrity

Fluorescein isothiocyanate with pisum sativum agglutininin (FITC – PSA) and propidium iodide (PI) fluorophores (Sigma, USA) was used to assess the acrosome integrity (Selvaraju *et al.,* 2015). Sperm sample (10 µL) from each incubation media was smeared on a clean grease-free glass slide and air-dried. The air-dried smear was then fixed in 100% methanol for 10 min. After fixation, freshly prepared fluorescent dye (50 µL) containing (FITC – PSA) and propidium iodide (PI) was flooded over the smear and incubated for 15 min. After incubation, the smear was washed thrice with PBS and air-dried in dark for 2-3 min. A minimum of 100 cells was counted under a 100x epifluorescence microscope (Eclipse 50i; Nikon) with an excitation filter of 510-560 nm and an emission filter of 505 nm. The sperm with smooth and bright green stained acrosome cap were considered as acrosome intact cells. The cells without acrosome cap or uneven, granulated, and damaged acrosome cap were considered as acrosome reacted. The percentage of sperm with intact acrosome were calculated.

3.8. Prediction of bull fertility based on osmoadaptive ability of sperm

The study aimed to classify bulls based on sperm osmoadaptive ability. The progressive motility of sperm obtained by CASA was used to compute two predictive measures of osmo-adaptation ability, namely

1. $$\text{Percent loss at 1 h} = \frac{(\text{PM \% before incubation (BI)} - \text{PM \% in HYP 1 at 1 h})}{\text{PM \% before incubation (BI)}} \times 100$$

2. $$\text{Percent loss at 4 h} = \frac{(\text{PM \% before incubation (BI)} - \text{PM \% in HYP 1 at 4 h})}{\text{PM \% before incubation (BI)}} \times 100$$

For the computation of the above predictive measures, HYP1 at uterine and oviductal pH were compared. The obtained osmo-sensitivity indices were correlated with the rejection rate of the bull. The medium with a better correlation with the rejection rate was selected for the prediction of osmoadaptive ability. The functional parameters were correlated with the predicted osmoadaption score of the sperm.

3.9. Biochemical estimation

3.9.1 Seminal plasma osmolality

The seminal plasma was separated from the semen by centrifugation at 250 g for 15 min. After centrifugation, the supernatant (seminal plasma) was collected and stored at -80 °C till evaluation. The seminal plasma osmolality was estimated using the osmometer (OSMOMAT 3000, Gonotec, Germany) employing the principle of freezing point depression. The osmometer was calibrated with standard (300 mOsm/kg) before the estimation of seminal plasma osmolality. Seminal plasma was thawed to room temperature and 100 µL of seminal plasma was transferred to osmometer tubes. The osmometer tube was placed on the slot and the osmometer probe was inserted into the tube and the osmolality was estimated.

3.9.2.Estimation of urea concentration

The urea concentrations of the seminal plasma (n=12) and serum (n=12) were measured using a urea test kit (SPAN diagnostics, India) by colorimetric method. The urea test kit was based on the Berthelot method. Briefly, 10 µL of the test sample was added to solution 1 and incubated at 37°C for 3 min followed by the addition of 1 mL of solution 2 and incubated at

37°C for 5 min. Then, 200 μL of the reaction mixture was transferred from each tube to a flat bottom 96 well plate and the absorbance was measured at 578 nm in the microplate reader (Multiskan FC Microplate photometer, Thermo Scientific, USA). The readings were taken in triplicates and the concentration of urea was calculated as follows:

$$\text{Urea concentration (mg/ dL)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 50$$

3.10. Expression of osmo-responsive genes in sperm

3.10.1 Sample preparation for RNA isolation

The neat semen samples (n=10) were centrifuged at 500 g at RT for 15 min and the supernatant (seminal plasma) were removed. The obtained sperm pellet was resuspended in PBS (pH 7.4) and snap-frozen in liquid nitrogen and stored at -80°C till further processing.

The frozen cells were thawed on ice and subjected to 50 % Bovipure (Nidacon, Sweden) density gradient centrifugation for the removal of somatic cells if any. The sperm pellet was resuspended in 2 mL of PBS (pH 7.4) and 1mL of sperm suspension was layered onto a 50% Bovipure gradient (2 mL of Bovipure and 2 mL of PBS) and centrifuged at 200 g for 20 min at RT. The obtained pellet was washed twice with 5 mL of PBS at 700 g for 10 min at 4°C. The resulting sperm pellet was resuspended in 1 mL of PBS, snap-frozen, and stored at -80°C till RNA isolation. The sperm concentration was evaluated using a haemocytometer to assess the percentage recovery of spermafter density gradient centrifugation.

For the control sample, the testis tissue was collected from the local slaughter house and made into small pieces and immersed in RNA later (Ambion, Life Technologies, USA), kept at 4°C overnight, and then stored in -80°C till further use.

3.10.2. RNA isolation

The sperm RNA was isolated by combining double lysis along with kit method (Purelink RNA mini kit, Invitrogen, USA) (Parthipan *et al.*, 2015). To the sperm (40 million), 0.5 mL of lysis buffer containing 5 μL of beta-mercaptoethanol was added and mixed thoroughly. To lyse the sperm, the suspension was homogenized15 times using a 2 mL syringe with 22 G needle. The homogenized mixture was vortexed for 2 min. Then 0.5 mL of TRIzol LS (Ambion, Life

Technologies, USA) was added to the mixture vortexed for 2 min and then incubated at room temperature for 5 min. To this, 200 µL of chloroform (Himedia, India) was added, mixed vigorously by hand for 15 s and incubated at room temperature for 5 min. The mixture was centrifuged at 12000 g for 20 min at 4 °C for phase separation. The top transparent aqueous layer was recovered carefully without disturbing the interphase and transferred to another microcentrifuge tube. Absolute ethanol (Merch, Germany) was added in the ratio of 1: 0.8 to the recovered volume of the aqueous layer and mixed by pipetting. The suspension has then transferred to the column and centrifuged at 12000 g for 30 s at RT. The column was washed with 0.7 mL of wash buffer I at 12000 g for 30 s and followed by 0.5 mL of wash buffer II twice at 12000 g for 30 seconds each at RT. The collection tube was changed and centrifuged at 12000 g for 1 min at RT. Then the RNA in the column was eluted with warm (60°C) nuclease-free water thrice (35 µL, 35 µL, and 25 µL) into separate tubes.

The 40 - 50 mg of testis tissue was homogenized using liquid nitrogen in mortar and pestle and RNA was isolated by the Trizol method.

3.10.3. RNA quantification

The isolated RNA was quantified using a spectrophotometer (NanoDrop, ND- 1000, Thermo Scientific, USA) by measuring the absorbance at 260nm and the purity was analyzed using ratios of 260/280 and 260/ 230. If the 260/280 ratio is not in the range of 1.8 to 2.0 indicates the presence of protein and when 260/230 ratio is not in the range of 2.0 to 2.2 indicates the salts and organic solvent contamination. Briefly, 1µL of the RNA sample was loaded in the NanoDrop (ND-1000, Thermo Scientific, USA) and the concentration was obtained in ng/µL. Further, the quantification of RNA and DNA were performed using Fluorometer (Qubit 2.0, Invitrogen) for more accuracy. For this assay, 1 µL of the sample was mixed with 199 µL of RNA working solution (1µL of Qubit RNA reagent + 199 µL of RNA buffer) and DNA working solution (1µL of Qubit DNA reagent + 199 µL of DNA buffer) separately in the dark The reaction mixture was then incubated at room temperature for 2 min and concentration was measured in Fluorometer (ng/µL) The DNA concentration was checked for the downstream DNase treatment.

3.10.4. DNase treatment

To remove the genomic DNA contamination from the isolated RNA, DNase treatment was done using the TURBO DNA-free kit (Ambion, Life Technologies, USA).Up to 2000 ng of the DNA (50 µL) contamination present in the RNA sample was digested by adding 2 U (1µL) of DNase.The mixture was mixed well and incubated for 15 min at 37 °C in a water bath. After incubating at 37 °C, 5 µL of DNase inactivating agent was added and incubated at room temperature for 5 min with intermittent stirring. Then, the DNase inactivating agent was removed by centrifuging at 10000 g for 1.5 min.The supernatant containing RNA free from DNA was recovered carefully and transferred to the new microcentrifuge tube. To the obtained RNA, 2 µL of the RNase inhibitor and dithiothreitol (DTT) to a final concentration of 10 mM was added and stored at -80°C.

3.10.5. Assessment of gDNA contamination

Intron-spanning *PRM 1* (Protamine 1) gene primer was used to detect genomic DNA contamination in the isolated RNA before cDNA synthesis using TB Green Premix Ex Taq II (Takara Bio, Japan) in q-PCR (StepOnePlus, Applied Biosystems, USA). The amplified product was confirmed by melt curve analyses and the product size by 2 % agarose gel electrophoresis.

3.10.6. cDNA synthesis

The complementary DNA (cDNA) was synthesized using the first-strand cDNA synthesis kit (SuperScript IV, Invitrogen, USA). Equal quantity (20 ng) of RNA from each sample was used for cDNA synthesis. The synthesis was carried out in 20µL reaction volume. First, an equal volume (0.5µL) of 50 µM random hexamer and 50 µM oligo d(T) primers were added (for better efficiency) to 20ng of RNA (11µL) and the mixture was maintained at 65 °C for 5 min for primer annealing, transferred to ice and incubated for 1 min. The reverse transcriptase reaction mix was then added to the mixture and transferred to the thermocycler (MJ Mini Thermal Cycler, BIO-RAD, USA) for cDNA synthesis. The PCR conditions set for cDNA synthesis were 23°C for 10 min, followed by 50°C for 10 min and inactivation at 80°C for 10 min.

3.10.7. Other cells RNA contamination

For the detection of RNA from other cell contaminants in the sperm total RNA, cell-specific gene primers, namely *CDH1* (Epithelial cadherin) for somatic cells, *CKIT* (Kit oncogene) for germ cells and *PTPRC* (Protein tyrosine phosphatase receptor type C) for leucocytes. If the samples had amplification for any of the primers, they were eliminated for further studies.

3.10.8. Primer design and synthesis

The primers for the selected genes were designed using Primer3Plus software.The gene sequences were retrieved from Ensembl and the primers were designed using the following criteria.

Table 16: Primer designing criteria adopted for synthesis of primers

Parameter	Range
Primer length	18 – 24 bp
Product size	80 – 200 bp
T_m	55- 65 °C
T_m difference between forward and reverse primer	Not more than 2°C
GC content	40 – 50 %
Complementarity	No

Using NCBI Primer-BLAST tool, the selected primers were also checked for its specificity of binding to the target gene. The oligonucleotides were synthesized (Eurofins Genomics India, Bangalore).

Table 17: The details of the primers used for gene expression study

Primer ID	Primer	Primer sequence (5' to 3')	Primer length (bp)	Product size (bp)	NCBI Accession No
SLC9C1	Forward	GGAACGCCTCGAATAAGCCT	20	149	XM_024994321.1
	Reverse	TCAGCTCAAAGTTGCTCCCT	20		
HSP90AB1	Forward	GTGACGATCTCCAACAGGCT	20	213	NM_001079637.1
	Reverse	GTCGTTTTTGTCCGCCTCTG	20		
EFHD1	Forward	AACGTGCCTCTACTTGGCAG	20	145	NM_001075832.1
	Reverse	TTAACATCACTGGCCTCCCG	20		
ENO1	Forward	ATGTCACCGAGCAGTGTGAG	20	212	NM_174049.2
	Reverse	GATACTTGGTGGGAGCGAGG	20		
NFAT5	Forward	ACCTCTTCCAGCCCTACCAT	20	170	XM_002694839.6
	Reverse	AAGACTGTGTGCCTCTTCGG	20		
MT-ND2	Forward	TCTCAGGCCAATGAACCGTA	20	127	NC_006853.1:4266-5307
	Reverse	ATGCCCTGTGTTACTTCTGGG	21		
MT-CO1	Forward	GTAACCGCACACGCATTTGT	20	217	NC_006853.1:5687-7231
	Reverse	GGTACACGGTTCAGCCTGTT	20		
ADAM1B	Forward	GAGTGGGAATGACAGGCTCA	20	119	NW_020192236
	Reverse	TGACAGAATCCCTCCTCCTAGT	22		
PRM1	Forward	AAGATGTCGCAGACGAAGGAG	21	222	NM_174156.2
	Reverse	GTGGCATTGTTCGTTAGCAGG	21		
UT-A	Forward	GGAAACGGGCATCTCCAAGA	20	148	NM_001144102.1
	Reverse	GGAAACGGGCATCTCCAAGA	20		
UT-B	Forward	TCAACATCACAGGGCCCATC	20	121	NM_001008666.1
	Reverse	CTGCCAGCCTGAAACAAACC	20		
RPL23	Forward	CAGCGGTGGTAATTCGACAAC	21	116	NM_001035014.2
	Reverse	GGCGGAACCTTTCATCTCG	19		
GAPDH	Forward	CTGAGGACCAGGTTGTCTCCTG	22	141	NM_001034034.1
	Reverse	CCCTGTTGCTGTAGCCAAATTC	22		
CDH1	Forward	CTGCATTCCTGGCTTTGGTG	20	171	NM_001002763.1
	Reverse	GTAAGCACGCCATCTGTGTG	20		
KIT	Forward	GAATAGCTGGCATCAGGGTG	20	224	AF263827.1
	Reverse	CCAGATCCACATTCTCTCCATC	22		
PTPRC	Forward	TGGACGAAATTGCATCCCTCAGGA	24	237	NM_174156.2
	Reverse	RTGGTCAGGACGTTTACAGCTCACA	24		

3.10.9. Real-time PCR (qPCR) experiment

RPL23, PRM1 and *GAPDH* were selected as an endogenous control and evaluated for stability using NormFinder and BestKeeper tools.The gene with best stability was used for normalizing the expression levels of sperm transcripts (Parthipan *et al*., 2017).The relative expression of sperm transcripts was studied with equal quantity (2 ng) of cDNA from each sample or animal in real time-PCR using TB Green Premix Ex Taq II (Takara Bio, Japan) (StepOnePlus, Applied Biosystems, USA). The primer concentration used was 2 µL of 1 µM per 10 µL reaction volume. The set qPCR conditions were: Initial denaturation at 95°C for 30 s; 40 cycles of 95°C for 0.03 s and 60°C for 1 min followed by melt curve: 95°C for 0.015 s, 70 °C for 1 min (increase in temperature up to 95°C with the rate of 3°C/s); 95°C for 0.015 s. The unique product was confirmed by melt curve analysis and the product size was verified by agarose gel (2.0%) electrophoresis. Relative gene expression levels were computed using the $2^{-\Delta\Delta Ct}$ method (Livak *et al*., 2001).

3.10.10. Agarose gel electrophoresis

The PCR products were resolved using agarose gel electrophoresis. Briefly, 2 % agarose was prepared by adding 2 g in 100 mL of 1x TBE (Tris Borate EDTA buffer, pH 8) and dissolved by heating in a microwave oven. After 2-3 min of cooling, 2 µL of Ethidium bromide was added and mixed slowly. Then it was poured slowly into the assembled gel casting tray with 20 well combs. The gel was allowed to solidify. The solidified gel was transferred to the electrophoresis tank and the comb was carefully removed. Around 650 mL of 1X TBE buffer was poured in the tank. 2 µL of DNA loading dye (6x DNA loading dye, Thermo Scientific) was added to 10 µL of PCR product mixed and 5µL of the sample was loaded into wells of the gel. Also, 5 µL of 100 bp ladder (NEX-GEN DNA Ladder) was loaded in the gel. The gel was run at 100 Volts for 45 min or till the dye crosses 2/3 of the gel. The gels were scanned using the gel documentation system (G BOX ichemi XR: Syngene, Cambridge, UK)

3.11. Statistical Analyses

The data were tested for normal distribution and sphericity (Mauchly test of sphericity). The log transformed data was subjected to statistical analysis. The data were back transformed for better graphical representation. Analyses were carried out using IBM SPSS version 20 and GraphPad prism 6. The sample size required for the experiment was computed using the effect size obtained from the pilot study (Progressive motility at 0 h) using the G power tool. The influence of osmolality and pH on sperm functional parameters at various time points was analyzed employing multiparametric analysis using repeat measure factorial ANOVA with B-TUKEY posthoc test. The functional parameters were considered as the individual dependent variable, and osmolality, pH, and incubation time were considered as dependent variables. The main effects of the independent variables and interaction effect of the same were analyzed. The effects of different concentrations of urea on sperm functional parameters at various time points were analyzed using repeat measure two-way ANOVA coupled with B-TUKEY posthoc test. The simple main effects and their interactions were also assessed. The student's t-test was used for analyzing the significant differences in sperm functional parameters between good and poor osmoadapters. The significance level was set at $p \leq 0.05$.ROC analysis and Linear regression analysis were employed for development of osmo-susceptibility based prediction of quality semen production capacity of bull. All the vales were presented as mean±SEM. Pearson correlation was employed for correlation analysis and significance was set at ($p<0.05$).

✍✍✍

Results

4 RESULTS

4.1. Estimation of seminal plasma osmolality and pH

The osmolality of HF bulls (n=20) seminal plasma was 292 ± 2.72 mOsm/kg with a range from 277 to 313 mOsm/kg. The pH of HF bulls semen (n=20) was 6.75 ± 0.05 with a range of 6.5 to 7.0.

4.2. Standardization of sperm transport and washing media

As the study aimed to explore the effect of osmolality and pH on sperm functional attributes, the development and or selection of an isotonic sperm transport and washing media were essential. In order to standardize these requirements, semen was transported in two isosmotic transportation media (mTEY and TBM, pH adjusted to 6.8) at 25-28°C and subsequently washed in three isosmotic washing media (Bovipure, TBM and mLBM).

The semen samples transported in both mTEY and TBM and subsequently washed in in TBM and mLBM resulted in significantly ($p<0.05$) higher percentage of concentration recovery of sperm as compared to Bovipure washing medium (Table 18).The semen samples transported in mTEY and washed in mLBM yielded a significantly ($p< 0.05$) higher progressive motility percentage than the Bovipure washing medium (Table 18). The percentage of sperm progressive motility (%) was non-significantly higher in semen transported in mTEY and washed in than TBM.The relative yield of the sperm (%) computed by considering both the parameters, concentration and progressive motility revealed non-significantly yield in sperm transported in mTEY than TBM. Similarly, the relative yield of the sperm (%) was significantly ($p< 0.05$)

higher when washed in mLBM than Bovipure washing media (Figure 11) and non-significantly higher than TBM washing media (Table18).

Table 18: The relative yield of sperm obtained from combinations of two transport media and three washing media. The semen samples (n=6) were transported in transportation media (mTEY and TLB) at 25-28°C and subsequently centrifuged in washing media (Bovipure, TBM and mLBM). (mTEY: modified isotonic Tris eggyolk extender, TBM: Tyrode basal medium, mLBM: modified low bicarbonate Tyrode medium)

Parameter	Transport media	Washing media		
		Bovipure	TBM	mLBM
Concentration recovered as compared to pre wash (%)	mTEY	60.6±1.49[a]	69.4±1.33[b]	67.4±1.93[b]
	TBM	57.8±1.46[a]	66.1±1.56[b]	67.5±1.54[b]
Progressive motility (%)	mTEY	40.5±7.07[a]	53.1±5.69[ab]	58.9±5.77[b]
	TBM	40.1±6.23	43.7±5.97	48.8±6.65
Relative yield (%)	mTEY	35.8±7.49[a]	50.6±7.48[ab]	60.1±7.78[b]
	TBM	36.6±6.82[a]	51.4±5.52[ab]	57.7±8.62[b]

Superscript bearing [a,b,c] in a row differ significantly (p<0.05)

4.3. Fixing of sampling time points for osmoadaptation study

The osmotic stress is expected to cause perturbation of cell volume and thus may transiently affect the motility of sperm. Since the window of this osmoadaptive behavior in sperm was not studied before, there was a necessity to conduct a pilot study to fix the sampling time points after subjecting to osmotic stress. The sperm was subjected to hyperosmotic stress (HYP1: 355mOsm/kg; HYP2: 420 mOsm/kg) with a control (ISO: 290 mOsm/kg). An aliquot of semen sample was taken immediately after incubation (0 h) and in equal intervals of 30 min (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 h) and subjected to motility analysis and HOS test.

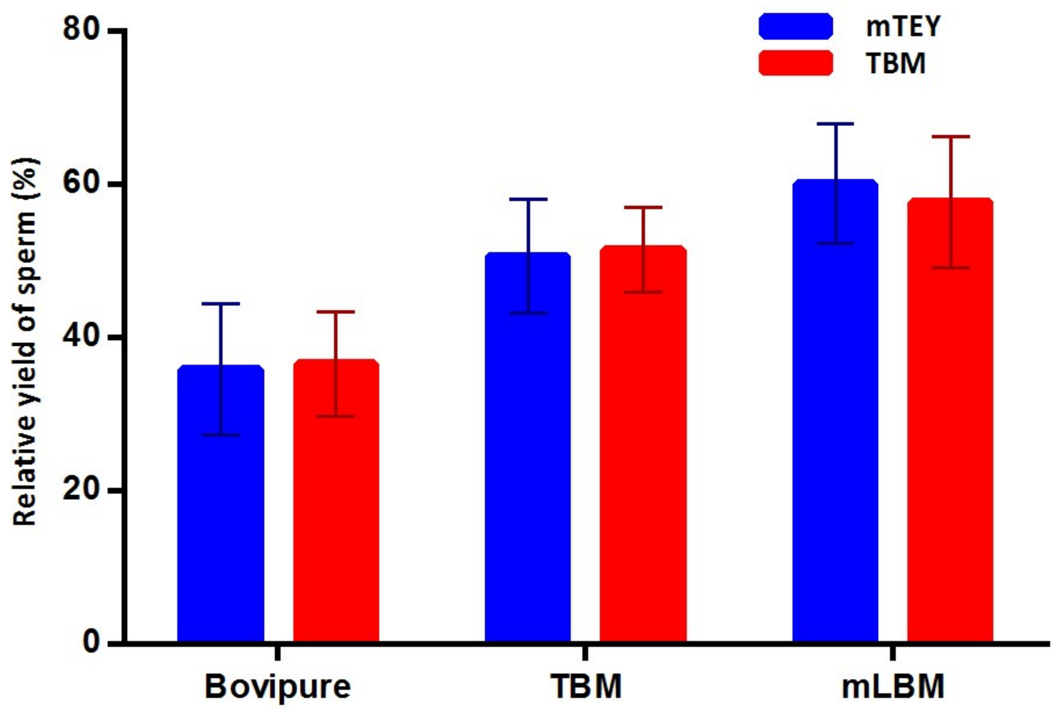

Fig. 11: The relative yield of sperm obtained from combinations of two transport media and three washing media (n=6).The relative yield of the sperm was significantly ($p < 0.05$) higher in sperm transported in mTEY and washed in mTLB. (mTEY: modified isotonic Tris eggyolk extender, TBM: Tyrode basal medium, mLBM : modified low bicarbonate Tyrode medium)

4.3.1. Progressive forward motility

The progressive motility (%) in the hyperosmotic media reduced significantly (p< 0.05) on immediate (0 h) exposure to stress and subsequently regained over a period of 1- 2 h (Table 19), whereas continuous drop was observed in the isosmotic medium. The time point at which maximum motility attained after a initial drop was considered as the regaining ability of that sample. Further, the time taken for regaining the motility varied from 1-2 h among the bulls. The regained progressive motility in the hyperosmotic medium (HYP1) significantly sustained well up to 4 h as compared to isosmotic medium (Table 13).

Table19: Standardization of duration of incubation for the experiment based on the changes in progressive motility (%) of sperm subjected to different osmotic media (n=6) (DOI: Duration of incubation; ISO: 290 mOsm/kg; HYP1: 355 mOsm/kg; HYP2: 420 mOsm/kg; BI: Before incubation)

DOI(h)	Progressive motility (%)		
	ISO 6.8	HYP1	HYP2
BI	88.1±1.33	88.1±1.33^{X}	88.1±1.33^{X}
0.0	85.5±1.71	65.3±3.27^{Y}	56.1±6.71^{Y}
0.5	78.0±4.31	76.9±3.78^{Z}	73.4±5.52^{Z}
1.0	76.1±3.51	77.7±3.65	77.2±4.85
1.5	73.5±3.28	77.2±2.91	74.3±4.13
2.0	71.6±3.91	75.0±2.84	76.2±3.83
2.5	65.5±5.14	74.5±2.75	69.1±5.50
3.0	63.5±4.71	72.2±4.17	63.5±4.78
3.5	58.4±3.67	66.4±4.68	60.1±5.48
4.0	54.6±3.69^{a}	64.5±5.18^{b}	56.0±6.48^{a}

Superscript bearing ^{a,b} in a row differ significantly (p<0.05)
Superscript bearing ^{X,Y,Z} in a column differ significantly (p<0.05)

4.3.2. Functional membrane integrity

The osmotic stress did not have adverse effect on percentage of sperm functional membrane integrity. Instead there was a non-significant conservative effect of functional membrane integrity in the hyperosmotic media especially in HYP1 as evidenced from 3 h of incubation (Table 20). The percentage of sperm progressive motility decreased immediately at

0 h and regained between 1-2 h, whereas the percentage of functional membrane integrity sustained in hyperosmotic media from 3 h. Based on these preliminary observation, it was decided to retrieve samples at 0, 1, 2 and 4 h for further studies.

Table 20: Standardization of duration of incubation for the experiment based on the changes in functional membrane integrity (%) of sperm subjected to hyperosmotic media(n=6).(DOI: Duration of incubation; ISO: 290 mOsm/kg; HYP1: 355 mOsm/kg; HYP2: 420 mOsm/kg; BI:Before incubation)

DOI(h)	Functional membrane integrity (%)		
	ISO 6.8	HYP1	HYP2
BI	84.0±1.34	84.0±1.34	84.0±1.34
0.0	82.3±1.17[a]	80.2±1.62[ab]	78.5±1.23[b]
0.5	79.3±2.33[a]	75.5±2.05[ab]	74.8±1.64[b]
1.0	70.8±1.49	70.2±1.64	68.3±2.03
1.5	68.5±1.65	65.5±2.72	62.8±2.95
2.0	63.2±3.19	62.2±2.89	59.7±3.29
2.5	56.0±4.60	57.0±3.37	58.2±4.43
3.0	48.3±4.88	52.0±3.76	52.5±4.30
3.5	43.0±4.84	46.0±3.07	47.7±3.83
4.0	37.5±4.98	42.2±2.50	41.7±3.33

Superscript bearing [a,b] in a row differ significantly (p<0.05)

4.4. Experiment 1: Influence of osmolality and pH on sperm functional attributes

4.4.1. Sperm kinematics

The percentages of total, progressive and rapid progressive motility were significantly (p<0.05) lower at 0 h (i.e., immediately after exposure) in hyperosmotic media and significant recovery was subsequently observed (Table 21). The percentages of progressive and rapid progressive motility sustained significantly (p < 0.05) higher at 4 h in HYP1 (355 mOsm/kg) as compared to ISO and HYP2 media at uterine pH, 6.8 (Figure 12). The same trend though observed in oviductal pH, it was non-significant. The hyperactive (%) and mucus penetration (%) did not differ significantly between the media differing osmolality and pH.

Table 21: Influence of osmolality and pH on kinematics of bovine sperm incubated *in vitro*. The semen samples were incubated at uterine (osmolality, 355 mOsm/kg and pH 6.8) and oviduct (osmolality, 355 mOsm/kg and pH 7.4) environment. The hyperosmotic (HYP1) uterine and oviductal environment significantly (p<0.05) decreased progressive forward motile spermatozoa (%) at 0 h and regained the same at 1 h of incubation. (DOI: Duration of incubation; ISO: 290 mOsm/kg; HYP1: 355 mOsm/kg; HYP2: 420 mOsm/kg; BI: Before incubation)

Parameters	DOI	Uterine pH 6.8			Oviduct pH 7.4		
		ISO	HYP1	HYP2	ISO	HYP1	HYP2
Total motility (%)	BI	97.6±0.97	97.6±0.97	97.6±0.97	97.6±0.97	97.6±0.97	97.6±0.97
	0 h	97.5±0.51[a]	91.8±0.70[b]	85.0±3.8[c]	96.3±1.44[a]	91.3±1.33[b]	82.3±4.16[c]
	1 h	91.3±1.49	93.6±1.98	91.1±2.71	93.1±1.45	93.9±2.38	92.9±1.87
	2 h	88.5±1.63	91.9±2.45	92.6±1.30	87.0±2.64	90.3±2.86	92.6±2.36
	4 h	81.7±3.60	87.2±3.17	84.4±4.42	82.7±3.63	86.2±3.44	88.4±2.89
Progressive motility (%)	BI	88.2±2.22	88.2±2.22	88.2±2.22	88.2±2.22	88.2±2.22	88.2±2.22
	0 h	87.1±1.32[a]	76.2±2.15[b]	67.0±4.70[c]	86.3±2.46[a]	75.5±3.06[b]	66.0±5.21[c]
	1 h	77.6±2.89	82.6±3.22	79.5±4.40	80.6±2.95	82.8±3.92	81.7±4.03
	2 h	72.9±2.68	79.8±3.89	78.4±3.36	72.1±4.29	77.5±4.28	80.9±4.15
	4 h	62.9±4.15[a]	73.4±4.46[b]	67.4±5.26[ab]	66.0±4.35	72.2±4.66	72.5±4.44
Rapid progressive (%)	BI	53.1±3.41	53.1±3.41	53.1±3.41	53.1±3.41	53.1±3.41	53.1±3.41
	0 h	58.3±3.27[a]	55.8±3.14[ab]	49.5±4.39[b]	56.7±3.03	55.2±3.33	51.0±4.60
	1 h	56.4±3.28	59.1±3.49	60.2±4.11	60.7±3.50	59.4±3.43	58.2±3.90
	2 h	53.3±2.62	58.9±3.22	58.0±3.31	55.2±4.47	55.2±3.26	59.8±4.52
	4 h	46.9±3.58[a]	55.7±3.42[b]	50.3±4.35[ab]	50.6±4.14	55.6±3.46	55.2±3.60
Hyperactive (%)	BI	7.13±1.65	7.13±1.65	7.13±1.65	7.13±1.65	7.13±1.65	7.13±1.65
	0 h	4.09±1.46	5.87±1.80	6.26±2.34	4.44±1.59	4.59±1.14	6.32±1.62
	1 h	6.65±1.96	7.35±2.10	6.33±1.12	5.01±1.47	7.14±1.57	7.41±2.14
	2 h	6.51±1.68	6.36±1.37	4.90±1.26	3.58±0.90	7.04±1.30	4.29±0.86
	4 h	3.16±0.53	2.75±0.77	2.42±0.61	2.92±0.56	3.34±1.02	2.63±0.62
Mucus penetration (%)	BI	43.5±3.54	43.5±3.54	43.5±3.54	43.5±3.54	43.5±3.54	43.5±3.54
	0 h	48.3±3.33	50.0±2.88	52.2±4.34	47.1±3.02	50.8±2.65	56.1±3.96
	1 h	52.8±3.72	56.1±3.70	58.9±3.86	56.2±3.34	54.3±2.57	55.0±4.26
	2 h	52.5±3.40	52.5±4.67	55.8±2.99	57.7±4.72	53.5±3.02	57.1±4.32
	4 h	51.8±2.99	54.6±2.32	52.0±3.12	53.8±4.01	56.1±3.26	55.4±2.24

Superscript bearing [a,b,c] for a parameter at a particular time point and pH differ significantly (p<0.05)
Superscript bearing [X,Y,Z] for a parameter at a particular osmolality and pH differ significantly (p<0.05)

4.4.2.Sperm head area

On immediate (0 h) exposure to osmotic stress, a significant ($p<0.05$) decrease in the sperm head area (μm^2) was observed (Table 22). Subsequently, a significant increase in sperm head area (μm^2) at 1 and 2 h indicated a regulatory volume increase, which was a sign of osmoadaptation. However, the recovery rate or adaptation was comparatively lower in HYP2 (420 mOsm/kg) than the HYP1 at both pH throughout the incubation period (Figure 13).

Table 22: Influence of osmolality and pH on sperm head area (µm2) in bovine sperm incubated *in vitro*. The semen samples were incubated at uterine (osmolality, 355 mOsm/kg and pH 6.8) and oviduct (osmolality, 355 mOsm/kg and pH 7.4) environment. The hyperosmotic (HYP1) uterine and oviductal environment significantly ($p<0.05$) decreased head area of spermatozoa at 0 h and regained the same at 1 h of incubation. (DOI: Duration of incubation; ISO: 290 mOsm/kg; HYP1: 355 mOsm/kg; HYP2: 420 mOsm/kg; BI:Before incubation)

Parameters	DOI	Uterine pH 6.8 Oviduct pH 7.4					
		ISO	HYP1	HYP2	ISO	HYP1	HYP2
Sperm Head area (μm^2)	BI	43.3±0.87	43.3±0.87	43.3±0.87	43.3±0.87	43.3±0.87	43.3±0.87
	0 h	43.2±0.78[a]	39.6±0.75[b]	38.6±0.71[b]	42.2±0.50[a]	38.9±0.85[b]	38.5±0.78[b]
	1 h	41.2±0.69	41.8±0.76	40.7±0.98	41.3±0.59	42.3±0.69	41.1±1.05
	2 h	39.3±0.92	41.2±0.84	40.6±0.68	39.5±0.91	41.1±0.73	41.0±0.85
	4 h	39.7±0.71	40.0±1.01	39.1±0.78	39.0±0.79	40.1±0.78	39.8±0.77

Superscript bearing [a,b] in a row for a particular pH differ significantly ($p<0.05$)

4.4.3. Sperm velocity and motility characteristics

The velocities (VCL, VAP and VSL) of sperm (µm/s) were significantly affected on immediate (0 h) exposure to hyperosmotic stress (Table 23). Subsequently, sperm velocities (µm/s) increased in the hyperosmotic media as compared to isosmotic medium throughout the incubation period (Table 17). The straight-line velocity (66.9±5.40 vs 54.5±3.99 µm/s) and average path velocity (79.7±5.97 vs 64.8±4.33µm/s) in HYP1 (355 mOsm/kg, 6.8 pH) were significantly ($p<0.05$) higher as compared to ISO media (Figure 14) from 2 h of incubation.The linearity was significantly ($p<0.05$) higher at 4 h in uterine pH in HYP1 as compared to ISO

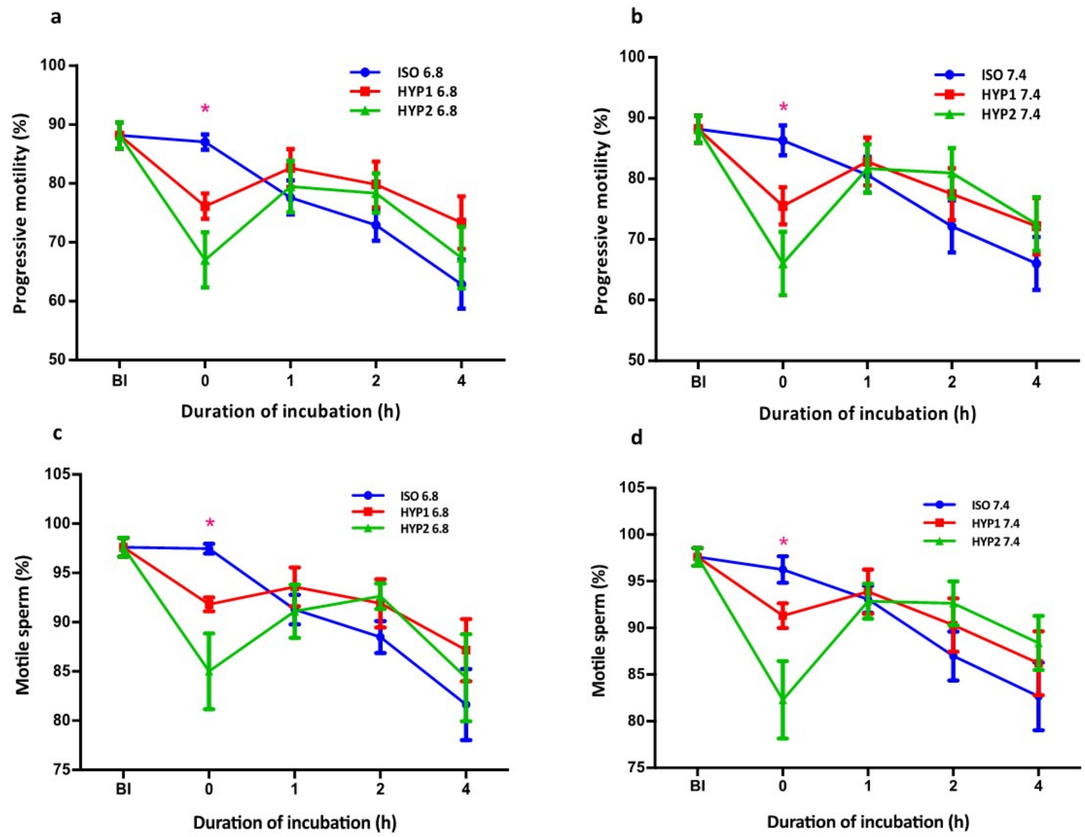

Fig. 12: Effect of osmolality and pH on sperm progressive motility (%) (a &b)and total motility (c & d) in bovine sperm incubated in vitro. The hyperosmotic (HYP1) uterine and oviductal environment significantly ($p<0.05$) decreased progressive forward motile spermatozoa at 0 h and regained the same at 1 h of incubation. (ISO: 290 mOsm/kg; HYP1: 355 mOsm/kg; HYP2 : 420 mOsm/ kg; BI:Before incubation)

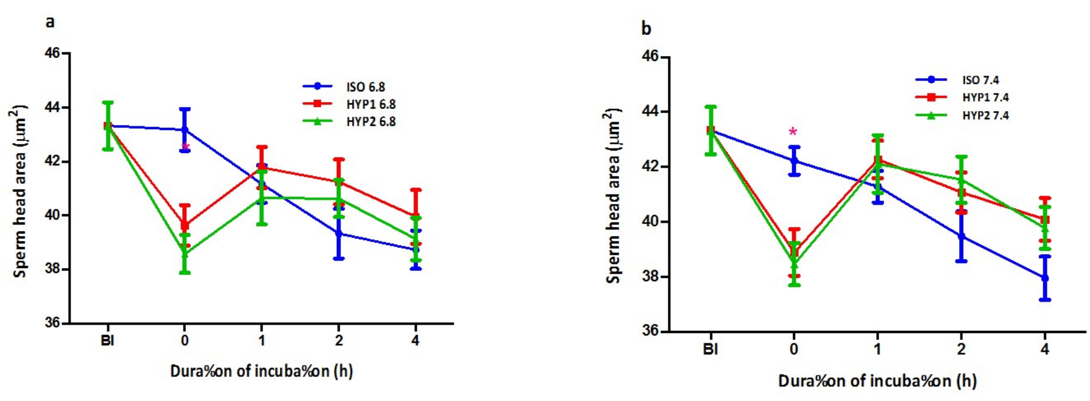

Fig. 13: Effect of osmolality and pH on sperm head area (µm2) in bovine sperm incubated in vitro. The hyperosmotic (HYP1) uterine (a) and oviductal (b) environment significantly ($p<0.05$) decreased head area of sperm at 0 h and regained the same at 1 h of incubation (ISO: 290 mOsm/kg; HYP1: 355 mOsm/kg; HYP2: 420 mOsm/kg; BI:Before incubation)

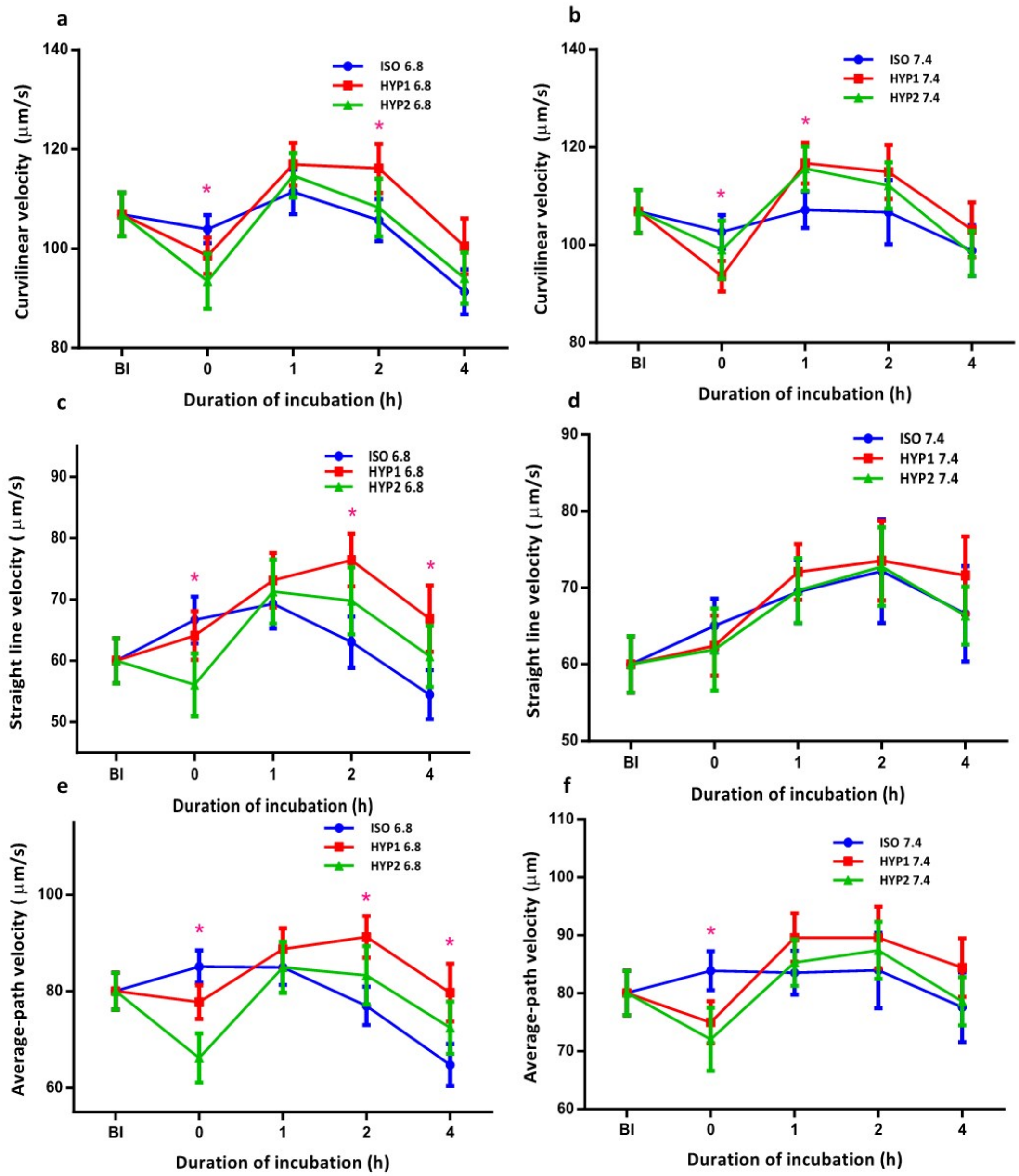

Fig. 14: Effect of osmolality and pH on sperm velocity (%)in bovine sperm incubated in vitro. The hyperosmotic (HYP1) uterine and oviductal environment significantly ($p<0.05$) increased VCL (a and b), VAP (e and f) post osmoadaptation at 2 hour of incubation characteristic of sperm activation. (ISO: 290 mOsm/kg; HYP1: 355 mOsm/kg; HYP2: 420 mOsm/kg; BI:Before incubation)

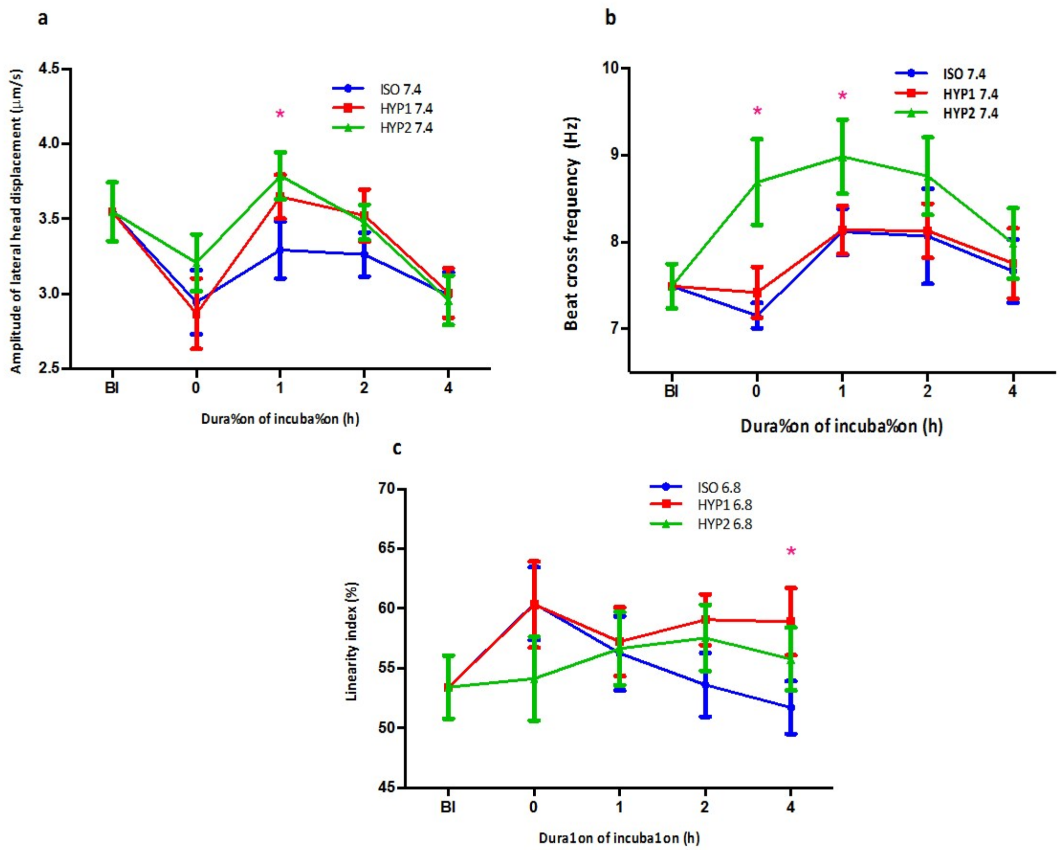

Fig. 15: Effect of osmolality and pH on sperm motility characteristics (BCF, ALH, Linearity index) in bovine sperm incubated in vitro. The hyperosmotic (HYP1 oviductal environment significantly (p<0.05) increased ALH (a), BCF (b) at 1 h.The hyperosmotic (HYP1 uterine environment significantly (p<0.05) increases linearity index at 4 h. (ISO: 290 mOsm/kg; HYP1 : 355 mOsm/kg; HYP2 : 420 mOsm/kg; BI:Before incubation)

media. The ALH (µm) was also significantly (p<0.05) higher in HYP1 medium than ISO (pH 6.8) at 1 hour (Figure 15).

Table 23: Influence of osmolality and pH on kinematic characteristics (VCL, VAP, VSL, BCF and ALH) of bovine sperm incubated *in vitro*. The semen samples were incubated at uterine (osmolality, 355 mOsm/kg and pH 6.8) and oviductal (osmolality, 355 mOsm/kg and pH 7.4) environment. The hyperosmotic (HYP1) uterine and oviductal environment significantly (p<0.05) increased the VCL and VAP post-osmoadaptation at 1 h of incubation. (DOI: Duration of incubation; ISO: 290 mOsm/kg; HYP1: 355 mOsm/kg; HYP2: 420 mOsm/kg; BI:Before incubation)

Parameters	DOI	Uterine pH 6.8			Oviduct pH 7.4		
		ISO	HYP1	HYP2	ISO	HYP1	HYP2
VCL (µm/s)	BI	106.9±4.41	106.9±4.41	106.9±4.41	106.9±4.41	106.9±4.41	106.9±4.41
	0 h	104.0±2.81^{a}	98.60±3.65^{bc}	93.50±5.53^{bc}	102.7±3.44^{a}	93.60±3.14^{b}	93.00±5.93^{b}
	1 h	111.40±4.5	117.0±4.26	114.8±4.47	107.2±3.69^{a}	116.7±4.17^{b}	115.6±4.54^{b}
	2 h	105.7±4.22^{a}	116.2±4.93^{b}	108.3±5.79^{ab}	106.7±6.57	114.9±5.53	112.2±4.72
	4 h	91.30±4.53	100.5±5.61	94.10±5.15	98.80±5.20	103.1±5.63	98.20±4.56
VAP (µm/s)	BI	80.1±3.83	80.1±3.83	80.1±3.83	80.1±3.83	80.1±3.83	80.1±3.83
	0 h	85.1±3.31^{a}	77.8±3.51^{b}	66.2±5.09^{c}	83.9±3.38^{a}	75.0±3.65^{bc}	72.1±5.47^{bc}
	1 h	85.0±3.64	88.8±4.27	85.0±5.27	83.6±3.78	89.6±4.19	85.3±4.00
	2 h	77.0±3.98^{a}	91.3±4.32^{b}	83.3±6.04^{ab}	84.0±6.53	89.6±5.33	87.4±4.92
	4 h	64.8±4.33^{a}	79.7±5.97^{b}	72.5±5.40^{ab}	77.6±6.02	84.4±5.04	78.6±4.15
VSL (µm/s)	BI	60.0±3.69	60.0±3.69	60.0±3.69	60.0±3.69	60.0±3.69	60.0±3.69
	0 h	66.7±3.82^{a}	64.1±3.98	56.1±5.10^{b}	65.1±3.51	62.5±3.92	62.0±5.36
	1 h	69.3±4.07	73.2±4.47	71.3±5.23	69.5±4.15	72.1±3.64	69.7±4.22
	2 h	63.1±4.19^{a}	76.5±4.32^{b}	69.8±5.50^{ab}	72.2±6.76	73.6±5.21	72.8±5.14
	4 h	54.5±3.99^{a}	66.9±5.40^{b}	60.7±4.98^{ab}	66.6±6.23	71.7±5.06	66.4±3.78
STR (%)	BI	69.1±1.82	69.1±1.82	69.1±1.82	69.1±1.82	69.1±1.82	69.1±1.82
	0 h	72.3±1.87	74.1±2.29	73.7±2.57	71.9±1.71	74.3±2.10	74.6±2.54
	1 h	72.6±2.27	73.7±2.25	75.6±2.28	74.8±2.05	72.7±1.50	73.9±2.42
	2 h	72.4±2.00	74.3±1.78	74.3±1.83	75.4±2.80	72.6±1.83	74.6±2.46
	4 h	72.0±1.76	73.9±1.80	72.3±1.83	73.2±2.44	74.3±1.79	74.4±1.83
LIN (%)	BI	53.4±2.64	53.4±2.64	53.4±2.64	53.4±2.64	53.4±2.64	53.4±2.64
	0 h	60.4±3.04	60.4±3.61	54.1±3.51	60.1±2.84	61.4±3.51	56.1±3.39
	1 h	56.3±3.11	57.2±2.88	56.7±3.07	59.6±3.15	56.6±2.17	55.6±2.70
	2 h	53.6±2.67	59.1±2.12	57.6±2.77	59.7±3.48	57.2±2.49	58.7±2.85
	4 h	51.7±2.20^{a}	58.9±2.82^{b}	55.8±2.64^{ab}	57.9±3.55	60.9±2.15	59.7±2.46

Table 23: Contd...

Parameters	DOI	Uterine pH 6.8			Oviduct pH 7.4		
		ISO	HYP1	HYP2	ISO	HYP1	HYP2
WOB (%)	BI	73.0±2.22	73.0±2.22	73.0±2.22	73.0±2.22	73.0±2.22	73.0±2.22
	0 h	79.1±2.43	76.3±3.02	67.6±3.01	79.2±2.30	76.9±2.93	69.4±3.07
	1 h	72.1±2.43	72.4±2.29	70.4±2.47	74.5±2.64	72.9±2.00	70.6±1.91
	2 h	68.7±2.19	73.9±1.55	72.1±2.42	73.3±2.46	73.1±1.80	73.6±1.98
	4 h	66.0±1.89	73.8±2.59	70.8±2.39	72.1±2.83	75.9±1.49	74.5±2.02
ALH (µm)	BI	3.55±0.20	3.55±0.20	3.55±0.20	3.55±0.20	3.55±0.20	3.55±0.20
	0 h	2.95±0.21	2.96±0.24	3.26±0.20	2.94±0.21	2.87±0.24	3.21±0.19
	1 h	3.49±0.22[a]	3.86±0.15[b]	3.69±0.09	3.29±0.19	3.65±0.15	3.79±0.16
	2 h	3.56±0.17	3.56±0.15	3.39±0.14	3.26±0.15	3.52±0.17	3.48±0.12
	4 h	3.24±0.14	3.02±0.15	2.96±0.13	2.99±0.15	3.01±0.17	2.96±0.16
BCF (Hz)	BI	7.49±0.26	7.49±0.26	7.49±0.26	7.49±0.26	7.49±0.26	7.49±0.26
	0 h	7.15±0.20[a]	7.20±0.31[a]	8.11±0.47[b]	7.15±0.15[a]	7.42±0.29[a]	8.69±0.49[b]
	1 h	8.51±0.45[a]	8.43±0.25[a]	9.48±0.43[b]	8.12±0.27	8.14±0.28	8.99±0.43
	2 h	8.67±0.33	8.66±0.34	8.69±0.45	8.07±0.55	8.13±0.31	8.76±0.45
	4 h	8.21±0.36	8.01±0.40	7.79±0.45	7.67±0.36	7.76±0.41	7.99±0.41

Superscript bearing [a,b,c] for a parameter at a particular time point and pH differ significantly (p<0.05)

4.4.4. Sperm capacitation and acrosome reaction status

The hyper-osmolality (HYP1 and HYP2) and pH (7.4) significantly increased (p<0.001) sperm capacitation (%) compared to isosmotic medium at 0, 1 and 2 h (Table 24). The hyperosmotic media (HYP1 and HYP2) induced capacitation in significantly higher percentage of sperm (86– 91%) on immediate exposure (0 h) as compared to 46-66 % in isosmotic media (Figure 16 and 17).The oviductal pH significantly (p<0.001) increased the percentage of sperm capacitation inisosmotic media (ISO 7.4) at 0 and 1 h but not in hyperosmotic (HYP1 and HYP2) media (Figure 18). Thus uterine and oviductal osmolality and pH (7.4) were found to have interaction effect (p<0.001) on sperm capacitation status.

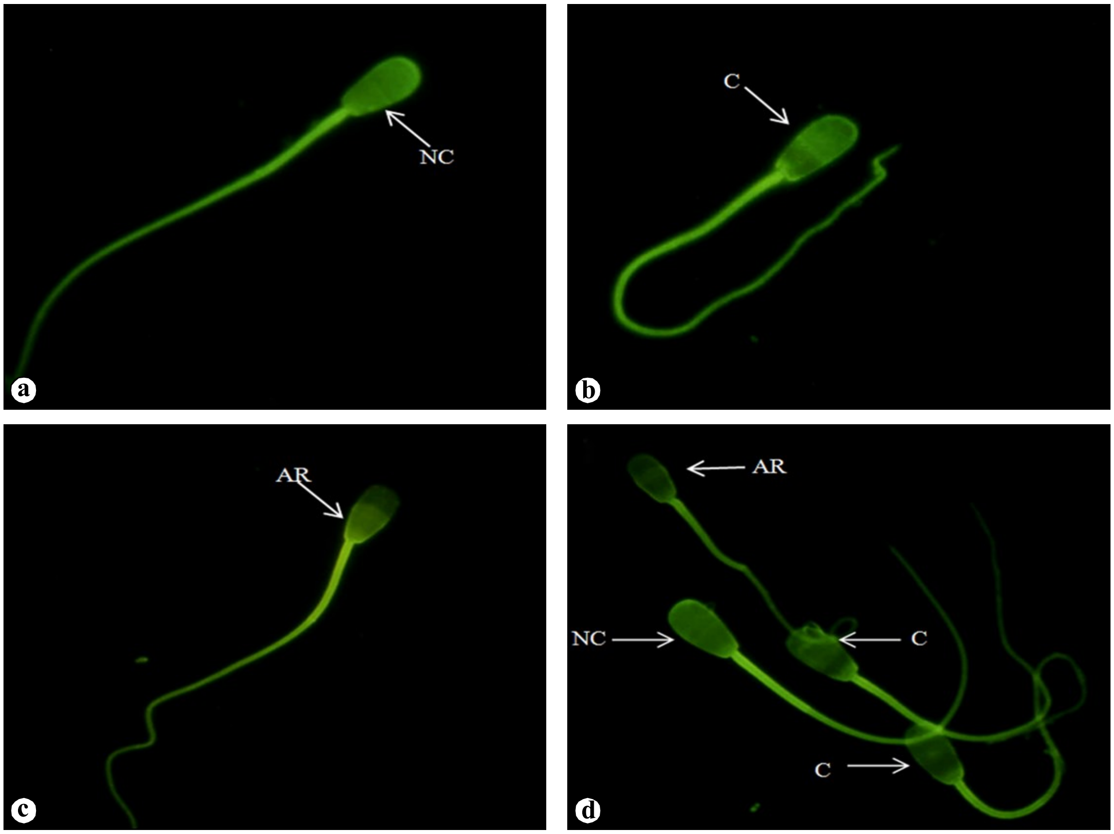

Fig. 16: The representative images showing the sperm capacitation status assessed by chlortetracycline assay. The patterns represented in the above figures indicate non capacitated (NC), capacitated (C), and acrosome reacted (AR)

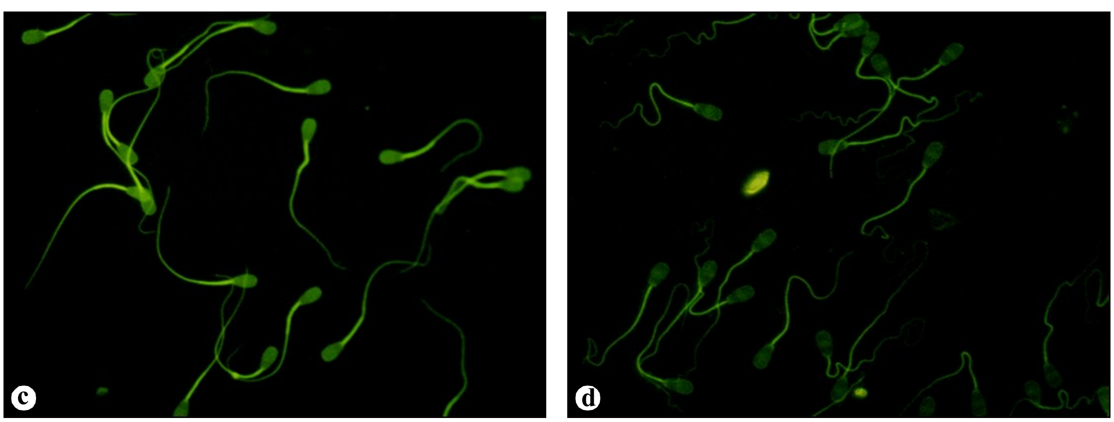

Fig. 17: Effect of osmolality on sperm capacitation. Capacitation was assessed by chlortetracycline assay. Representative images indicate (a) major representation of non-capacitaed sperm in the isosmotic (ISO 6.8) medium and (b) major representation of capacitated sperms in the hyperosmotic (HYP1 6.8) media

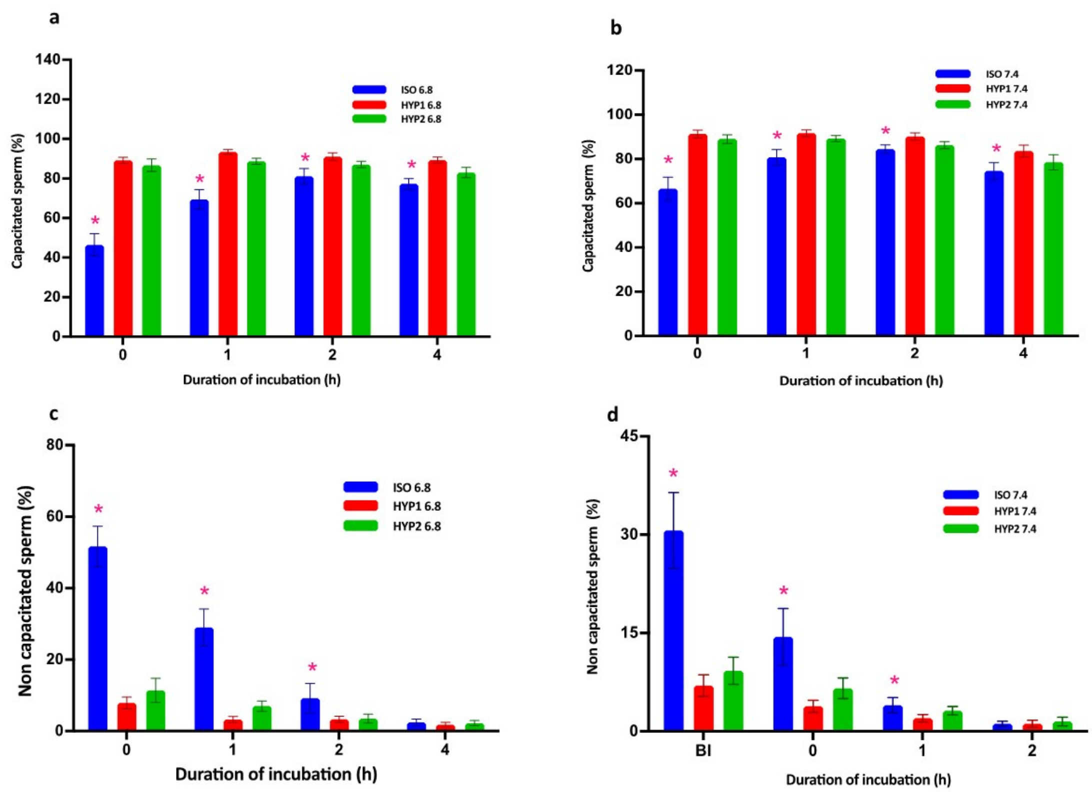

Fig. 18: Effect of osmolality and pH on sperm capacitation status in bovine sperm incubated *in vitro*. The hyperosmotic (HYP1) uterine (a and c) and oviductal (b and d) environment significantly (p<0.05) increased capacitation of sperm and protected acrosome integrity. (ISO: 290 mOsm/kg; HYP1: 355 mOsm/kg; HYP2 : 420 mOsm/kg;BI:Before incubation)

Table 24: Influence of osmolality and pH on capacitation status and acrosome reaction of bovine sperm incubated *in vitro* based on CTC assay. The semen samples were incubated at uterine (osmolality, 355 mOsm/kg and pH 6.8) and oviduct (osmolality, 355 mOsm/kg and pH 7.4) osmolality and pH. The hyperosmotic (HYP1) uterine and oviductal environment significantly (p<0.05) increased the percentage of capacitated sperm. (DOI: Duration of incubation: ISO: 290 mOsm/kg; HYP1: 355 mOsm/kg; HYP2: 420 mOsm/kg; BI:Before incubation)

Parameters	DOI	Uterine pH 6.8			Oviduct pH 7.4		
		ISO	HYP1	HYP2	ISO	HYP1	HYP2
Non capacitated (%)	0 h	51.8±5.78[ap]	7.90±1.62[bc]	11.4±3.38[bc]	30.6±5.79[aq]	7.00±1.64[bc]	9.20±2.06[bc]
	1 h	26.7±5.55[ap]	3.40±0.83[bc]	7.00±1.45[bc]	14.4±4.30[aq]	3.80±0.93[bc]	6.60±1.56[bc]
	2 h	9.30±4.14[a]	3.30±0.81[bc]	3.50±1.23[bc]	4.00±1.14	2.00±0.55	3.30±0.60
	4 h	2.50±0.84	1.80±0.64	2.30±0.69	1.20±0.41	1.20±0.53	1.50±0.66
Capacitated (%)	0 h	46.5±5.65[ap]	90.0±1.40[bc]	86.6±3.12[bc]	66.6±5.20[aq]	91.3±1.75[bc]	89.0±1.92[bc]
	1 h	69.5±4.88[ap]	93.5±1.23[bc]	88.8±1.51[bc]	80.6±3.45[aq]	91.8±1.54[bc]	89.3±1.38[bc]
	2 h	81.2±3.77[a]	91.1±1.92[bc]	87.1±1.61[bc]	84.8±2.06	90.3±1.64[b]	86.9±1.50
	4 h	77.3±2.77[a]	89.3±1.50[b]	83.0±2.54[c]	74.5±3.88	83.6±2.68	78.5±3.43
Acrosome reacted (%)	0 h	1.80±0.43	2.10±0.62	2.00±0.60	2.90±1.05	1.70±0.48	1.90±0.58
	1 h	3.80±1.24	3.10±0.74	4.30±1.18	5.10±1.49	4.40±1.21	4.30±0.79
	2 h	9.50±1.63[a]	5.90±1.31[b]	9.40±1.20[a]	11.3±2.05	7.80±1.53	9.80±1.48
	4 h	20.3±3.16[a]	8.80±1.78[b]	14.8±2.78[c]	24.3±4.05[a]	15.30±2.9[b]	20.0±3.71[ab]

Superscript bearing [a,b,c] for a parameter at a particular time point and pH differ significantly (p<0.05)
Superscript bearing [p,q] for a parameter at a particular time point and osmolality differ significantly (p<0.05)

4.4.5. Sperm viability, functional membrane integrity, acrosome integrity, mitochondrial membrane potential, chromatin distribution and morphology

The sperm viability was significantly higher in HYP1 medium in uterine pH after 1 h of incubation (Table 25). The uterine osmolality and pH also significantly (p<0.05) protected the functional membrane integrity (Figure 19) at 4 h of incubation (Table 25 and Figure 24). The sperm mitochondrial membrane potential (Figure 20) on exposure to uterine osmotic stress was significantly (p<0.05) higher at 1and 4 h,as compared to isosmotic and HYP2 media at uterine pH. But the increase in the mitochondrial membrane potential was not observed in 420 mOsm/kg (HYP2 media) at both pH. The chromatin distribution (Figure 21) was not influenced by pH and osmolality in our study. The acrosome integrity (Figure 22) assessed by FITC –

PSA assay revealed presence of significantly ($p<0.05$) higher percentage of acrosome intact sperm in uterine and oviductal osmolality and pH from 2 h of incubation (Table 25). Accordingly, uterine and oviductal environment were found to significantly increase sperm capacitation and protect acrosome as compared to isosmotic media at both pH (Figure 24). Sperm morphology (Figure 23) was not affected by the range of osmotic stress and pH employed in our study.

Table 25: Influence of osmolality and pH on functional membrane integrity, acrosome integrity, mitochondrial membrane potential and chromatin distribution in bovine sperm incubated *in vitro*. The semen samples were incubated at uterine (osmolality, 355 mOsm/kg and pH 6.8) and oviduct (osmolality 355 mOsm/kg and pH 7.4) environment. The hyperosmotic (HYP1) uterine and oviductal environment significantly (p<0.05) protected membrane integrity, acrosome integrity and mitochondrial membrane potential (DOI: Duration of incubation; ISO: 290 mOsm/kg; HYP1: 355 mOsm/kg; HYP2: 420 mOsm/kg; BI:Before incubation)

Parameters	DOI	Uterine pH 6.8			Oviduct pH 7.4		
		ISO	HYP1	HYP2	ISO	HYP1	HYP2
Sperm	BI	93±1	93±1	93±1	93±1	93±1	93±1
viability (%)	0 h	92±1[a]	87±1[b]	83±4[b]	91±1[a]	87±1[b]	86±4[c]
	1 h	83±1[a]	88±2[b]	84±3[ab]	86±1	87±2	86±2
	2 h	78±2[a]	84±2[b]	84±1[bc]	77±3	82±3	85±2
	4 h	70±4[a]	78±3[b]	74±4[ab]	71±4	77±3	78±3
Acrosome	0 h	94.4±1.03	95.9±0.84	95.2±0.75	95.2±0.63	94.8±0.82	93.6±1.18
integrity (%)	1 h	89.7±0.79[a]	93.7±0.73[b]	90.2±1.10[a]	87.8±1.92[a]	92.3±0.62[b]	88.2±1.19[a]
(FITC PSA assay)	2 h	83.0±1.29[a]	90.7±0.97[b]	86.3±1.21[c]	80.7±1.93[a]	88.1±0.71[b]	82.2±1.75[a]
	4 h	73.0±2.95[a]	85.0±1.84[b]	74.5±3.59[a]	68.7±4.10[a]	79.8±2.67[b]	73.2±3.64[a]
Functional	0 h	72.0±4.37	70.8±3.81	64.0±4.59	67.7±5.56	69.4±4.23	58.7±6.62
membrane &	2 h	47.7±6.11	55.4±5.43	45.2±5.32	48.7±5.07[ab]	55.5±4.87[a]	43.8±5.87[b]
integrity (%)	4 h	28.2±4.43[a]	37.9±4.56[b]	25.5±4.20[a]	29.3±3.79[ab]	34.3±4.25[a]	24.0±3.92[b]
Mitochondrial	0 h	85.5±2.68	85.3±2.97	82.1±2.96	82.9±2.90	85.5±3.54	83.2±3.33
membrane	2 h	79.8±4.18	86.6±2.86	77.8±5.75	83.3±4.21	83.4±4.31	76.9±3.91
potential (%)	4 h	69.2±4.92[ab]	77.9±4.75[a]	66.8±6.86[b]	77.1±5.05	79.8±5.14	66.8±5.79
Morphology	0 h	98.3±1.37	98.2±2.21	96.3±1.78	98.3±2.18	98.3±1.56	95.7±2.19
(Normal %)	2 h	98.3±1.37	97.8±2.83	96.3±3.03	97.7±2.02	97.3±2.27	95.8±2.73
	4 h	96.6±3.48	96.8±3.1	94.3±5.55	96.0±4.13	96.3±3.52	95.2±5.67
Chromatin	0 h	97.4±0.76	96.9±0.85	96.8±0.73	97.2±0.73	96.5±0.61	96.6±0.56
distribution (%)	4 h	96.4±0.60	96.5±0.72	95.9±0.75	96.7±0.61	96.8±0.66	96.7±0.73

Superscript bearing [a,b,c] for a parameter at a particular time point and pH differ significantly ($p<0.05$)

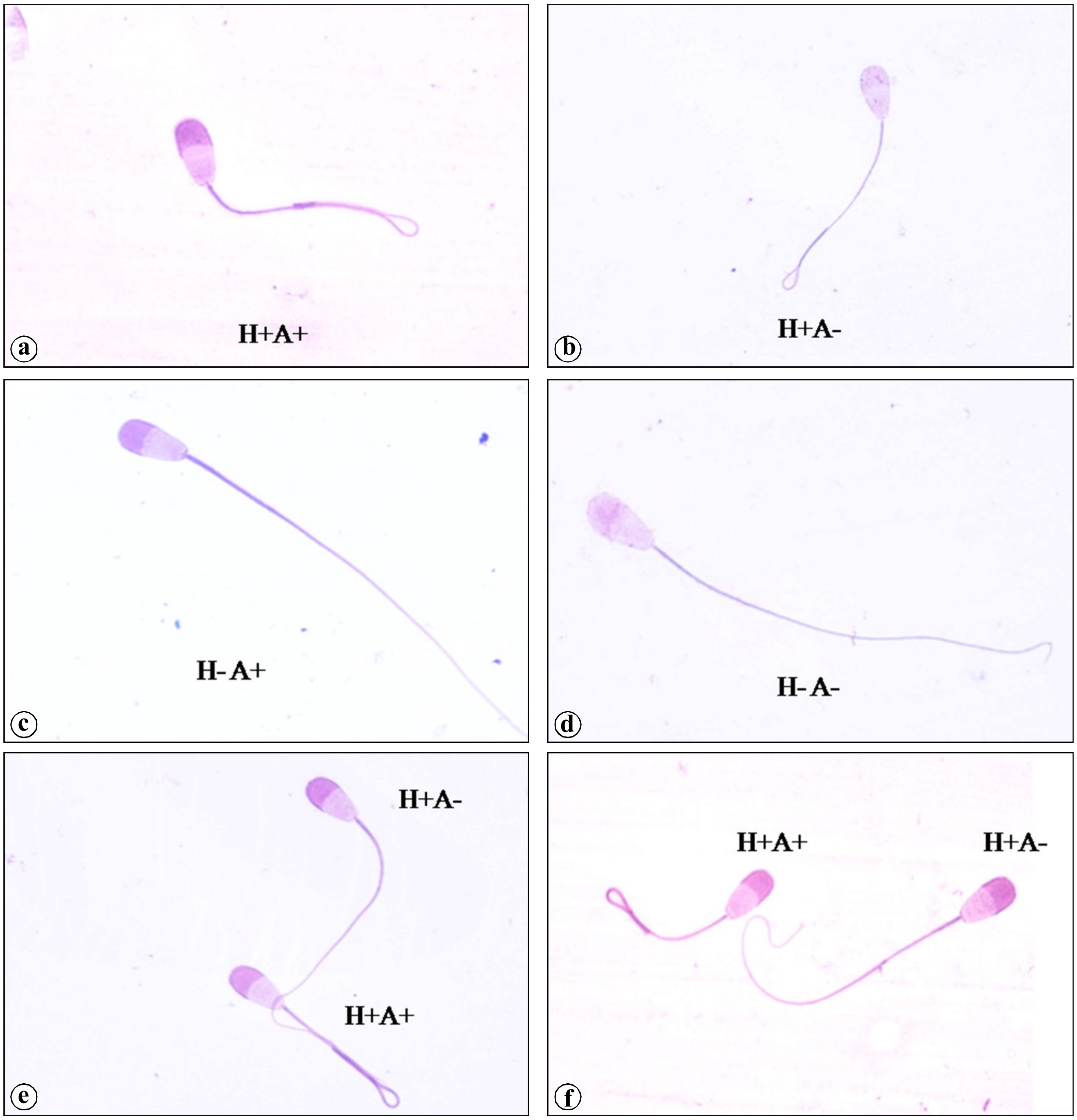

Fig. 19: **The representative images showing thefour subpopulations (a,b,c,d) assessed in the hyposmotic swelling – Giemsa test. Figure a) HOS positive and acrosome positive (H+A+), b) HOS positive and acrosome negative (H+A-), c) HOS negative and acrosome positive (H-A+), d) HOS negative and acrosome negative (H-A-)**

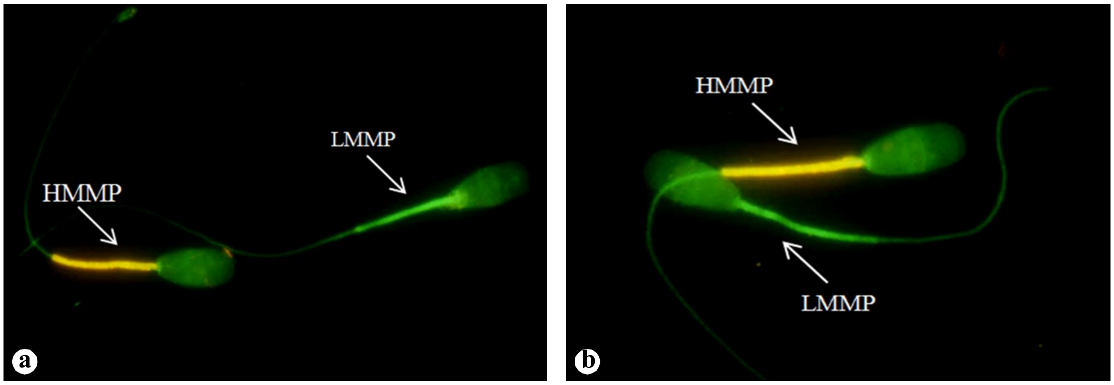

Fig. 20: **The representative images showing the mitochondrial membrane potential status of sperm assessed by JC-1 dye. The sperm with high membrane potential (HMMP) represented by orange to yellow fluorescence in the midpiece and sperm with low mitochondrial membrane potential (LMMP) represented by green fluorescence in the midpiece**

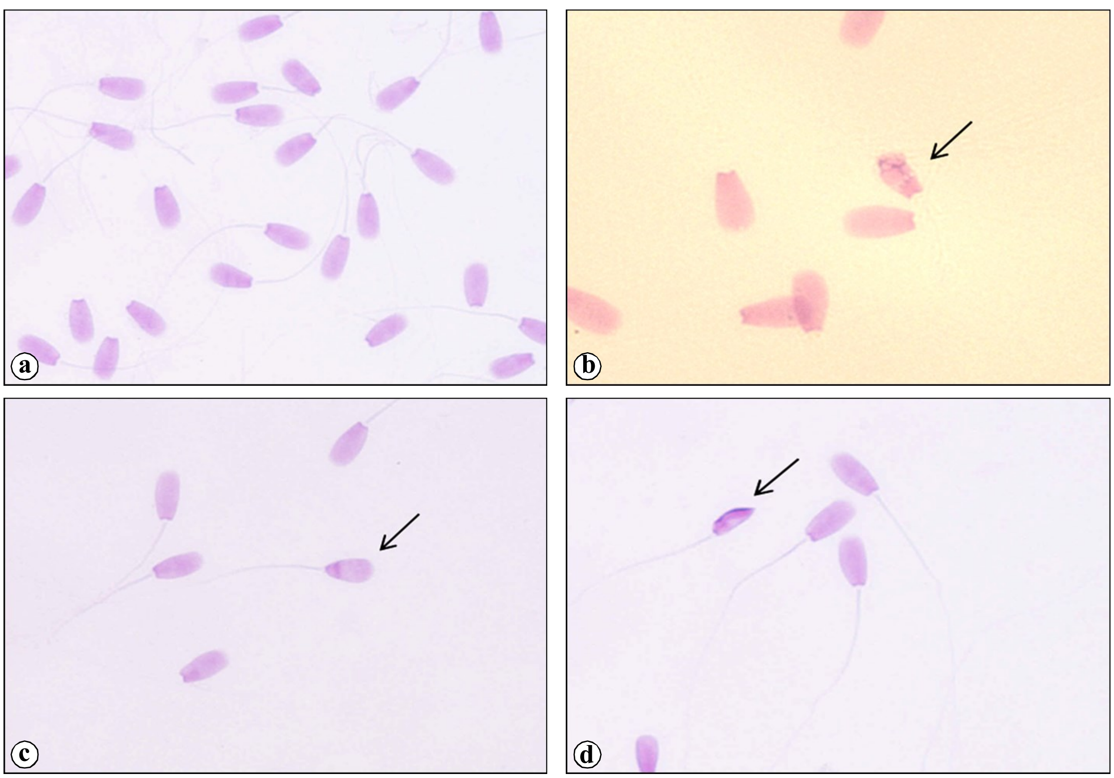

Fig. 21: **The representative images showing the chromatin distribution of the HF bull sperm assessed by Feulgen's assay. (a) Normal chromatin (b) diadem defect (c) swollen nucleus and (d) folded chromatin**

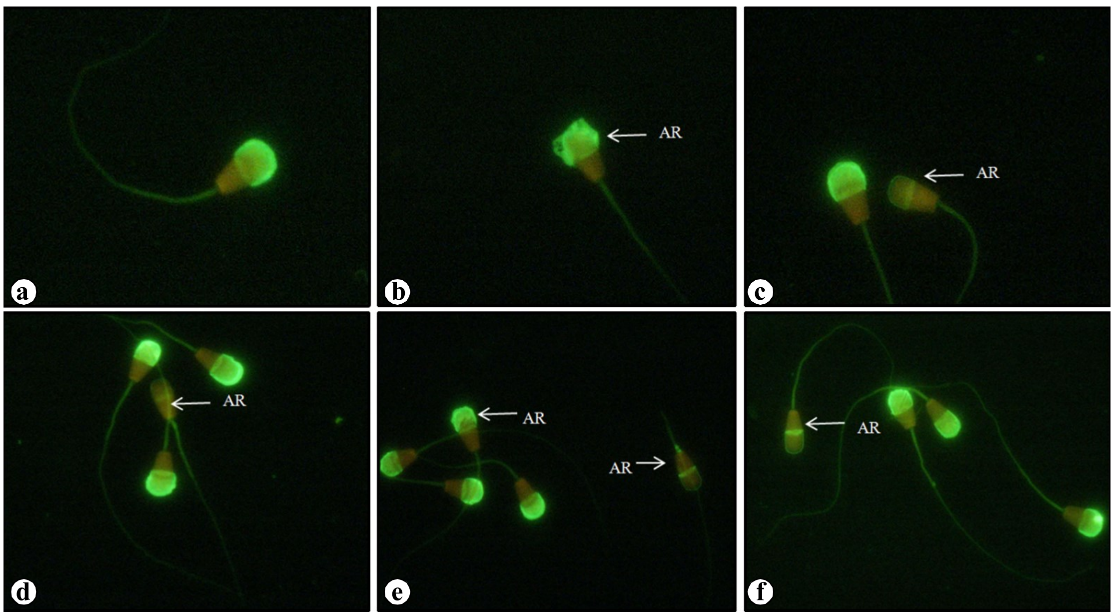

Fig. 22: The representative images showing the sperm acrosome status assessed by FITC-PSA and PI flurophores. (a) The sperm without arrow represent acrosome intact sperm with even green fluorescence inthe acrosome cap. (b-f) different pattern of acrosome reacted sperm either with ruffled acrosome cap or without acrosome cap(white arrows)

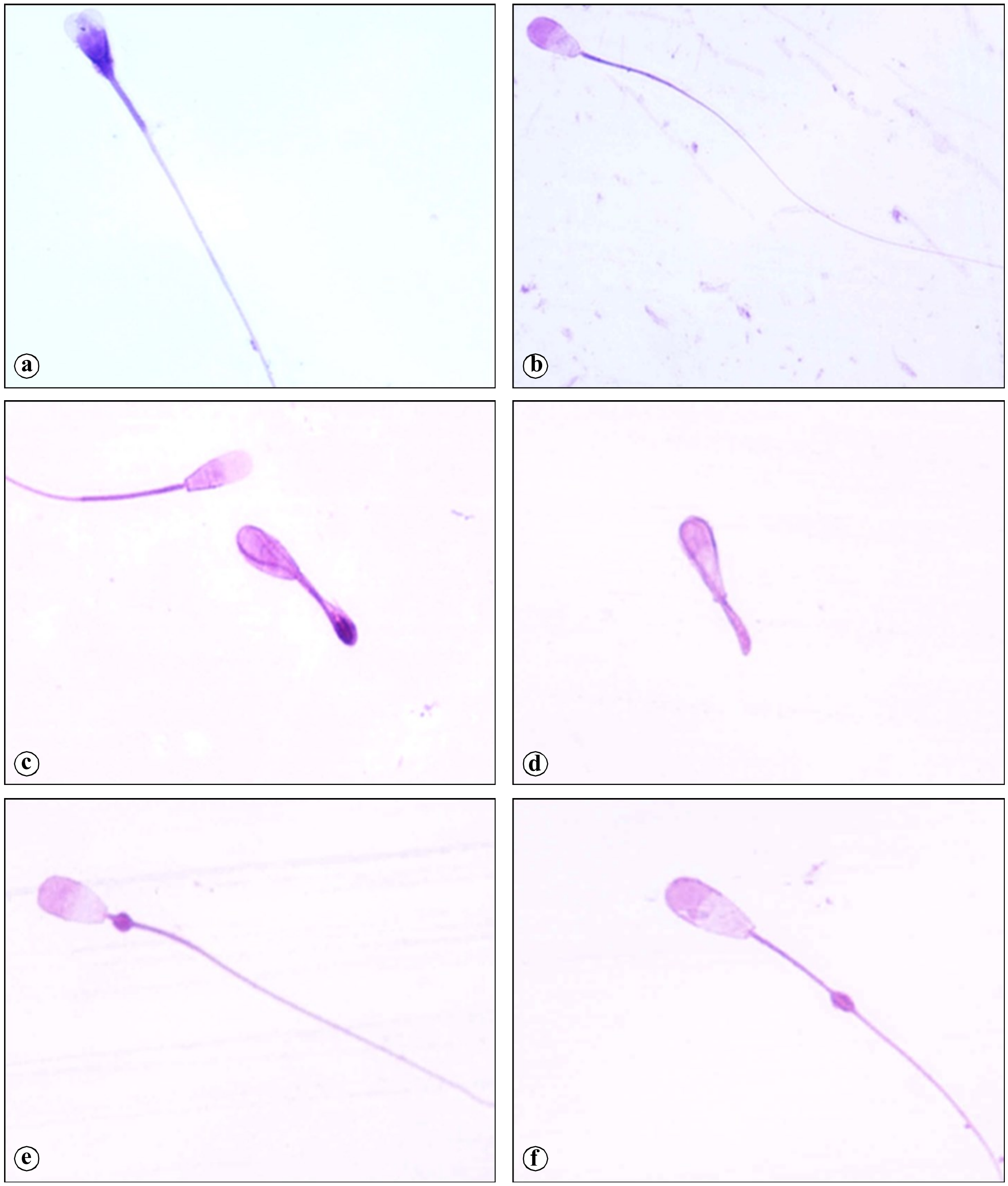

Fig. 23: The representative images of sperm with abnormal morphology. (a) double headed sperm, (b) sperm with long thread-like tail, (c) sperm with protoplasmic droplet and folded tail. (d) sperm with bent and coiled tail from midpiece, (e) sperm with proximal protoplasmic droplet and (f) sperm with distal protoplasmic droplet

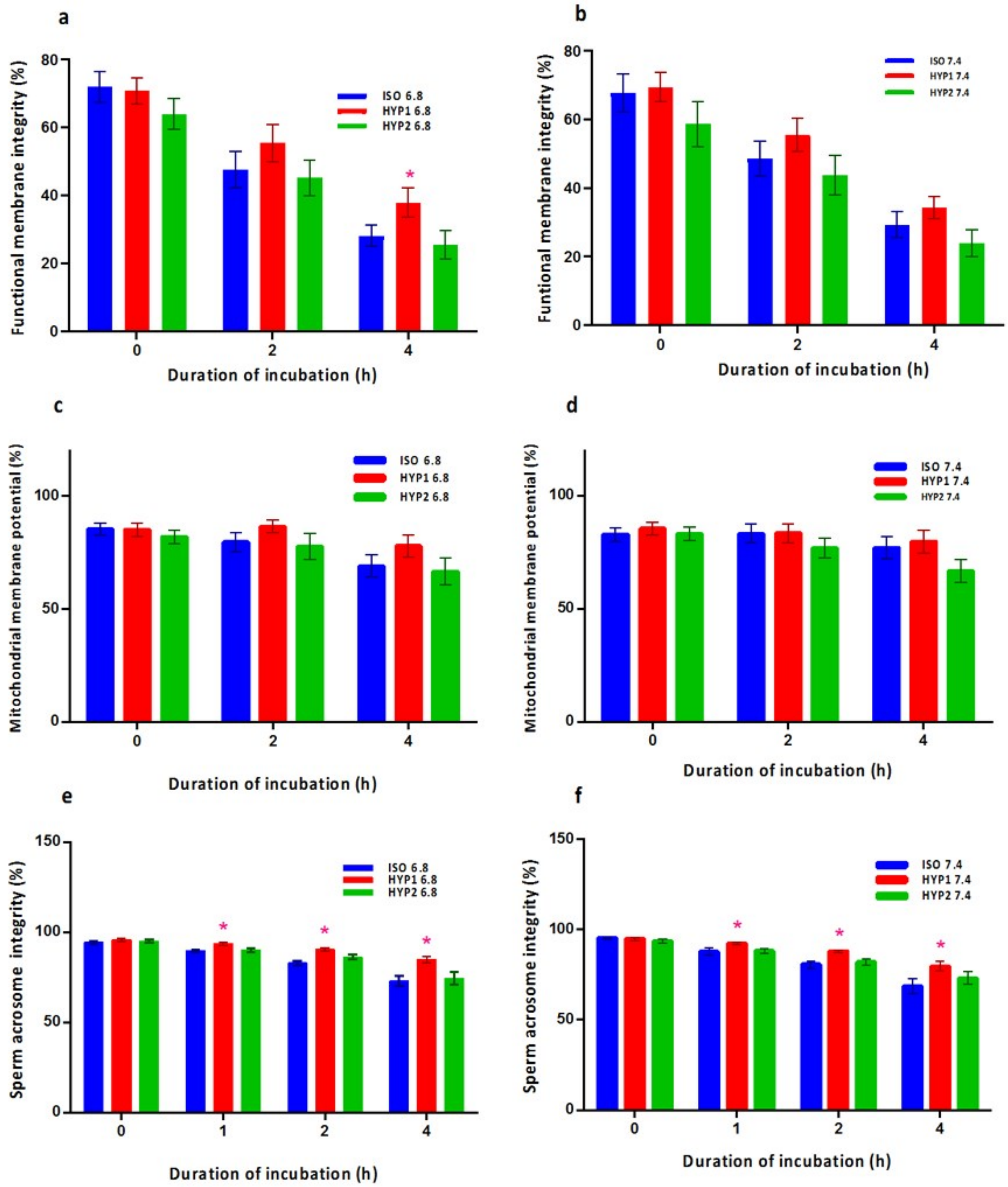

Fig. 24: Effect of osmolality and pH on sperm functional membrane integrity (a and b), mitochondrial membrane potential (c and d) and acrosome integrity (e and f). The hyperosmotic (HYP1) uterine and oviductal environment significantly ($p<0.05$) protects membrane integrity and acrosome integrity. (ISO: 290 mOsm/kg; HYP1: 355 mOsm/kg; HYP2: 420 mOsm/kg; BI: Before Incubation)

4.4.6. Effect of osmoarity and pH

The repeat measure factorial ANOVA results suggested significant (p<0.05) influence of osmolality on sperm functional attributes including progressive motility, sperm velocities (VAP, VCL and VSL), head area, ALH, BCF, capacitation, acrosome integrity and mitochondrial membrane potential. Further the osmolality had a quadratic effect on progressive motility, functional membrane integrity, and capacitation. The pH had significant (p<0.05) influence on sperm capacitation. A significant (p<0.05) interaction effect of osmolality and pH was also observed on sperm capacitation.

4.5. Prediction of quality semen production behavior of bull based on sperm osmoadaptation ability

The predictive ability of quality semen production behavior of bulls with the sperm functional parameters obtained after correlating the changes in sperm functional attributes to different incubation media simulating bovine reproductive tract osmolality and pH (Table 26). The results revealed the existence of strong negative correlation between sperm mitochondrial membrane potential, functional membrane integrity and acrosome reacted with semen rejection rate in hyperosmotic medium (HYP1) than ISO and HYP2 media (Table 26). Hence the two hyperosmotic medium (HYP 1) at uterine and oviduct pH were considered for developing osmoadaptation scores predictive of bull fertility.

Table 26: The correlation of sperm functional parameters at different incubation media simulating bovine reproductive tract osmolality (355 mOsm/kg) and pH (6.8 and 7.4) with semen rejection rate.

Parameter	**Correlation coefficient (*r*) of sperm functional parameters with rejection rate** (p < 0.05)											
	ISO 6.8		**HYP1 6.8**		**HYP2 6.8**		**ISO 7.4**		**HYP1 7.4**		**HYP2 7.4**	
	1 h	**4 h**	**1 h**	**4 h**	**1 h**	**4 h**	**1 h**	**4 h**	**1 h**	**4 h**	**1 h**	**4 h**
PM (%)	-0.58											
TM (%)	-0.56											
MUP (%)				-0.80								
MMP(%)	-0.60	-0.57	-0.67	-0.60	-0.50		-0.56		-0.73	-0.73	-0.63	
MI (%)	-0.57		-0.60	-0.65	-0.58	-0.59	-0.58	-0.59	-0.59	-0.69	-0.60	
AR (%)			0.72		0.76		0.76		0.77		0.70	

(PM – progressive motility, TM – total motility, MUP – mucus penetration, MMP – mitochondrial membrane potential, AR- acrosome reacted)

The percent progressive motility loss at 1 and 4 h (Table 27) in HYP1 medium was used as measure of osmoadaptation ability.

Table 27: The progressive motility of sperm incubated in hyperosmotic medium (uterine and oviductal fluid osmolality- 355 mOsm/kg) at both uterine and oviductal pH (6.8 and 7.4, respectively). The progressive motility (%) was obtained using a computer assisted sperm analyzer. (HYP1-355 mOsm/kg; BI: Before incubation)

Animal ID	BI	Progressive motility (%)									
		HYP1, pH 6.8		Motility loss		UTSI	HYP1, pH 7.4		Motility loss		OVTS
		1 h	4 h	1 h (UTI 1)	4 h (UTI 2)		1 h	4 h	1 h (OVI 1)	4 h (OVI 2)	
01	95.53	93.60	85.16	2.02	10.8	12.9	90.88	80.22	4.87	16.0	20.9
02	84.56	87.85	68.78	-3.88	18.6	14.8	83.60	66.03	1.13	21.9	23.0
03	85.06	88.30	78.47	-3.81	7.74	3.9	91.18	83.92	-3.19	1.33	-1.86
04	92.94	86.81	82.45	6.59	11.7	17.9	90.70	82.43	2.40	11.3	13.7
05	95.21	90.45	83.47	4.99	12.3	17.3	91.17	85.41	4.24	10.2	14.5
06	87.31	80.02	70.02	8.34	19.8	28.2	80.79	69.02	7.46	20.9	28.4
07	77.86	65.96	55.83	15.2	28.2	43.6	65.03	55.31	16.4	28.9	45.4
08	80.50	80.48	70.74	0.02	12.1	12.2	77.44	53.23	3.80	33.8	37.6
09	89.58	82.19	68.80	8.25	23.2	31.5	88.82	61.66	0.85	31.1	32.0
10	95.09	92.32	85.04	2.91	10.5	13.5	92.65	86.75	2.57	8.77	11.3
11	93.03	91.62	85.69	1.51	7.89	9.41	90.11	89.78	3.14	3.49	6.64
Average (%)				**3.8**	**14.7**	**18.6**			**3.9**	**17.1**	**21**

(Osmo-susceptibility indexs UTI 1 (percent loss at 1h in HYP1 media at uterine pH), UTI 2 (percent loss at 4h in HYP1 media at uterine pH), UTSI (summation of percent loss at 1 and 4 h in HYP 1 media at uterine pH), OVS1 (percent loss at 1h in HYP 1 media at oviduct pH), OVS 2 (percent loss at 4 h in HYP1 media at oviduct pH) and OVSI (summation of percent loss at 1 and 4 h in HYP 1 media at oviduct pH)

The percent loss at 1 and 4 h (n=11) were 3.8 %, 14.7 % in HYP1 media at uterine pH and 3.9 %, 17.1 % in HYP1 media at oviductal pH.The obtained osmo-susceptibility indices UTI 1 (percent loss at 1h in HYP1 media at uterine pH) and UTI 2 (percent loss at 4h in HYP1 media at uterine pH), UTSI (summation of percent loss at 1 and 4 h in HYP 1 media at uterine pH), OVI 1 (percent loss at 1h in HYP 1 media at oviduct pH), OVI 2 (percent loss at 4 h in HYP1 media at oviduct pH, Figure 25) and OVSI (summation of percent loss at 1 and 4 h in HYP 1 media at oviduct pH, Figure 26) were correlated with the rejection rate. Of

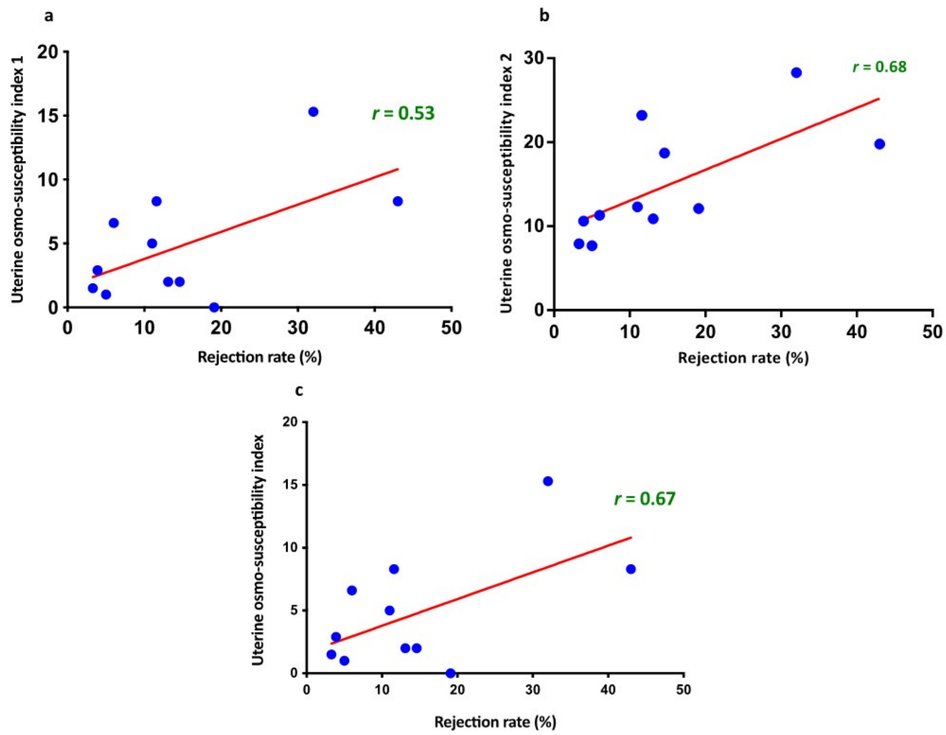

Fig. 25: Correlation between predicted uterine osmo-susceptability indices (uterine osmo-susceptability indices at 1 h (UTI 1- a), at 4 h (UTI 2 - b) and total score (UTSI - c) with rejection rate

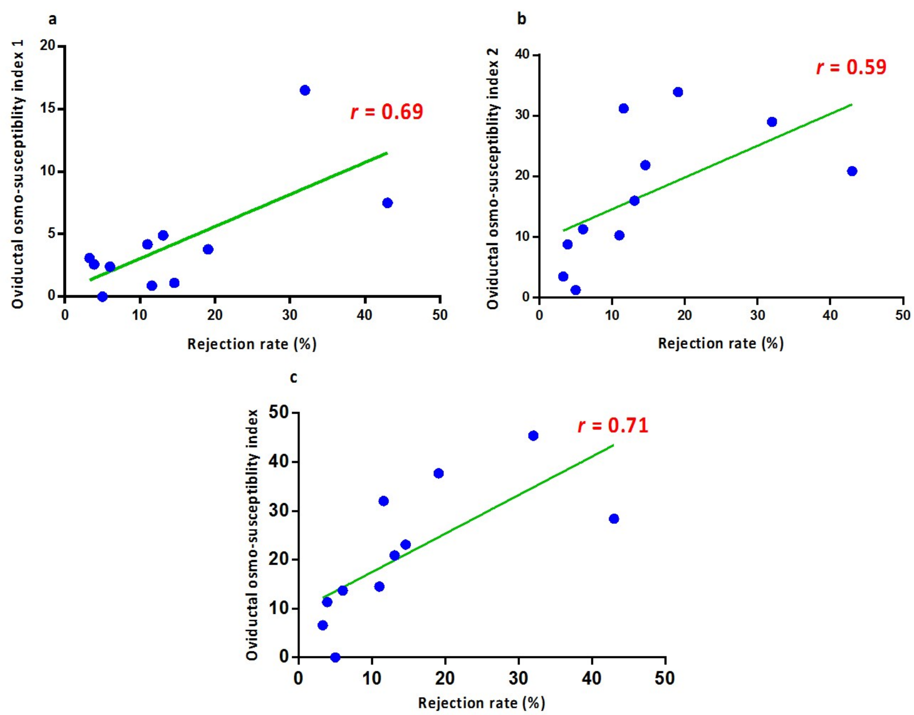

Fig. 26: Correlation between predicted oviductal osmo-susceptability indices (oviductal osmo-susceptability indices at 1 h (OVI 1- a), at 4 h (OVI 2- b) and total score (OVSI- c)) and rejection rate.Thetotalosmo-susceptibility index (OVSI) arrived using HYP 1 media withoviductal pH (7.4) was strongly negatively correlated (r = 0.71) with rejection rate (p<0.05)

the six osmo-susceptibility indices, OVSI (summation of percent loss at 1 and 4 h in HYP 1 media at oviduct pH) had better positive correlation (r = 0.71) with semen rejaction rate than other indices (Figures 26). ROC analysis was done to determine the cut off for oviductal osmo-susceptibility index (OVSI). The OVSI at 18 % had a maximum likelihood ratio of 6, and at this chosen cut-off, the specificity was 83.3% and sensitivity was 100%.

The obtained OVSI cut-off was validated in randomly selected semen samples (n=6). In these samples when classified based on the cut-off of 18%, samples with less than 18% OVSI had a rejection rate of 3-11 % and samples with more than 18% OVSI, had rejection rate of 15 – 22 % (Table 28). Thus, the chosen OVSI cut-off segregated the samples effectively. Further, a regression equation based on the OVSI was developed for the prediction of semen rejection rate (Figure 32). The coefficient of determination (R^2) of the regression equation was 0.70. The developed regression equation was,

$$\boldsymbol{Y} = 0.4198\,\boldsymbol{X} \times 2.742$$

Where X is the predictor variable, oviductal osmo-susceptibility index (OVI) and Y is the response variable, semen rection rate.

Table 28: The validation of oviductal osmo-susceptibility index cut-off in randomly chosen semen samples. The chosen OVSI cut-off 18% segregated the samples effectively

Animal ID	OVI	Rejection rate
1	8.51	3.3
2	17.81	5
3	2.63	9.1
4	12.02	11
5	38.99	15.7
6	50.32	21.6

With the developed regression equation, the bulls with less than 18% OVI were predicted to have rejection rate less than 10%. The predicted semen rejection rate was found to have strong correlation (r=0.79) with actual semen rejaction rate. Besides, the predicted oviductal osmoadaption score correlated with several other sperm functional attributes of immense importance in fertilization (Table 29).Thus, it was concluded that the predicted oviduct osmoadaptation score (OVSI) can be employed to predict semen rejection rate (Figure 27).

Table 29: The correlation of predicted oviductal osmo-susceptibility index with rejection rate (%) and sperm functional parameters ($p < 0.05$)

Parameter	DOI	Correlation coefficient (r) of OVTS with rejection rate and sperm functional parameters
Rejection rate		**0.71**
Progressive motility (%)	0 h	-0.67
	1 h	-0.62
	2 h	-0.72
	4 h	-0.81
Total motility (%)	2 h	-0.71
	4 h	-0.83
Head area (μm^2)	4 h	-0.62
ALH (μm)	1 h	-0.60
	2 h	-0.67
	4 h	-0.68
BCF (Hz)	1 h	-0.67
Mucus penetration (%)	4 h	-0.59
AR(%)	1 h	0.59
MMP (%)	0 h	-0.75
	2 h	-0.74
	4 h	-0.92
FMI (%)	0 h	-0.74
	2 h	-0.83
	4 h	-0.58
AR (%)	1 h	0.59

(ALH – amplitude of lateral head displacement, BCF – beat cross frequency, AR – acrosome reacted, MMP – mitochondrial membrane potential, FMI – functional membrane integrity)

4.6. Differential expression of osmo-responsive genes for prediction of QSP of bull

The differential expression levels of osmo-responsive genes between good (n=5) and poor osmoadapters (n=5) were studied in bull sperm. The average total RNA yield as obtained using flurometer were18.2fg/sperm and 9.02fg/sperm before and after DNase treatment, respectively (Table 30). The purity of the RNA samples was assessed from 260/280 and 260/280 ratios and were found to be 1.93 and 1.95, respectively in spectrophotometer.

Table 30: The sperm total RNA concentration and purity obtained for gene expression study. The quality of the RNA was checked using spectrophotometer and the quantity was estimated using fluorometer

Animal ID	**RNA Quality**		**RNA concentration before DNase treatment**		**RNA concentration after DNase treatment**
	Spectro-photometer absorbance		Spectro-photometer	Fluorometer	Fluorometer
	260/230	260/280	(ng/μL)	(ng/μL)	(ng/μL)
01	2.20	1.89	341.2	11.0	6.10
02	1.96	1.90	305.8	12.1	4.80
03	2.27	1.89	194.7	4.55	2.11
04	2.29	1.89	152.5	8.88	2.58
05	1.72	1.88	267.0	8.54	5.64
06	1.26	1.84	178.0	5.24	2.27
07	2.23	1.83	96.39	6.32	2.50
08	1.80	2.14	242.3	9.18	2.67
09	1.80	2.30	185.4	8.68	6.94
10	2.04	1.77	72.18	6.48	2.40
Average	**1.95**	**1.93**	**203.5**	**8.09**	**3.80**

The obtained total RNA did not have genomic DNA contamination as assessed by PCR using intron spanning primer *PRM1*.Similarly, the RNA was free of RNA contamination from somatic cells, germ cells and leucocytes (Figure 28).

The three genes *RPL23*, *PRM1 and GAPDH* were assessed for stability using their expression levels. The *RPL 23* was found to have better stability compared to *PRM1* and *GAPDH* using both NormFinder and BestKeeper and hence chosen as house keeping gene (Table 31).

Table 31: The stability value calculated using NormFinder and BestKeeper tool for selecting house-keeping genes for the gene expression studies in sperm.

Gene name	NormFinder		BestKeeper	
	Standard error	Stability factor	Standard error	Coeffecient of correlation (*r*)
RPL23	2.7	0.53	1.5	0.87
PRM1	1.6	1.08	1.2	0.73
GAPDH	2.6	11.02	1.9	0.71

Subsequently the expression levels were normalized with *RPL23* (Figure 29).The fold change was calculated by normalizing the gene expression levels of good osmoadapter group to poor osmoadapter group. The relative expression levels for the genes *NFAT5, ADAM1B* and *SLC9C1* were significantly (p<0.05) higher in good osmoadapter group (Figures 36 and 37). Interestingly, the expression levels of *NFAT5, ADAM1B* genes were consistently non-detectable in the poor osmoadapter group (Figure 30).Similarly,the expression of *ENO1* and *EFHD1* genes were observed only in two out of 5 samples in poor osmoadapter group. *HSP90AB1* and *GAPDH* were upregulated in good osmoadapter group with a fold change of 2.34 and 1.68 respectively (Figure 31). *MT-CO1* and *PRM1* were abundantly expressed in poor osmoadapter group when compared to good osmoadapter group. The relative expression levels of *MT-ND2* gene expressed abundantly in both groups and not altered between groups (Table 32).

Further the correlation of gene expression levels of the osmoresponsive genes with the sperm functional attributes revealed existence of significant correlation.The expression levels of *NFAT5, ADAM1B, SLC9C1* and *HSP90AB1* were significantly (p<0.05) positively correlated and *MT-CO1, EFHD1* and *ENO1*were significantly (p<0.05) negatively correlated with sperm functional attributes (Table 33).

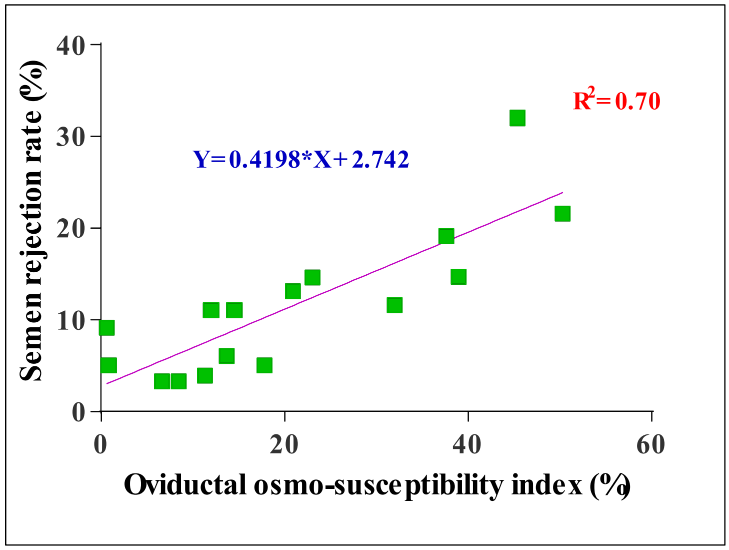

Fig. 27: Correlation between predicted oviductal osmo-susceptability indices (oviductal osmo-susceptability indices at 1 h (OVI 1- a), at 4 h (OVI 2- b) and total score (OVSI- c)) and rejection rate.Thetotalosmo-susceptibility index (OVSI) arrived using HYP 1 media withoviductal pH (7.4) was strongly negatively correlated (r = 0.71) with rejection rate (p<0.05)

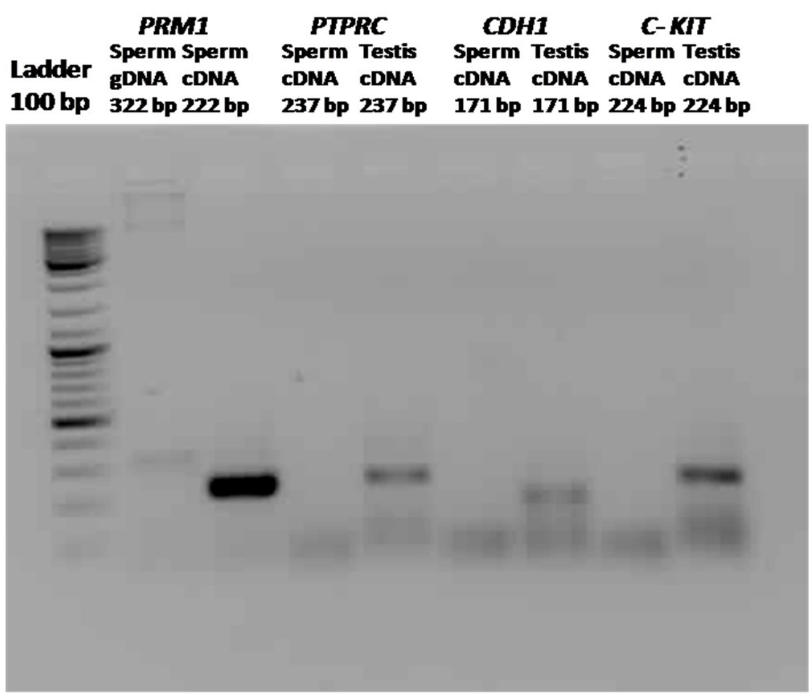

Fig. 28: The representative image showing the quality assessment of obtained sperm RNA. Intron spanning PRM1 primer was used for genomic DNA contamination. PTPRC, CDH1, and C-KIT primers were employed for detection of RNA contamination from leucocytes, somatic cells, and germ cells respectively. Testis cDNA was used as positive control. The product size was confirmed using 1.5 % agarose gel electrophoresis

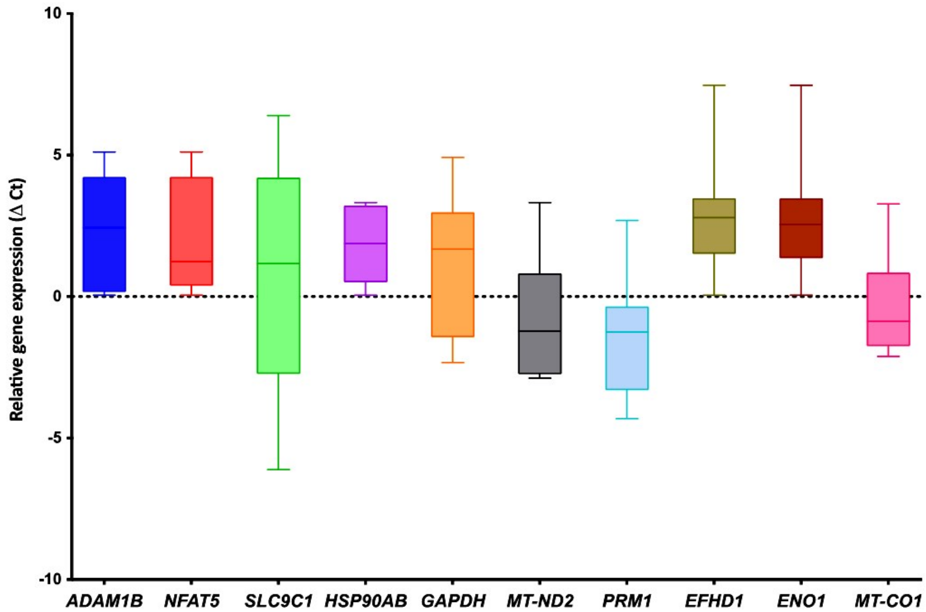

Fig. 29: The relative gene expression levels of osmo-responsive genes normalised to house keeping gene *RPL 23* (ΔCt). The Box Whisker plot shows the distribution of gene expression levels (n=10)

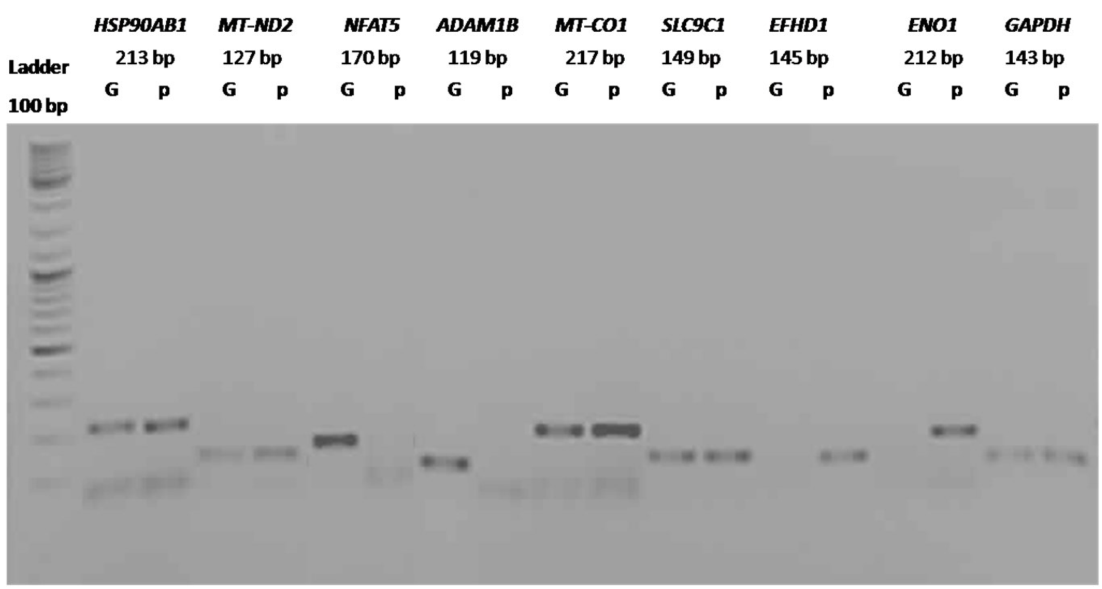

Fig. 30: Comparison of expression levels of osmo-responsive genes between good and poor osmoadapters, and their product size resolved in agarose gel electrophoresis from their respective PCR products

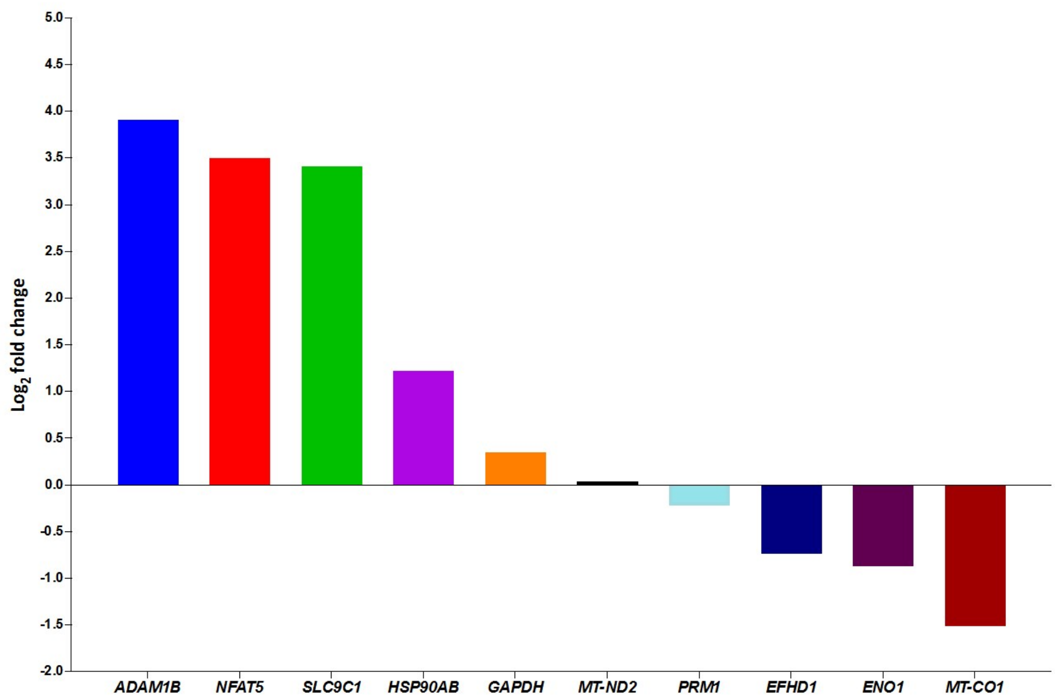

Fig. 31: Log2 fold change of the osmo-responsive genes ingoodosmoadapterasnormalized to poor osmoadapter group. The genes *NFAT5, ADAM1B* and *SLC9C1* were significantly (*p*<0.05) abundantly expressed in good osmoadapter group

Table 32: The relative gene expression levels of osmo-responsive genes normalised to house-keeping gene RPL23. The fold change in the gene expression levels of good osmoadapter group was compared to poor osmoadaptor group

Genes	ΔCt – Poor	ΔCt - Good	ΔΔCt (G-P)	Fold change
ADAM1B	3.48	-0.42	-3.91	15.0
NFAT5	3.48	-0.01	-3.49	11.3
SLC9C1	2.41	-0.99	-3.41	10.6
HSP90AB1	2.41	1.18	-1.22	2.34
GAPDH	1.15	0.79	0.87	1.68
Mt - ND2	-0.65	-0.69	-0.04	1.03
PRM1	-1.52	-1.31	0.73	0.86
EFHD1	2.29	3.17	-0.35	0.55
ENO1	2.43	3.17	0.21	0.60
MT-CO1	-1.10	0.40	1.51	0.35

(ΔCt - gene expression levels normalised with house-keeping gene *RPL23* expression levels, ΔΔCt - gene expression levels of good osmoadapter group normalised with poor osmoadapter group)

Table 33: The correlation of relative gene expression levels (ΔCt) of osmoresponsive genes with sperm functional parameters

	Correlation coefficient (*r*) of relative gene expression levels with sperm functional parameters and rejection rate ($p < 0.05$)							
Parameter	***HSP90AB1***	***MT-ND2***	***NFAT5***	***ADAM1B***	***SLC9C1***	***MT-CO1***	***EFHD1***	***ENO1***
PM (%)						-0.48	-0.46	-0.46
Rapid PM (%)						-0.54	-0.63	-0.62
VAP (μm/s)							-0.58	-0.57
VSL (μm/s)							-0.53	-0.59
LIN (%)	-0.47				-0.47		-0.55	-0.57
Hyperactive (%)			0.51					
Head area (μm^2)			0.64	0.62		-0.45		
ALH (μm)	0.45							
BCF (Hz)							-0.55	
Mucus penetration (%)						-0.51	-0.68	-0.67
Non capacitated (%)			0.5	0.58	0.59			
Acrosome intact (%)				0.57	0.68			
MMP (%)			0.48	0.46		-0.55		-0.48
FMI (%)		-0.49				-0.51		-0.47
CD (%)			0.5				-0.45	
RR (%)	-0.51		-0.74	-0.71	-0.49			

(PM – progressive motility, VAP – average path velocity, VSL – straight-line velocity, LIN- linearity index, ALH – amplitude of lateral head displacement, BCF – beat cross frequency, MMP – mitochondrial membrane potential, FMI – functional membrane integrity, CD – chromatin distribution, RR – rejection rate)

4.7. Experiment 2: Influence of urea on bovine sperm functional attributes

4.7.1. Sperm kinematics

Sperm were incubated in media mimicking uterine and oviductal micro environment (osmolality and pH) with three different concentrations of urea (physiological and pathological levels) and control (without urea). The sperm functional attributes in the urea containing medium were compared with the control medium. The U3 medium significantly ($p<0.05$) decreased the percentages of motile and progressive motile sperm at 1 h of incubation (1, 2 and 4 h). It also significantly decreased ($p<0.05$) percentage of rapid progressive sperm at 4 h of incubation (Table 34). The osmotic stress as an inherent component of sperm transport in bovine reproductive tract, the motility, though dropped immediately on exposure to stress, subsequent recovery was evidenced at 1 h in control, U1 and U2 media, but not in U3 medium (Figure 32). Thus, the higher urea concentration affected the osmoadaptive behavior of sperm. Besides, U3 medium also significantly decreased ($p<0.05$) the hyperactive sperm percentage at 4 h of incubation. The mucus penetration percentage significantly ($p<0.05$) decreased in U3 medium at 4 h as compared to 0 h of incubation, whereas in other media sustainability of mucus penetration was observed from 0- 4 h of incubation.

4.7.2. Sperm velocity and motility characteristics

In consistent with the sperm motility, U3 media significantly ($p<0.05$) decreased sperm average path velocity (µm/s) and straight line velocity (µm/s) at 4 h and curvilinear velocity (µm/s) from 1 h of incubation (Table 35). A significant decrease in the sperm velocity (VCL, VSL and VAP) in U3 media was observed as compared to control media at 4 h of incubation (Figure 33).Besides there was no significant effect of urea on sperm linearity and straightness index.

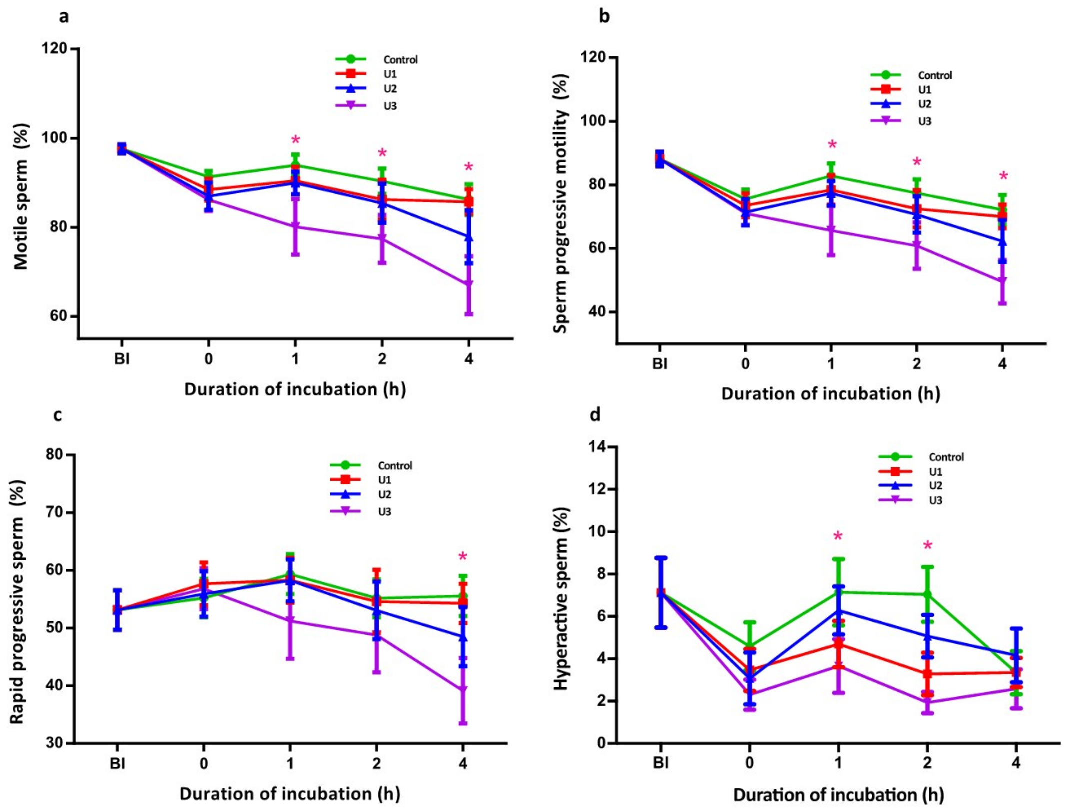

Fig. 32: Effect of urea on sperm motility and hyperactivation (d) in bovine sperm incubated at oviductal osmolality (355 mOsm/kg) and pH (7.4) *in vitro*. The U3 medium significantly (*p*<0.05) decreases total motile (a) and progressive forward motile (b) spermatozoa from 1 h of incubation. Similarly, significantly (*p*<0.05) decreases rapid progressive sperm at 4 h (c). (Control: No urea was added, U1: 0.04 gof urea /L, U2: 0.13 gof urea /L U3: 0.43gof urea/L; BI:Before incubation)

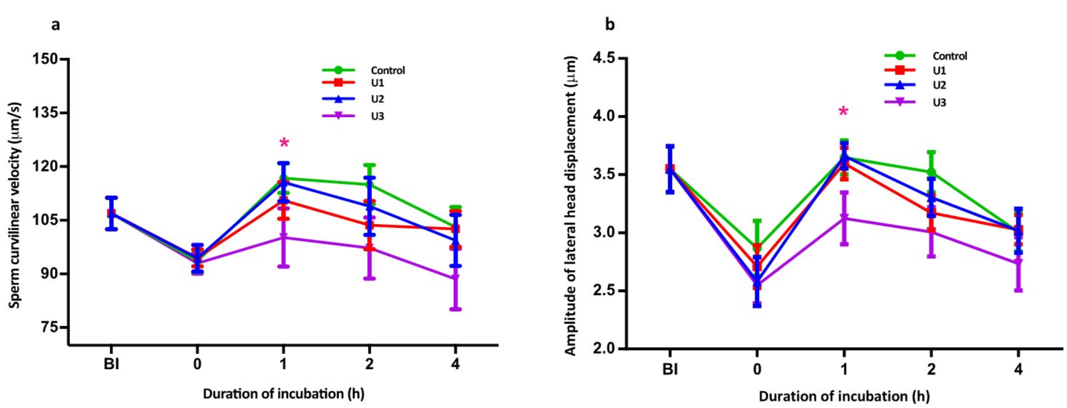

Fig. 33: Effect of urea on on sperm velocity (%) and motility characteristics in bovine sperm incubated at oviductal osmolality (355 mOsm/kg) and pH (7.4) *in vitro*. a) sperm curvilinear velocity b) amplitude of lateral head displacement. The U3 medium significantly (*p*<0.05) decreases sperm curvilinear velocity (a) from 1 h of incubation (Control: No urea was added, U1 : 0.04 gof urea /L, U2 : 0.13 gof urea /L, U3: 0.43gof urea/L; BI:Before incubation)

Table 34: Influence of urea on the percentages of motility and mucous penetration properties of bovine sperm incubated at oviductal osmolality (355 mOsm/kg) and pH (7.4) *in vitro*. The semen samples were incubated at three different concentrations of urea. The total motile and progressive forward motile spermatozoa significantly (p<0.05) decreased from 1 h of incubation in the U3 medium having highest urea concentration (0.43 g of urea /L of medium). (DOI: Duration of incubation; Control: No urea was added, U1: 0.04 g/L of urea, U2: 0.13g/L of urea, U3: 0.43 g/L of urea; BI:Before incubation)

Parameters	DOI	Control	U1	U 2	U3
Total motility (%)	BI	97.6±0.97	97.6±0.97	97.6±0.97	97.6±0.97
	0 h	91.3±1.33	88.5±2.45	87.0±3.03	86.2±2.50
	1 h	93.9±2.38^{a}	90.5±3.09^{a}	89.9±2.53^{a}	80.1±6.24^{b}
	2 h	90.3±2.86^{a}	86.2±4.44^{ab}	85.4±4.37^{ab}	77.4±5.37^{b}
	4 h	86.2±3.44^{a}	85.8±2.80^{a}	77.9±5.93^{ab}	67.0±6.51^{b}
Progressive motility (%)	BI	88.2±2.22	88.2±2.22	88.2±2.22	88.2±2.22
	0 h	75.5±3.06	73.6±3.78	71.4±4.16	71.1±3.64
	1 h	82.8±3.92^{a}	78.4±4.63^{ab}	77.4±3.82^{a}	65.6±7.75^{b}
	2 h	77.5±4.28^{a}	72.5±5.84^{ab}	70.7±5.70^{ab}	60.9±7.29^{b}
	4 h	72.2±4.66^{a}	70.0±3.72^{a}	62.3±6.55^{ab}	49.5±6.85^{b}
Rapid progressive (%)	BI	53.1±3.41	53.1±3.41	53.1±3.41	53.1±3.41
	0 h	55.2±3.33	57.7±3.71	55.9±3.94	56.8±3.56
	1 h	59.4±3.43	58.3±3.90	58.3±3.62	51.2±6.56
	2 h	55.2±3.26	54.6±5.47	53.1±4.98	48.8±6.47
	4 h	55.6±3.46^{a}	54.3±3.40^{a}	48.5±5.12^{ab}	39.1±5.67^{b}
Hyperactive	BI	7.10±1.65	7.10±1.65	7.10±1.65	7.10±1.65
	0 h	4.60±1.14	3.50±0.99	3.10±1.23	2.30±0.71
	1 h	7.10±1.57	4.70±1.10	6.30±1.13	3.70±1.28
	2 h	7.00±1.30^{a}	3.30±1.00^{b}	5.10±1.01^{ab}	1.90±0.51^{c}
	4 h	4.30±1.02^{a}	3.40±0.70^{a}	4.20±1.27^{a}	2.60±0.92^{b}
Mucus penetration	BI	43.5±3.54	43.5±3.54	43.5±3.54	43.5±3.54
	0 h	50.8±2.65^{X}	54.6±2.96^{X}	55.0±2.91^{X}	56.8±2.18^{X}
	1 h	54.3±2.57	57.2±3.38	57.7±3.14	55.1±5.73
	2 h	53.5±3.02	56.5±4.87	54.1±4.75	55.00±5.9
	4 h	56.1±3.26^{X}	55.4±3.42^{X}	54.4±3.67^{X}	48.5±5.31^{Y}

Superscript bearing ^{a,b,c} for a parameter at a particular time point and pH differ significantly (p<0.05)
Superscript bearing ^{X,Y,Z} for a parameter at a particular osmolality and pH differ significantly (p<0.05)

Table 35: Influence of urea on sperm velocity (%) and motility characteristics (BCF, ALH) of bovine sperm incubated at oviductal osmolality (355 mOsm/kg) and pH (7.4) *in vitro*. The semen samples were incubated at three different concentrations of urea. The U3 medium significantly (p<0.05) decreased sperm velocity (VCL, VSL and VAP) from 1 h of incubation. (DOI: Duration of incubation; Control: No urea was added, U1: 0.04 g/of urea L, U2: 0.13 g of urea /L, U3: 0.43g of urea/L; BI:Before incubation)

Parameters	DOI	Control	U1	U 2	U3
VCL (µm/s)	BI	106.9±4.41	106.9±4.41	106.9±4.41	106.9±4.41
	0 h	93.60±3.14	94.50±2.41	94.30±3.79	93.00±2.90
	1 h	116.7±4.17[a]	110.6±5.12	115.6±5.31[a]	100.2±8.12[b]
	2 h	114.9±5.53[a]	103.6±6.75[ab]	108.9±8.03[ab]	97.20±8.53[b]
	4 h	103.1±5.63[a]	102.6±5.03[a]	99.40±7.15[ab]	88.60±8.50[b]
VAP (µm/s)	BI	60.0±3.69	60.0±3.69	60.0±3.69	60.0±3.69
	0 h	62.5±3.92	65.9±3.11	67.3±4.38	66.7±2.77
	1 h	72.1±3.64	69.3±4.39	74.0±5.45	66.4±6.97
	2 h	73.6±5.21	69.4±6.78	72.6±7.13	65.4±8.05
	4 h	71.6±5.06[a]	69.7±5.80[ab]	67.4±6.42[ab]	59.7±6.37[b]
VSL (µm/s)	BI	80.1±3.83	80.1±3.83	80.1±3.83	80.1±3.83
	0 h	75.0±3.65	76.8±2.74	78.4±4.06	77.1±2.97
	1 h	89.6±4.19	83.7±4.55	88.1±5.51	77.7±7.47
	2 h	89.6±5.33	81.8±6.90	86.0±7.54	77.2±8.13
	4 h	84.4±5.04[a]	82.0±5.63 [a]	78.3±6.78[ab]	68.8±7.84[b]
STR (%)	BI	69.1±1.82	69.1±1.82	69.1±1.82	69.1±1.82
	0 h	74.3±2.10	76.4±2.35	76.3±2.15	77.0±1.83
	1 h	72.7±1.50	73.5±1.99	74.6±1.86	72.1±3.91
	2 h	72.6±1.83	73.8±2.71	73.2±2.47	71.3±4.33
	4 h	74.3±1.79	73.4±2.05	73.2±2.58	69.4±4.12
LIN (%)	BI	53.4±2.64	53.4±2.64	53.4±2.64	53.4±2.64
	0 h	61.4±3.51	63.7±3.51	64.6±3.65	65.1±2.96
	1 h	56.6±2.17	56.6±2.14	57.6±2.46	56.1±4.86
	2 h	57.2±2.49	58.3±4.03	57.8±3.16	55.9±5.01
	4 h	60.9±2.15	58.9±3.06	57.9±3.54	53.6±4.80
WOB (%)	BI	73.0±2.22	73.0±2.22	73.0±2.22	73.0±2.22
	0 h	76.9±2.22	77.5±2.79	78.9±1.87	78.8±3.26
	1 h	72.9±2.22	71.7±2.98	72.0±2.03	70.0±2.48
	2 h	73.1±2.22	73.0±2.70	72.8±4.04	69.6±3.96
	4 h	75.9±1.49	73.7±2.45	71.8±3.14	67.9±4.12

Table 35: Contd...

Parameters	DOI	Control	U1	U 2	U3
ALH (µm)	BI	3.50±0.20	3.50±0.20	3.50±0.20	3.50±0.20
	0 h	2.90±0.24	2.70±0.19	2.60±0.21	2.50±0.10
	1 h	3.60±0.15[a]	3.60±0.14[ab]	3.70±0.11[ab]	3.10±0.22[b]
	2 h	3.50±0.17	3.20±0.15	3.30±0.16	3.00±0.21
	4 h	3.00±0.17	3.00±0.13	3.00±0.19	2.70±0.23
BCF (λ)	BI	7.50±0.26	7.50±0.26	7.50±0.26	7.50±0.26
	0 h	7.40±0.29	7.40±0.24	7.30±0.23	7.50±0.21
	1 h	8.10±0.28	8.40±0.48	8.70±0.31	7.70±0.61
	2 h	8.10±0.31	7.80±0.48	8.00±0.56	7.20±0.63
	4 h	7.80±0.41	7.80±0.30	7.70±0.44	7.10±0.69

Superscript bearing [a,b,c] for a parameter at a particular time point and pH differ significantly ($p<0.05$)

4.7.3. Sperm head area

The sperm head area (μm^2) was shown as an indicator of regulatory volume increase post- hyperosmotic stress in our study. The decrease in the sperm head size was observed in sperm incubated in all media and subsequent recovery of the head size was evidenced in control, U1 and U2 but not in U3 medium (Table 22). There was a significant increase ($p<0.05$) in sperm head size at 1 h in comparison to 0 h in control, U1 and U2 media, but not in U3 medium (Figure 34). Further, the sperm head size in U3 medium remained significantly lower ($p<0.05$) at 2 and 4 h of incubation (Table 28).

4.7.4. Capacitation status and acrosome integrity

The percentage of capacitated sperm at 1, 2 and 4 h of incubation was significantly ($p<0.05$) lower in U3 medium (Figure 35).This decrease was due to the faster shifting of cells to acrosome reacted status from capacitated status in U3 medium. Similarly, U3 medium significantly ($p<0.05$) decreased acrosome integrity at 4 h of incubation (Table 37).

Table 36: Influence of urea on sperm head area in bovine sperm incubated at oviductal osmolality (355 mOsm/kg) and pH (7.4) *in vitro*. The semen samples were incubated at three different concentrations of urea. The U3 medium significantly (p<0.05) affected the regulatory volume increase in sperm head from 1 h of incubation (DOI: Duration of incubation; Control: No urea was added, U1: 0.04 gof urea /L, U2: 0.13 gof urea /L, U3: 0.43gof urea/L; BI:Before incubation)

DOI	Head area(µm²)			
	Control	U1	U 2	U3
BI	43.3±0.87^{X}	43.3±0.87^{X}	43.3±0.87^{X}	43.3±0.87^{X}
0 h	38.9±0.85^{Y}	39.5±0.78^{Y}	39.6±0.76^{Y}	38.7±0.72^{Y}
1 h	42.3±0.69^{Za}	42.1±0.75^{Za}	41.6±0.89^{Za}	39.2±0.76^{b}
2 h	41.1±0.73^{a}	40.6±0.81^{ab}	39.3±1.07^{ab}	39.2±0.90^{b}
4 h	40.1±0.78^{a}	40.1±0.58^{a}	39.9±1.01^{a}	37.3±0.88^{b}

Superscript bearing ^{a,b,c} for a parameter at a particular time point and pH differ significantly (p<0.05)
Superscript bearing ^{X,Y,Z} for a parameter at a particular osmolality and pH differ significantly (p<0.05)

Table 37: Influence of urea on capacitation status and acrosome reaction status of bovine sperm incubated at oviductal osmolality (355 mOsm/kg) and pH (7.4) *in vitro*. The semen samples were incubated at three different concentrations of urea. The U3 medium significantly (*p*<0.05) decreased the sperm capacitation and acrosome integrity from 1 h of incubation (DOI: Duration of incubation; Control: No urea was added, U1: 0.04 g of urea /L, U2: 0.13 gof urea /L, U3: 0.43g of urea/L; BI:Before incubation)

Parameters	DOI	Control	U1	U 2	U3
Non capacitated (%)	0 h	7.00±1.64	6.80±1.55	7.20±2.23	9.50±2.71
	1 h	3.80±0.93	5.00±1.36	7.00±1.64	10.5±2.25
	2 h	2.00±0.55	2.60±0.92	3.30±1.18	13.3±6.69
	4 h	1.20±0.53	1.10±0.45	1.20±0.44	2.30±0.90
Capacitated (%)	0 h	91.3±1.75	90.8±1.94	90.9±2.15	89.8±2.47
	1 h	91.7±1.52^{a}	90.7±1.39^{a}	87.6±1.49^{ab}	84.0±2.19^{a}
	2 h	90.2±1.70^{a}	90.7±1.73^{a}	87.6±2.23^{ab}	84.0±3.24^{b}
	4 h	83.6±2.68^{a}	85.0±2.73^{a}	82.8±2.85^{a}	72.7±4.29^{b}
Acrosome reacted (%)	0 h	1.70±0.48	2.30±0.80	1.80±0.69	2.80±0.42
	1 h	4.40±1.21	3.50±1.03	5.40±0.93	5.60±1.52
	2 h	7.80±1.53^{a}	9.20±1.47^{a}	9.30±1.34^{a}	14.3±1.61^{b}
	4 h	15.3±2.96^{a}	13.8±2.80^{a}	16.1±2.88^{a}	23.8±4.65^{b}

Superscript bearing ^{a,b,c} for a parameter at a particular time point and pH differ significantly (p<0.05)

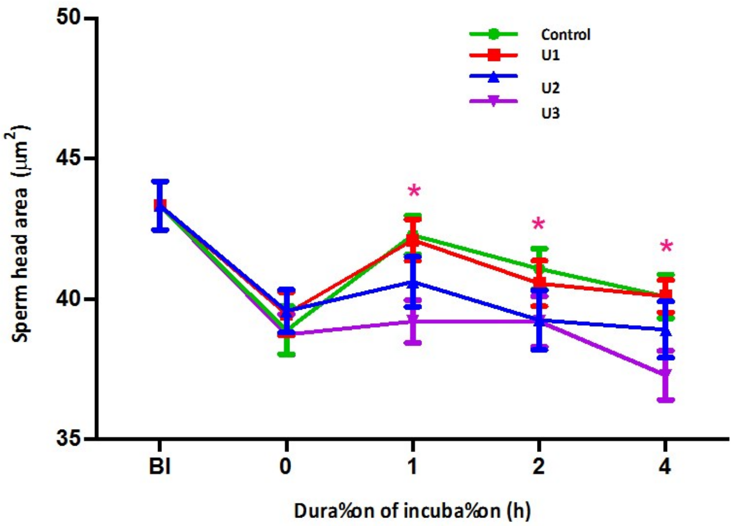

Fig. 34: Effect of urea on sperm head area in bovine sperm incubated at oviductal osmolality (355 mOsm/kg) and pH (7.4) *in vitro*. The U3 medium prevents increase in sperm head size significantly (p<0.05) from 1 h of incubation (DOI: Duration of incubation; Control: No urea was added, U1 : 0.04 gof urea /L, U2 : 0.13 gof urea /L U3: 0.43gof urea/L; BI:Before incubation)

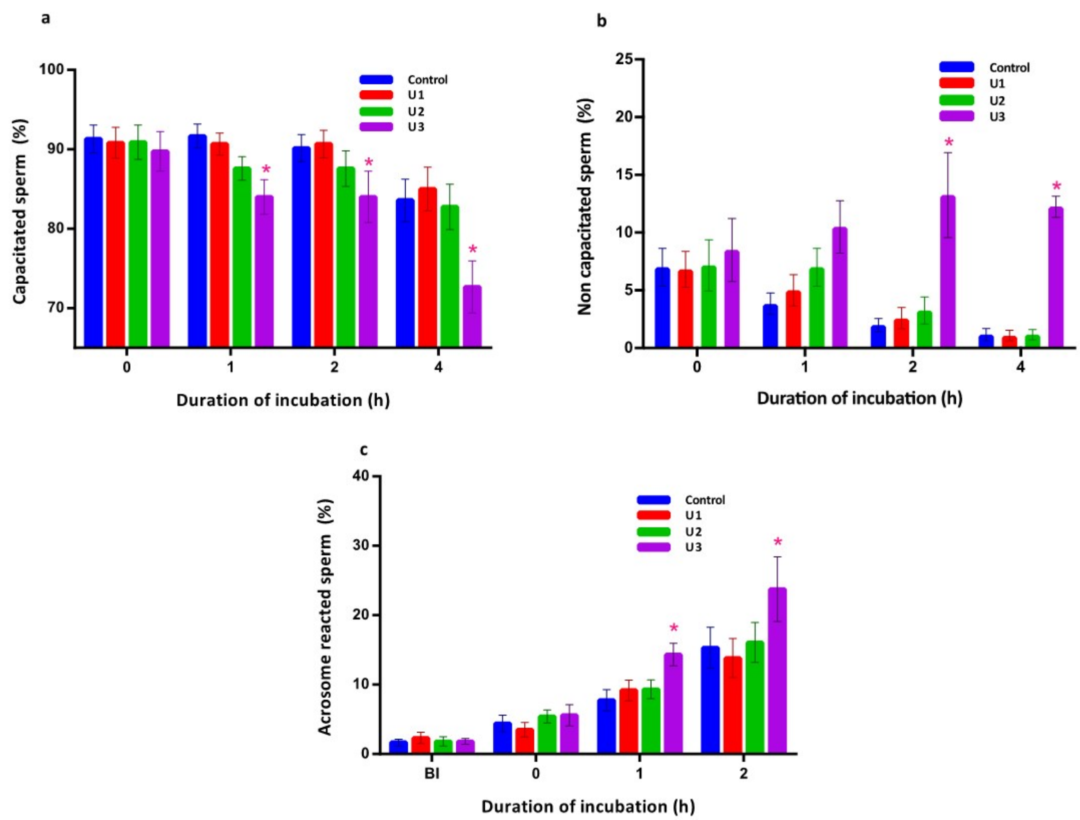

Fig. 35: Effect of urea on capacitation status in bovine sperm incubated at oviductal osmolality (355 mOsm/kg) and pH (7.4) *in vitro*. The U3 medium significantly (p<0.05) decreases sperm capacitation (a) and acrosome integrity (c) from 1 h of incubation (DOI: Duration of incubation; Control: No urea was added, U1 : 0.04 gof urea /L, U2 : 0.13 gof urea /L, U3: 0.43gof urea/L)

4.7.5. Sperm viability, functional membrane integrity, acrosome integrity, mitochondrial membrane potential, chromatin distribution and morphology

The pathologically higher concentration of urea in the U3 medium significantly (p<0.05) affected the percentages of sperm viability and functional membrane integrity from 1 h and 2 h of incubation, respectively (Figure 36). The percentage of acrosome integrity was significantly

Table 38: Influence of urea on functional membrane integrity, mitochondrial membrane potential and chromatin distribution in bovine sperm incubated at oviductal osmolality (355 mOsm/kg) and pH (7.4) *in vitro*. The semen samples were incubated at three different concentrations of urea. The U3 medium significantly (p<0.05) decreased functional membrane integrity and mitochondrial membrane potential. (DOI: Duration of incubation; Control: No urea was added, U1: 0.04 g of urea /L of urea, U2: 0.13 g of urea /L, U3: 0.43g of urea/L; BI:Before incubation)

Parameters	DOI	Control	U1	U 2	U3
Sperm viability (%)	BI	93±1	93±1	93±1	93±1
	0 h	82±1[a]	79±2[a]	77±3[b]	75±2[c]
	1 h	87±2[a]	83±3[a]	82±3[a]	70±6[b]
	2 h	82±3[a]	77±4[a]	76±4[a]	65±5[b]
	4 h	76±3[a]	75±3[a]	67±6[a]	54±7[b]
Functional membrane	0 h	69.4±4.23	64.8±4.60	62.1±4.55	57.5±5.85
integrity (%)	2 h	55.5±4.87[a]	46.8±5.42[ab]	44.8±5.51[b]	35.9±5.21[c]
	4 h	34.3±4.25[a]	30.0±4.17[a]	28.4±3.84[a]	18.4±3.79[b]
Mitochondrial membrane	0 h	85.5±3.54	84.8±3.65	85.6±2.66	82.3±3.86
potential (%)	2 h	83.4±4.31	83.1±4.70	80.4±4.90	77.3±5.10
	4 h	79.8±5.14[a]	78.0±5.11[a]	74.6±5.02[a]	64.2±5.77[b]
Chromatin distribution (%)	0 h	97.2±0.61	96.6±0.76	96.8±0.58	95.7±0.62
	4 h	96.7±0.66	96.1±0.74	95.8±0.69	95.5±0.61
Acrosome integrity (%)	0 h	94.8±0.82[a]	92.3±0.92[a]	88.1±0.71[b]	79.8±2.67[c]
(FITC PSA assay)	1 h	94.1±1.09[a]	90.2±0.81[b]	85.4±1.22[c]	80.3±2.08[d]
	2 h	93.0±1.51[a]	88.6±1.04[b]	84.1±1.74[c]	76.2±3.11[d]
	4 h	93.3±1.52[a]	86.8±1.31	79.1±2.81	61.6±4.72[b]
Morphology (Normal %)	0 h	97.7±2.02	96.7±2.90	97.6±2.11	97.0±3.16
	2 h	97.3±2.27	97.9±1.51	97.0±2.17	96.3±2.90
	4 h	95.8±2.73	97.2±1.99	95.6±3.37	94.3±3.28

Superscript bearing [a,b,c] for a parameter at a particular time point and pH differ significantly (p<0.05)

(p<0.05) decreased immediately after incubation in U3 medium containing the pathological levels of urea (Figure 26, Table 38).Similarly, a significantly (p<0.05) lesser subpopulation of sperm (%) with high mitochondrial membrane potential was observed at 4 h of incubationin U3 medium. However, urea concentration did not have a significant effect on percent sperm chromatin distribution and morphology in our study.

4.8. Urea transporter

The presence of urea transporters (*UT-A and UT-B*) were evaluated in sperm transcriptome using qPCR. Interestingly, both *UT-A and UT-B* transporters were detected in testes cDNA and only *UT-B* transporter expression was detected in sperm cDNA (n=5). The product was confirmed from the melt curve analysis and dissolving the product in agarose gel electrophoresis (Figure 37).

4.9. Urea concentration in serum and seminal plasma and its effect on semen quality

The urea concentration was estimated in the serum and seminal plasma collected during the period of semen collection. The urea concentration in seminal plasma (31.4±0.78 mg/dl) was non-significantly different from serum urea concentration (27.2±0.56 mg/dl) (Table 39).

Table 39: The concentration of urea and urea nitrogen in the serum and seminal plasma of HF bulls (n=12)

Parameters	Serum	Seminal plasma
Urea(mg/dl)	27.2±0.56	31.4±0.78
Urea Nitrogen(mg/dl)	12.7±0.26	14.7±0.37

The relationship between urea concentration and semen quality was studied by classifying the bulls in to two groups based on the rejection rate.The seminal plasma urea concentration was significantly (p<0.05) higher in semen samples with high rejection rate (Table 40) semen as compared to good quality semen. However, the serum urea concentration did not differ significantly between good and poor semen producers.

Table 40: The relationship of serum and seminal plasma urea concentration with semen quality in HF bulls (n=12)

Biological fluid	Parameter	Good (n=6)	Poor (n=6)
Seminal plasma	Urea(mg/dL)	29.6±0.91[a]	33.2±0.80[b]
	Urea N(mg/dL)	13.8±0.43[a]	15.5±0.37[b]
Serum	Urea(mg/dL)	27.8±0.43	26.3±0.94
	Urea N(mg/dL)	12.2±0.20	13.2±0.44

Superscript bearing [a,b] for a parameter differ significantly ($p<0.05$)

The seminal plasma urea concentration was inversely correlated with the sperm functional attributes (Figure 38) Progressive motility (r = -0.55), functional membrane integrity (r = -0.70) and mitochondrial membrane potential (r = -0.64). Moreover, strong positive correlation (r = 0.69) was found between semen rejection rate and seminal plasma urea concentration (Table 41).

Table 41: The correlation of seminal plasma urea concentration with sperm functional parameters and ejaculate rejection rate in HF bulls

Parameters	*r*
Progressive motility	-0.55
Total motility	-0.58
Functional membrane integrity	-0.70
Mitochondrial membrane potential	-0.64
Rejection rate	0.69

✍✍✍

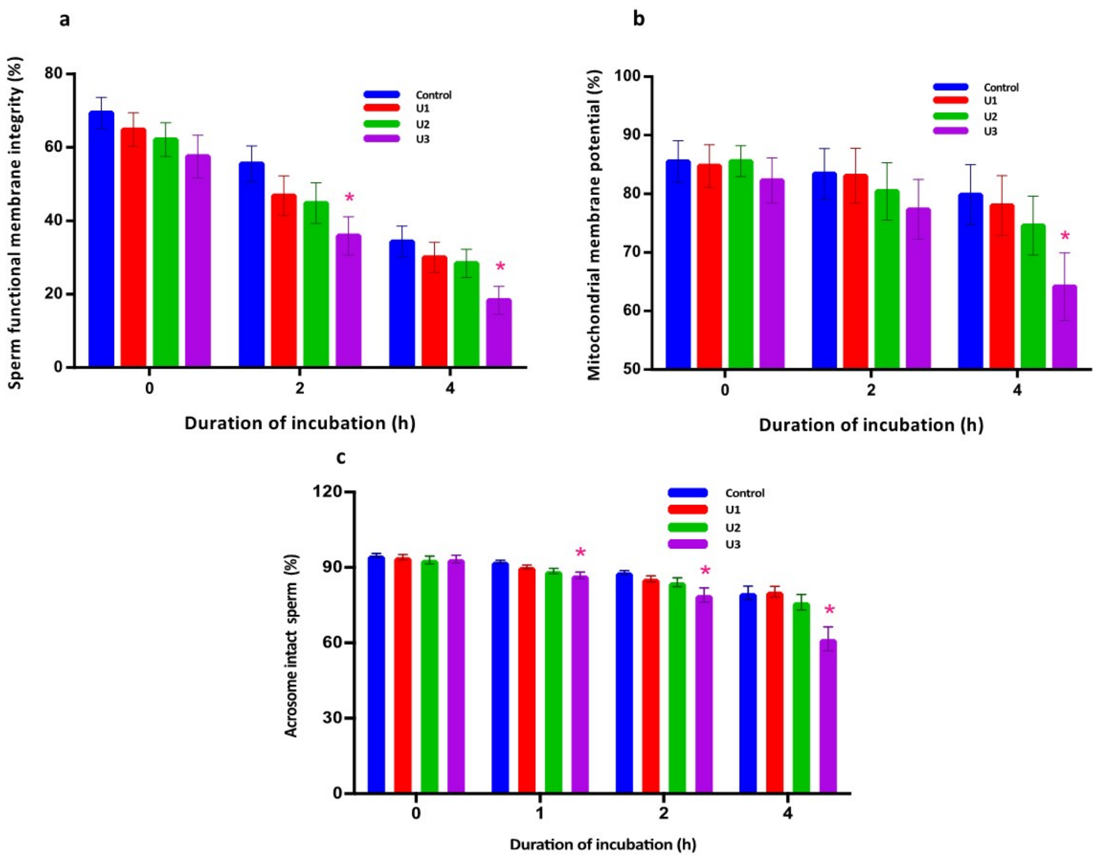

Fig. 36: Effect of urea on functional membrane integrity(a), mitochondrial membrane potential (b) and acrosome integrity(c) in bovine sperm incubated at oviductal osmolality (355 mOsm/kg) and pH (7.4) *in vitro*. The U3 medium significantly (*p*<0.05) decreases functional membrane integrity and mitochondrial membrane potential. (DOI: Duration of incubation; Control: No urea was added, U1: 0.04 gof urea /L, U2: 0.13 gof urea /L, U3: 0.43gof urea/L; BI:Before incubation)

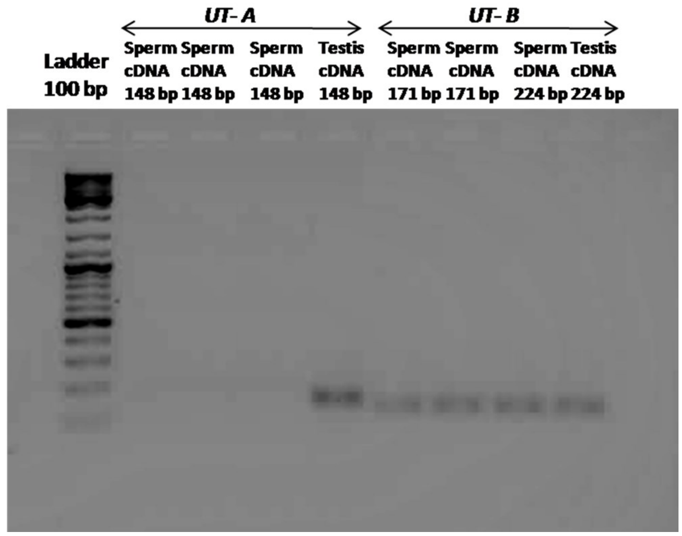

Fig. 37: **The representative image showing the presence of *UT-B* ureatransporter expression in sperm. Testis cDNA was used as positive control. Both *UT-A and UT-B* urea transporter expression were seen in testes. The product size was confirmed using 1.5 % agarose gel electrophoresis.**

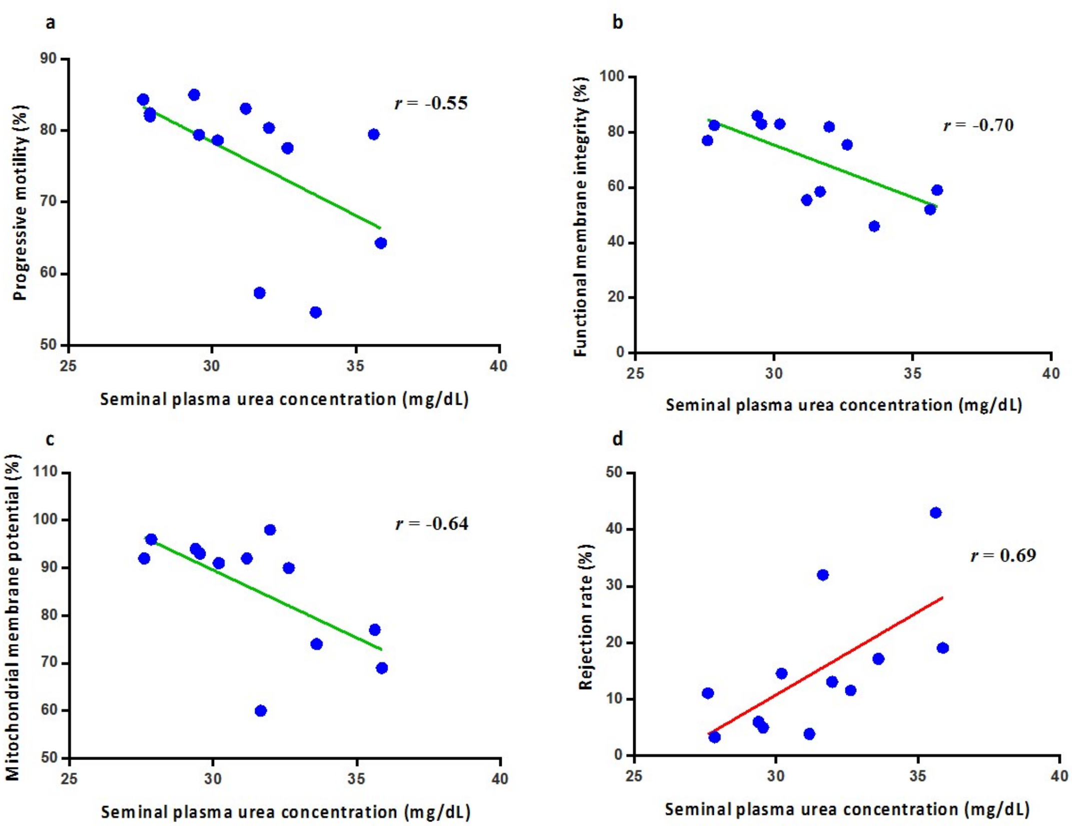

Fig. 38: **Correlation of seminal plasma urea concentration with sperm functional attributes and semen rejection rate**

Discussion

5 DISCUSSION

The present study aimed at exploring the effects of inevitable osmotic and pH excursions imposed on the sperm during their quest to fertilize the ovum. The detrimental effects of osmotic stress on the sperm functions were perceived largely from the cryopreservation point of view. The significance of the physiological range of osmotic stress intrinsical to the tightly regulated reproductive tract microenvironment on sperm function has not been paid much attention. Moreover, the information on the adaptive ability of sperm to these changes and possible impact on their functional competence are scanty. Hence, the study was designed to decipher the effect of physiological pH and osmotic stress prevailing in the bovine uterus and oviduct on the ejaculated bull sperm in preparation for fertilization.As the bulls vary in their fertility status, they were also expected to differ in osmoadaptation ability and concomitantly the sperm functional attributes. Such osmoadaptation behavior of sperm was studied for predicting the quality semen production capacity of a bull.

The elevated blood urea nitrogen concentration has long been imputed as a cause for subfertility in the cattle. Though many hypotheses were proposed for elucidating the BUN mediated infertility due to deterioration of ovum and embryo, not much work has been carried out on the male gamete. The bull sperm stay for at least 12 - 24 h in the female reproductive tract during final maturational changes to accomplish the fertilization process.The effects of different concentrations of blood derived urea in the uterine and oviductal fluids on the sperm are unknown *hitherto*.In this regard, in the present study by mimicking the *in vivo* physiological as well as toxic levels of urea, the sperm functional attribute were studied.

5.1. Seminal plasma osmolality and development of isosmotic sperm transport and washing media

The measured bull seminal plasma osmolality of 292 mOsm/kg was in agreement with the earlier findings (Drevius *et al*., 1972). The seminal plasma osmolality was isosmotic to bovine plasma (Radostits *et al*., 2007), and hypoosmotic to epididymal plasma (Verberckmoes *et al.,* 2003) and female reproductive tract (Olds *et al*., 1957) fluids (uterus and oviduct). The conventional tris egg yolk extender (with and without glycerol) is hyperosmotic and induced alterations in sperm motility patterns, characteristic of hyperactivation. Considering the seminal plasma osmolality, the development or selection of isosmotic sperm transport and washing media became highly essential. The isosmotic tris egg yolk extender preserves of the innate sperm motility characteristics. The low bicarbonate Tyrode medium reduced the incidence of bicarbonate mediated sperm hyperactivation, and facilitated a relatively higher yield of sperm than the conventional Tyrode medium as reported earlier (Mortier *et al*., 2000).

5.2. Effect of physiological osmolality and pH on sperm functional attributes

The total, progressive, and rapid progressive motility of sperm declined sharply as an immediate response to osmotic stress. The sudden drop in motility can be due to shrinkage of cells in response to hyperosmotic stress as evidenced by significantly lower sperm head area. Subsequently, restoration of both cell size and motility were appreciated.The hyperosmotic stress altered sperm cell volume by the outward displacement of cellular water in less than 1 s (Goa *et al*., 1995) and sperm motility (Liu *et al*., 1998; Goa *et al*., 1995). The sperm in turn activated the osmoregulatory mechanisms in favor of osmoadaptation and cell protection was reflected by regained cell volume and motility. Sucha regulatory volume increase in sperm has been reported to correlate with the non-return rate (Petrunika, 2001); however, restoration of motility has not been established clearly in earlier studies probably be due to the single time point evaluation after exposure to stress. Besides, regaining ability in hyperosmotic media also varied among bulls both temporally (1 to 2 h) and in magnitude.Whereas, in the isosmotic control medium, acontinuous drop in motility was observed throughout the incubation period.

Interestingly, significantly higher percent of sperm were capacitated in uterine and oviductal osmolality than that of the isosmotic medium. These results, though not reported earlier, the high ionic strength (HIS) medium or modified Brackett and Oliphant medium (BO) of 380 mOsm/kg has been developed as a capacitation media for guinea pigs, rabbits and later in bovine (Brakett *et al.*, 1978). The high ionic nature of the medium was attributed to be the cause of effective capacitation as it efficiently removes the decapacitating factor, cholesterol from the sperm surface. The hyperosmotic stress was similarly reported to enhance tyrosine phosphorylation of sperm proteins, a hallmark of sperm capacitation, and zonapellucida binding capacity of human sperm (Liu *et al.*, 2006). The bovine sperm stored in 320 mOsm/kg TEST egg yolk extender at 4°C for 4 h were capable of zona penetration (Ijaz and Hunter, 1989). The cryopreservation mediated osmotic stress also results in capacitation like changes in sperm (Cormier *et al.*, 1997). Consistent with these findings, the sperm velocities were significantly affected on exposure to osmotic stress.The recovery of sperm cell size was associated with improved motility andvelocity attributes such as VSL, VCL and VAP. Besides, the velocity acquired on osmoadaptation at 2 h was significantly higher than that of the control medium and even the same medium before incubation. A sharp increase in sperm motility is often attributed as a characteristic feature of capacitated spermatozoa (Yanagimachi *et al.*, 1969; Bavister *et al.*, 1973). In the present study, the ALH and BCF also had a substantial increase at 1h in the hyperosmotic medium which can be attributed to hyperactivation (Yanagimachi *et al.*, 1970, Fraser, 1977). But, the significant sustenance of these attributes was not observed in our study which may be due to inadequate bicarbonate levels of 12 mM in the incubation medium. It is reported that at least 25 mM bicarbonate is necessary to cause sperm hyperactivation (Chen *etal.*, 2000).The extracellular hyperosmolality resulting in cell shrinkage activates chloride bicarbonate exchanger as a part of volume regulatory measure (Reusch *et al.*, 1995) favoring the influx of bicarbonate. Thus, it is highly likely that the altered sperm motility characteristics during capacitation (increase in VSL, ALH, BCF) are the results of physiological osmotic stress prevailing in the uterus and oviduct. Such altered motility could be sustained in the presence of adequate bicarbonate.Though both uterine and oviductal osmolality induce capacitation, the oviductal pH (7.4) contributes significantly higher than the uterine pH in isosmotic medium. The oviductal pH having a capacitating effect on the isosmotic medium has been widely accepted in many species (Parrish *et al.,* 1989; Mishra *et al.,* 2018). However,the capacitating effect of oviduct pH was inappreciable in the hyperosmotic medium. The interaction effect of osmolality and pH was found significant in regulating capacitation, in the present study. This implicates that the reproductive tract fluid osmolality and pH may possess a significant synergistic role in prompting the sperm capacitation process.

The instantaneous appearances of CTC-pattern B indicating capacitated sperm were observed on exposure (0 h) to hyperosmotic medium (Figure 14). The minimal capacitation time required for bovine sperm being 3- 4 h, this immediate effect can be explained in light of osmotic stress. The chlortetracycline assay uses a calcium probe to identify the morphological pattern changes occurring during the course of capacitation (Perry *et al*., 1995). The calcium influx shifts the sperm from pattern F to pattern B. This calcium influx is an initial event in the capacitation process (Breitbart, 2002) and does not indicate the completion. The osmotic stress signaling in somatic cells is also mediated by initial calcium influx (Erickson *et al*., 2001).The uterine osmolality significantly protected sperm viability both in terms of structural and functional membrane integrities which was contradictory to previous findings (Liu *et al*., 1998). The same trend though seen in oviductal osmolality remained insignificant. Though the membrane integrity was reported to be not significantly affected in hyperosmolality as high as 500 mOsm/kg, it was reported to be optimally maintained between 200 – 300 mOsm/kg (Liu *et al*., 1998).These earlier observations could be due to single time point evaluation, as the hyperosmolality mediated (HYP 1) conservative effect was evident only after 2 h of incubation in our study. The assumptions of the linear effect of osmolality on sperm functions would have caused erroneous observations. In the present study, as we observed a significant quadratic effect of osmolality on several sperm functions like progressive motility, functional membrane integrity, and capacitation, there could be a discrete protective function of physiological osmolality on sperm function. In addition, the hyperosmotic medium as compared to the isosmotic medium significantly prevented premature acrosome reaction and protected acrosome integrity. This is similar to the results of Bielfeld (1993). The hyperosmotic stress cause cells to undergo actin polymerization and reorganization as a defense strategy. This cytoskeletal reorganization enables cell membrane protection, maintenance of cell shape, and promote cell motility (Yamamoto *et al*., 2006: Shukla *et al*., 2004). The actin polymerization has been reported to occur in sperm (Breitbart *et al*., 2005) during capacitation. This actin mediated cytoskeletal reorganization not only protects the plasma membrane integrity, but also facilitates the fusion of the sperm plasma membrane and outer acrosomal membrane. The sperm, while encountering the zonapellucida,intracellular calcium signaling is initiated that inturn activates actin scavengers favoring acrosome reaction (Breitbart *et al*., 2005). As the sperm were not challenged with acrosome reaction inducers in our study, the hyperosmolality induced protection of plasma membrane and acrosome integrities were evident. Moreover, the higher and longer sustenance of progressive motility in the hyperosmotic medium can be attributed to the actin polymerization mediated promotion of sperm motility (Gervasi *et al*., 2008)

The mitochondria are the stress perceiving organelles and their crosstalk with the nucleus decide the fate of cell survivability (Galluzi *et al*., 2012). The mitochondrial membrane depolarization and elevation of membrane potential in response to osmotic stress is a determinant of cellular adaptation (Pastor *et al*., 2009). Similar findings were observed in our study as the percent subpopulation of sperm with high membrane potential was significantly higher in the good osmoadapter group indicating an efficient osmoadaptation process. The sperm mitochondria membrane potential considerably influences motility and fertilizing potential (Kasai *et al*., 2002). Further, evidence for retrograde signaling of mitochondrial translated nulear encoded proteins in bovine sperm (Gur and Breitbart, 2006) and transcription, translation of mitochondrial genome in boar sperm (Zhu *et al*.,2019) has made us revisit the conventional belief of transcriptionally and translationally inactive sperm. In addition, the recent evidence of existence of unfolded protein response pathway in mature sperm (Santiago *et al*., 2019) and osmotic stress-induced mitochondrial function allows us to speculate that the mitochondrial functions are essential beyond being a mere energy reservoir in bovine sperm (Storey *et al*, 2008). It is also important to note that when the sperm were subjected to higher magnitude of osmotic stress like cryopreservation in deterioration of the sperm mitochondrial membrane potential resulted in stallion (Garcia *et al* 2011) and mouse (Kim *et al*., 2013) sperm.

The morphology and chromatin distribution pattern were not affected by the uterine and oviductal osmolality (Table 25). Chromatin compaction and decondensation were described as definite outcomes of hyperosmotic (Olins *et al.*, 2020) and hypoosmotic stress (Lima *et al.*, 2018), respectively. As we examined only abnormal distribution patterns, there was no effect of uterine hyperosmolality on chromatin distribution patterns in bovine sperm.

Altogether, the results of the study suggest that the uterine and oviductal osmolality though initially hampers the sperm motility for a while, improvement in kinematics were observed subsequently. The physiological osmolality protects the structural and functional membrane integrity coupled with mitochondrial membrane potential. In addition, it favors the capacitation of sperm in terms of favouring calcium influx and prevents premature acrosome reaction. These findings suggest that the physiological osmotic stress and pH excursions imposed on sperm are more of appreciative nature towards the fertilization process.

These findings may have two significant impacts on reproductive success a) cryptic female choice and b) phenotypic plasticity. The literature survey revealed the existence of osmotic gradient between the seminal plasma and uterine fluid of most species (human, bovine, porcine, caprine, mouse, rat) (Table 1) which in turn can be one of the selection strategies employed by the female towards selecting the superior sperm for fertilization. The changes in the sperm behavior such as chemotaxis and hyperactivation in an alkaline environment in response to external stimuli support phenotypic plasticity. This phenotypic plasticity seems to be conserved across the species in evolution. For instance, the sperm activation to osmotic stress has been well documented in the lower order of vertebrates like fishes and frogs (Caisson *et al.*, 2009). Both the cryptic female choice in the form of osmotic stress and phenotypic plasticity of sperm as a response to the osmotic stress may have immense importance in the regulation of sperm functions and fertilization events.

Dinstinctly, the results from the present study and earlier published results suggest that the osmotic stress is an important regulator of many biological functions as it modulates both nuclear and mitochondrial functions apart from regulating the cell metabolic state via epigenetic changes. As the sperm also deliver an epigenitically marked nucleus which influences the developmental programming of the embryo (Teperek *et al*., 2016), deciphering the effects of intrinsic osmotic stress further may reveal potential implications of the fertilization process.

5.3. Prediction of quality semen production capacity of bull

In spite of the several sperm functional assessments carried out, predicting semen fertility is still challenging for the reproductive biologist. Those bulls with identical sperm functional attributes differ in field fertility to a considerable extent (Larson and Miller, 2000). This uncertainty is of serious concern in the dairy industry as one bull through AI can impregnate thousands of females. Hence predicting bull fertility and subsequently removing subfertile from herd or semen bank can greatly improve dairy cattle fertility. The efficiency of predicting bull fertility can be effectively addressed by the including molecular techniques (genomics, transcriptomics, proteomics, and metabolomics) with a routine array of tests.

In the present study, we found that the osmotic stress enroute the perilous journey of the sperm markedly influences the sperm functional attributes and thereby fertility. Based on the results, it is reasonable to speculate that osmotic stress can determine the endurance of sperm subpopulation capable of fertilization. The sperm functional attributes assessed in different combinations of osmolality and pH (uterine and oviductal) was subjected to correlation analyses with the semen rejection rate, a measure of quality semen production capacity of bull. Interestingly, the sperm functional attributes like functional membrane integrity, acrosome integrity, mitochondrial membrane potential, and mucus penetration in hyperosmotic media (HYP1 and HYP2) were significantly correlated with rejection rate than isosmotic media (Table 27).

The sperm progressive motility and mitochondrial function significantly differed between the groups classified based on rejection rate and obviously predictive of semen rejection rate. Based on these preliminary findings, osmoadaptation scores were developed by incorporating the sperm progressive motility in hyperosmotic media (HYP1 and HYP2). Later to address the pH influence on sperm function, both the pH and osmolality were incorporated in developing osmoadaptation predictive measures. The progressive motility was significantly influenced by the osmotic stress and it improved with regulatory volume increase. This recovery of motility also differed temporally and in magnitude between bulls and hence preferred as the suitable parameter representing sperm osmotolerance behavior than that of mitochondrial membrane potential. Since significant difference in the progressive motility was observed between the hyperosmotic and isosmotic media at 0 h and 4 h, the loss in progressive motility at 1 and 4 h in hyperosmotic media (HYP 1 and HYP 2) were considered as osmoadaptation ability (Table 28). Higher the loss in progressive motility, more the osmo-susceptibility index, and poorer the sperm osmoadaptation ability and semen quality.To analyze the efficiency of different osmo-susceptibility indices to predict the semen quality, they were subjected to correlation with the semen rejection rate. Of the six osmo-susceptibility indices developed, oviductal osmo-susceptibility index (OVSI) showed a significantly strong positive correlation (*r*) of 0.71 with the rejection rate (Figures 15 and 16). The cut-off for oviductal osmo-susceptibility index (OVSI), was arrived with ROC analysis. The OVSI at 18 % cutt-off had the maximum likelihood ratio of 6. At the choosen cut-off the specificity was 83.3% and sensitivity was 100%.

The choosen cut–off was also validated in samples of known rejection rate. Subsequently, a linear regression equation developed for the prediction of semen rejection rate had a coefficient of determination (R^2) of 0.70. The predicted semen rejection rate had high correlation (r=0.79) with actual semen rejection rate (n=18).Further, the obtained OVSI was strongly positively correlated with several sperm functional attributes like progressive motility, total motility, mucus penetration, ALH, BCF, head area, functional membrane integrity, mitochondrial membrane potential, and acrosome integrity (Table 29). This indicated that the sperm functional attributes in hyperosmotic media prevailing in the reproductive tract are better correlated with semen quality than isosmotic media routinely used for bull fertility prediction tests. Hence, the developed OVSI can be used as a convenient semen quality predictive measure forefficiently predicting the quality semen production capacity of a bull. This results also suggest a reconsideration of the traditional use of isosmotic media for routine semen analysis as *in vitro* fertilization is achievable over a wide range of osmolality between 308 to 372 mOsm/kg in the mouse and 292 to 392 mOsm/kg in hamster (Miyamato and Chang, 1973) and insignificantly higher *in vitro* fertilization was achieved in hyperosmotic media (340 mOsm/kg) over isosmotic medium in bovine (Miller and Hunter, 1986).

5.4. Osmoregulatory gene expression levels in sperm

In order to assess the osmoregulatory capacity of the bovine sperm, some of the genes reported to regulate osmotic behavior of a cell and are relevant to the sperm functions were studied. The selected genes were reported to influence the following functions: oxidative phosphorylation (*MT-ND2, MT-CO1*), glycolysis (*ENO1, GAPDH*), mitochondrial calcium sensor (*EFHD1*/mitocalcin), chaperone (*HSP90AB1*), a transcription factor acting as the major controller of osmoresponsive genes (*NFAT5*), an osmotic stress effector ion channel (*SLC9C1*), and a disintegrin and metalloprotease (*ADAM1B*).

Since the sperm are transcriptionally and translationally silent, the sperm are expected to synthesize and carry these osmotolerence proteins also well in advance. The presence of RNA in sperm thus reflects the sperm capability in adapting to the micro environment prevailing in the male and female reproductive tract. Nevertheless, the recent research evidence on the mitochondrial translation of nuclear encoded proteins (Gur and Braiebart, 2006), nuclear shuttling of transcription factors and their localization on histone retained regions (Dadoune *et al*., 2005), mitochondrial transcription and translation in boar sperm (Zhu *et al*., 2019) and paternal RNA contribution for embryo development unlocked the new possibilities and scope for gene expression in sperm.

The expression levels of the genes *NFAT5, HSP90AB1, SLC9C1, ADAM1B,* and *GAPDH* were upregulated in the good osmoadapter group. But, the expression levels of the genes *MT-CO1, PRM1, ENO1,* and *EFHD1* were down regulated in the good osmoadapter group. The expression levels of *MT-ND2* was found unaltered between the groups. A few of the genes in the above list though reported earlier in sperm with respect to functional attributes, their changes in relation to osmotic stress have not been reported.

The ADAM1B expression level was 15-fold upregulated in the good osmoadapter group.ADAM1 is a membrane-anchored protease and mediate osmotic stress signaling (Fischer *et al*., 2004).ADAM1 undergoes cleavage at its extracellular proheparin binding EGF ligand during osmotic stress and thereby activates downstream kinases MAPK, ERK, and JUNK (Fischer *et al*., 2004). These kinases are of vital importance in sperm as they mediate post-translational modification of sperm proteins during capacitation (Silva *et al*., 2015). Further, ADAM1 was localized in the sperm head plasma membrane and found to mediate sperm transport and sperm- zona binding (Yamaguchi *et al*., 2009; Leahy *et al*., 2020). The knock out studies in mice confirmed the involvement of *ADAM1* in embryo development by regulating the NOTCH pathway (Weber *et al.,* 2011). We found that *ADAM1* is positively associated with sperm head size thereby reflecting osmoadaptation status, mitochondrial membrane potential, and acrosome integrity (Table 39).Similarly, it was strongly, but negatively correlated with semen rejection rate (Table 39).The abundance of *ADAM1* in the good osmoadapter group suggests the possibility of osmotic stress mediated activation of kinases by *ADAM1* and thereby involved in the regulation of sperm capacitation and sperm-zona binding.

NFAT5 also known as tonicity responsive enhancer binding protein (TonEBP) was 11.3-fold upregulated in the good osmoadapter group.*NFAT5* has an intrinsically disordered region (IDR) that can directly sense osmotic stress mediated macromolecular crowding. It is also activated by the ROS produced by hyperosmolality induced oxidative stress. The osmoprotective genes has an osmotic response element sequence (ORE) located in front of their gene in DNA. The hyperosmotic stress activated *NFAT5* binds to the ORE and hence known as osmotic response element sequence binding protein (OREBP). It acts as a transcription factor for several effectors of osmotic stress adaption process namely aquaporins, aldolase reductase, sodium myoinositol transporter (Lopez Rodriguez *et al*., 2014), taurine transporter, betaine transporter (Miyakawa *et al*., 1998), heat shock proteins (Navarro *et al*., 2008) and urea transporter (Nakayama *et al*., 2000). The gene-specific knock out studies in mice revealed severe embryonic mortality (Mak *et al*., 2011) revealing its incompensable role in reproduction. Though these functions of *NFAT5* has not been elucidated in sperm before, the differential expression of *NFAT5* between semen of good and poor osmoadapters, its positive association with sperm head area, mitochondrial membrane potential, percent hyperactive sperm and strong negative correlation with semen rejection rate (Table 39) in our study implicates strong evidence for *NFAT5* mediated osmoadaptation process in sperm and the remarkable influence on the fertilizing ability of sperm.

SLC9C1 was found to be 10.6-fold upregulated in the good osmoadapter group. SLC9C1 is a solute carrier protein which acts as an effector protein during osmotic stress and responsible for the accumulation of solute inside the cell to overcome cell shrinkage. SLC9C1is also known as sperm-specific sodium proton exchanger (sNHE) and is of vital importance in regulating sperm intracellular pH (Ruiz-Martínez *et al*., 2011). NHE (sodium proton channels) have sites that are sensitive to hyperosmotic stress and proton gradient, which are involved in the exchange of intracellular protons to extracellular sodium thereby reestablishing cellular volume (Bianchini *et al*., 1995; Lacroix *et al*., 2008).sNHE in sperm was shown to regulate intracellular pH, by extrusion of proton and consequently involved in the capacitation and hyperactivation aiding in the fertilization process (Grienstein *et al*., 1992 ; Ruiz-Martínez *et al.,* 2011).Moreover, *SLC9C1* knock out mice revealed absolute male infertility (Wang *et al*., 2007).*sNHE* expression levels were significantly negatively correlated with sperm linearity in our study which is in favor of facilitating hyperactivation. *sNHE* expression levels were also positively associated with acrosome integrity and negatively associated with semen rejection rate (Table 39). Therefore, *SLC9C1*may promote sperm regulatory volume increase by extrusion of protons and facilitate intracellular alkalization and thereby favor the capacitation and hyperactivation of sperm.

HSP90AB1 was found to be 2.34-fold upregulated in the good osmoadapter group. The hyperosmotic stress causes instantaneous cell volume shrinkage which causes macromolecular crowding inside the cell. The hydration status of proteins and nucleus gets affected which ultimately affects the protein functions. Heat shock proteins are molecular chaperones recruited under stress conditions to protect these cellular proteins and degrade the misfolded proteins (Lackie *et al*., 2017).The heat shock proteins also modulate histone deacetylases and DNA methyltransferases and thereby regulate gene expression for reestablishing the cellular functions during stress (Mazaira *et al*., 2018).The *HSP90* has been reported to regulate sperm motility, calcium influx, and capacitation process (Li *et al*., 2014). In our study, we found that *HSP90AB1*expression levels are positively associated with ALH and negatively associated with sperm linearity index (Table 39). This supports the involvement of *HSP90AB1* in sperm hyperactivation. Recently, their role in the remodeling of human sperm chromatin was also revealed (Beeram *et al.,* 2019).Thus, upregulation of *HSP90AB1* in good osmoadapter group suggests that HSP90AB1may protect sperm proteins during osmotic stress and thereby positively influence the sperm motility.

GAPDH is primarily involved in the glycolysis pathway. The expression levels of *GAPDH* were1.6 fold upregulated in the good osmoadapter group. Under the osmotic stress conditions, the cellular energy network switches from glycolysis to oxidative phosphorylation. The predominant energy production pathway in bull sperm was also oxidative phosphorylation rather than glycolysis (Storey *et al*., 2008). Hence under osmotic stress, the cellular metabolic reprogramming recruits the constitutive proteins for other cellular functions of necessity. Such multi-tasking proteins are called moonlighting proteins and GAPDH performs such functions under osmotic stress (Muronetz *et al*., 2020). Henceforth it is logical to speculate the specialized functions like ensuring mRNA stability (Zhou *et al*., 2008), facilitating nuclear RNA transport (Dastoor and Duyer *et al*., 2001), and intracellular membrane trafficking of proteins (Sirover *et al*., 2012) are carried out by *GAPDH* in sperm. The growing evidence of nuclear-encoded protein synthesis in mitochondria (Gur and Breitbart *et al*., 2006) and aggregation of transcription factors in the nucleus (Doudone *et al*., 2005) in the presence of osmotic stress support this speculation to be worthy, however further research is needed for confirmation in sperm.

This gene encodes for another structural component of mitochondrial electron transport chain namely NADH dehydrogenase enzyme (Complex I subunit 2), which is major energy deriving oxidative phosphorylation pathway for the cells under osmotic stress.In the present study, *MT-ND2* expression levels didnot alter between the good and poor osmoadaptergroup. However, *MT-ND2* expression levels were negatively associated with sperm functional membrane integrity (Table 39). Active transcription and translation of mitochondrial genes in boar sperm were recently demonstrated (Zhu *et al*., 2019). Our findings of nondifferential expression of *MT-ND2* suggest nonosmotic- responsiveness of *MT-ND2* in bovine sperm.

The expression levels of *EFHD1* were 0.55 fold down-regulated in the good osmoadapter group. *EFHD1*codes for the mitochondrial calcium sensor protein mediating the apoptosis via the mitochondrial flash activation process resulting in cell death (Hou *et al*., 2016). However, in our study the expression was observed only in two samples of the poor osmoadaptation group. The expression levels of *EFHD1* were strongly negatively correlated with sperm progressivity and velocity (Table 39). Further, the samples with *EFHD1*abundance had significantly compromised mitochondrial membrane potential in our study. As the functions of *EFHD1*in sperm are yet to be elucidated, our primary findings study suggests the negative role of *EFHD1*in the sperm osmoadaptation process.

The expression levels of *ENO1* was 0.6 fold down-regulated in good osmoadapter group.*ENO1* codes for enolase enzyme which is a component of the glycolysis pathway. *ENO1* expression was upregulated under oxidative and osmotic stress (Didiasova *et al*., 2019) suggesting the possible role of *ENO1*in regulating osmoadaptation process.But interestingly the expression of this gene was observed only in the semen samples of two bulls belonging to the poor osmoadaptation group. There asons for the inconsistent expression of *ENO1* in different semen samples are not clear. However,the abundant expression of ENO1 in the poor quality semen samples of idiopathic infertile male and the correlation of the protein abundance with poor quality embryos (Jodar *et al*., 2020) have been reported.Similarly, strong negative relationship of *ENO1*expression levels was observed with sperm motility, functional membrane integrity, and mitochondrial membrane potential in our study (Table 39).ENO1 has been found as a facilitator of the apoptotic processby surface expression under stress (Ucker *et al*., 2012). As the functions of *ENO1* in sperm remain elusive, our primary findings suggest potential negative effects of *ENO1* abundance on sperm function.

The expression levels of *MT-CO1* in our study were 0.35 fold downregulated in the semen samples with good osmoadaptation ability. This gene encodes for a structural component of mitochondrial electron transport chain, cytochrome c oxidase (complex IV). Though, the mitochondrial function is vital under osmotic stress, the *MT-CO1* expression is reported to be downregulated under osmotic stress (Mansilla *et al*., 2018).Active transcription and translation of mitochondrial genes in boar sperm were recently demonstrated (Zhu *et al*., 2019). The MT-CO1 release from inner mitochondrial membrane into the cytoplasm is a determinant of cell death. This finding is in agreement with our results as the expression of *MT-CO1* was downregulated in the good osmoadapter group. Though MT-CO1 mediated apoptotic cell death has not been elucidated in sperm, it was reported in testes (Liu *et al.*, 2006). In our study, we found that *MT-CO1* expression levels were negatively associated with sperm motility, mucus penetration ability, functional membrane integrity, and mitochondrial membrane potential (Table 39). In light of these observations we speculate abundant expression of MT-CO1 proteins in the sperm might also activate caspase cascade favoring apoptosis in the osmo-stress sensitive sperm.

The osmoresponsive genes selected from the literature survey were elucidative of the osmoadaptation process. The genes were validated in our study, in context with sperm osmoadaptation ability. Overall, in sperm upregulation of *HSP90AB1, SLC9C1, ADAM1B* and *NFAT5* in good osmoadapter group and down regulation of *MT-CO1* in the poor osmoadapter group describes the preparedness of sperm to osmotic stress. Further, the gene expression levels of osmoresponsive genes were in accordance with the bulls classified by the oviductalosmo-susceptibility index (OVTS). This confirms the utility of the oviduct alosmo-susceptibility index (OVTS) as a predictor of semen quality in bulls.

Overall, the subcellular changes associated with capacitation like cholesterol efflux, tyrosine phosphorylation, calcium influx, hyperpolarization of the plasma membrane, and elevation of intracellular pH, ROS production are also found to be homogenous with the established osmoadaptation process (Figure 40). The upregulated *HSP90AB1, SLC9C1,* and *ADAM1B* genes in the good osmoadapter group are also reported to be associated with the sperm capacitation process. These findings with the universal existence of osmotic stress in the reproductive tract of most vertebrates suggest an incompensable role of osmotic stress in preparing the sperm for fertilization.

5.5. Influence of urea on sperm functions

The urea was added to the incubation medium simulating oviductal osmolality and pH as the oviduct milieu supports sperm survival for a longer time *in vivo* (Miller *et al*., 2015). It was hypothesized that urea may impede sperm function by a) alteration of pH b) alteration of osmolality and c) own deleterious effects.

The U3 medium containing the pathological concentration of urea significantly reduced the sperm total motility and progressive motility from 1 h of incubation. Though a steep drop in motility was observed in all media as a immediate response to osmotic stress, the motility recovered in all except U3 media. The urea being an osmotically active substance support red blood cells to ameliorate hyperosmotic stress in the medulla of the kidney (Weiner *et al*., 2015). But at higher concentration, urea hampered the osmoadaptation process in the sperm as the sperm motility recovery was hindered in the U3 medium Further, the regulatory volume increase was observed in all media (1 h), but not in the U3 medium. The sperm velocity was also significantly retarded in the U3 medium. The VCL, ALH and the kinematics related to sperm hyperactivation were hampered from 1 h of incubation. In addition, VAP and VSL were also significantly compromised at 4 h of incubation.Overall, sperm kinematics in urea containing medium was significantly affected and is in accordance with the previous findings in rat (Turner & Howard, 1978) and human (Kim *et al*., 1998) sperm. Similarly, the mucus penetration rate was significantly lower at 4 h of incubation in the urea containing medium (U3) and is in accordance with the preliminary findings of Hossain (1993) and Breau (1985).

The sperm structural and functional membrane integrity were significantly affected from 0 h of incubation in the U3 medium. This destruction of membrane integrity would have compromised the downstream sperm functions like motility.Urea at pathological concentration significantly induced premature acrosomal reaction from 2 and 4 h of incubation respectively. In addition, urea at pathological concentration significantly affected the percentages of sperm subpopulation with high mitochondrial membrane potential at 4 h of incubation. Urea concentration did not have a significant effect on percent chromatin distribution.

The elevated urea concentration in the medium had pronounced detrimental effects on both sperm structure and function in our study.The urea is transported across the biological membranes by facilitated diffusion. Two class of mammalian urea transporters, UT-A and UT-B are involved in the urea transport. But the presence of urea transporters in sperm was unexplored, however existance of such transporters in rat testis has been reported (Fenton *et al.,* 2002). The urea transporter knockout male mice reported to exhibit early maturation due to increased expression of FSHR and ABP receptors in the Sertoli cells (Yang *et al*., 2014).

Since the urea caused severe detoriative effect of sperm functional parameters, we evaluated the presence of *UT-A* and *UT-B* transcripts in sperm. Though the transcripts for both transporters were present in bovine testis, only the *UT-B* transcript was expressed in bovine sperm. This first report documented the presence of urea transporter protein-B in mature bovine sperm.The physiological functions of urea transporter-B in sperm and regulation of male fertility needs to be unraveled.

The urea is one of the most abundant metabolites in bovine semen (Velho *et al.,* 2018). The translation of naive proteins and the existence of urea cycle enzymes in sperm suggest that urea can be the end product of protein metabolism during sperm maturation process. The urea transporter protein (UT-B) can be involved in the transport of urea through the sperm membrane. Further the external medium osmolality and urea concentration has been reported as regulators of urea transporter protein (UT-B) in mammalian cell lines (Farrell and Stewart, 2019). Together, it can be hypothesized that the increased urea concentration outside the cell may prevent the urea clearance from the cell and may even promote urea uptake into the cells. This accumulation of urea can be attributed to the protein denaturation effect of urea (Rossky *et al*., 2008; Lim *et al.,* 2009), as the post-translation modification of proteins (Samanta *et al*., 2016) and naive mitochondrial protein synthesis (Zhu *et al*., 2019) occupy an inevitable role in sperm functions and accomplishing fertilization.

The urea concentration in the seminal plasma was nonsignificantly higher than the serum. The observed levels were in accordance with the previous findings for serum (Javid *et al*., 2008) and seminal plasma (Cevik *et al*., 2008) in bovine.The close evaluation of the individual values revealed that higher urea concentration of about 4 35 mg/dL in two of the bulls in poor quality semen producer group and others had had similar serum urea concentrations of that of good quality semen producer group.

The bulls classified based on the semen rejection rate revealed that the bulls with a higher rejection rate had a significantly higher seminal plasma urea concentration than with low rejection rate. The semen functional parameters from two bulls with the higher urea concentration also had suboptimal membrane integrity, mitochondrial membrane potential, and motile sperm.

Hence the abnormal urea concentration in the seminal plasma was attributed to have a severe effect on the sperm. This was in accordance with the previous study wherein the poor semen characteristics were associated with elevated seminal plasma urea levels (Moradi *et al*., 2017). Since the serum urea concentration did not differ between the groups, the elevated seminal plasma urea concentration can be attributed by the local secretion from the reproductive tract rather than the feed effect in our study.

The present study revealed that higher urea concentration either in the seminal plasma or in the incubation medium (U3) significantly deteriorated the bull sperm functional attributes (Figure 40). The analysis of the urea mediated effects reveals a) structural damage observed within1 h (significantly lower structural membrane integrity, functional membrane integrity, and sperm chromatin distribution) followed by b) functional deterioration later at 2-4 h (premature acrosomal reaction/acrosomal loss, motility, and mitochondrial membrane potential).

It is also worthy to mention that urea may impair fertility by altering reproductive tract pH and osmolality apart from the direct toxic effects for the urea on gametes' functions. We also evaluated the changes in pH and osmolality after the addition of urea to the medium in vitro, however, no major physiochemical changes of the medium were observed. In animals with higher BUN concetration had no change in pH and osmolality in the uterus during estrus (Elrod and Butler, 1993; Amundson *et al*., 2016). Hence, *in vivo*, the urea mediated sperm functional impairment could be one of the major reasons for low fertility.

Conclusively, the present study reveals that urea affects the vital sperm functional attributes and organelles especially mitochondrial functions which are essential for fertilization success. The present study strongly suggests that higher BUN levels in uterine and oviductal microenvironment might impair fertility due to the toxic effect on sperm structure and function. Such impairment may ultimately induce either fertilization failure or early embryonic lethality.

Summary and Conclusions

6 SUMMARY AND CONCLUSIONS

The present study aimed at exploring the effects of the osmotic and pH excursions on sperm during their transport in the female reproductive tract.The osmolality and pH of HF bulls seminal plasma were 292 ± 2.72 mOsm/kg and 6.75 ± 0.05, respectively. In this study, isosmotic modified tris egg yolk extender (mTEY) and modified low-bicarbonate Tyrode (mLBT) media were developed as sperm transport and washing media, respectively for maintaining innate sperm motility.

The results of the study revealed that the sperm motility declined sharply on exposure to osmotic stress. The sudden drop in motility was due to shrinkage of cells in response to hyperosmotic stress as evidenced by significantly lower head area. Subsequent restoration of cell size, motility and velocities (VSL, VCL, and VAP) were appreciated. The velocity acquired on osmoadaptation (2 h) was significantly higher than the control medium and even the same medium before incubation. A sharp increase in sperm motility is a characteristic feature of capacitated sperm. Similarly, a significantly higher percentage of sperm were capacitated in uterine and oviductal osmolality as compared to the isosmotic medium. The oviductal pH also had a capacitating effect on the isosmotic medium. The interaction effect of osmolality and pH was found significant in regulating capacitation.The uterine and oviductal osmolality significantly protected sperm viability both in terms of structural and functional membrane integrity and promoted mitochondrial function.

Further the sperm functional attributes in hyperosmotic media prevailing in the reproductive tract were better correlated with semen quality than isosmotic media used for bull fertility prediction tests. Accordingly, the oviductal osmo-susceptibility index (OVI) was developed and can be used as a convenient and efficient predictor of quality semen production capacity of a bull. The expression levels of the osmo-responsive genes *NFAT5, HSP90AB1, SLC9C1, ADAM1B,* and *GAPDH* were found upregulated in the good osmoadapter group. The expression levels of the genes *COX1, PRM1, ENO1,* and*EFHD1*were downregulated in the good osmoadapter group.

This study revealed the osmotic and pH adaptation ability of sperm and their influence on functional attributes in HF bulls. These findings suggest that the cryptic female choice in the form of osmotic stress and phenotypic plasticity of sperm as a response to the osmotic stress may have immense importance in the regulation of sperm functions and fertilization events.

The elevated blood urea nitrogen level has long been prognosticated as a cause for subfertility in cattle. The present study also aimed to explore the influence of urea on bovine sperm functional attributes *in vitro* by mimicking the *in vivo* physiological as well as toxic levels of urea.

The urea induced structural damage (structural membrane integrity, functional membrane integrity, and acrosome integrity) immediately after exposure and is significantly evident within 1 h followed by functional deterioration (capacitation, motility, and mitochondrial membrane potential) at 2-4 h. As the urea affects the vital sperm functional attributes and mitochondrial functions, either the fertilization failure or early embryonic lethality becomes inevitable. In order to elucidate the mechanism of urea transportation process, the expression levels of urea transporters (*UT-A* and *UT-B*) were assessed in sperm. While transcripts for both transporters were expressed in bovine testis, only *UT-B* transcript was observed in bovine sperm. This finding suggests the presence of urea transporter protein-B in mature bovine sperm which may regulate urea transportation in and out of the cell. However, the physiological functions of urea transporter in testis and sperm on regulating male fertility remain elusive hitherto.

The study concludes that,

1. Bull sperm in relation to motility and cell size adapt well at 355 mOsm/kg
2. Hyperosmolality protects better sperm membrane integrities and mitochondrial membrane potential better than isosmotic (300mosm) and 420 mOsm

3. A positive interaction effect of pH and osmolality was observed in promoting capacitation
4. Urea at higher concentration either in the seminal plasma or incubation medium (0.4347g/L) significantly affect sperm motility, membrane integrity and mitochondrial membrane potential
5. The oviductal osmo-susceptibility index and expression of osmoregulatory genes - *NFAT5*, *HSP90AB1*, *SLC9C1* and *ADAM1B* can be used to identify good quality semen producing bulls

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Mini Abstract

7 MINI ABSTRACT

The sperm are exposed to diverse micro-milieu in the reproductive tract and are prone to more stress than the oocyte. The reproductive tract fluids vary in the osmolality, pH and ionic concentration, which play a vital role in regulating sperm function. However, the influence of osmolality and pH on the adaptive ability and functional competence of the bovine sperm are scanty. The sperm were subjected to 300, 355 and 420 mOsm media with two different pH, 6.8 and 7.4. The sperm motility, velocities including VSL, VCL and VAP, and head area were significantly affected upon exposure to physiological hyperosmotic stress. The magnitude and duration of recovery due to osmoadaptation ability differed among bulls. The velocity regained upon osmoadaptation (2h) was significantly higher than the control medium. Similarly, the percentages of sperm capacitated in uterine and oviductal hyperosmolality were significantly higher as compared to the isosmotic medium.The osmolality and pH interaction was significant in regulating capacitation. The hyperosmolality significantly protected sperm structural and functional membrane integrities and mitochondrial function.The oviductal osmo-susceptibility index (OVI) developed in the study was efficient in predicting the quality semen production capacity of a bull, however this needs to be validated in large numbers of animals.The expression levels of osmo-responsive genes, *ADAM1B, NFAT5* and *SLC9C1* were higher in sperm with good osmo-adaptation ability.The urea induced structural damage was observed within 1 h followed by functional deterioration in motility, and mitochondrial membrane potential at 2-4 h of exposure. These effects may be possible through urea transporter, as *UT-B* expression was observed in the sperm. This study revealed that the osmotic and pH adaptation ability varies among the semen samples. The oviductal osmo-susceptibility index can be used to predict quality semen production capacity of a bull. The urea mediated deterioration of sperm functional attributes and mitochondrial functions may lead to infertility either by affecting fertilization process or early embryonic survivability.

लघु सारांश

8 लघु सारांश

प्रजनन पथ में शुक्राणु विभिन्न सूक्ष्मपरिवेश के संपर्क में आता है और डिम्बाणु की तुलना में अधिकत तनाव प्रवृत्ति होता है। प्रजनन पथ के तरल पदार्थ परासरणीयता, अम्लता और आयनिक एकाग्रता में भिन्न होते हैं जो शुक्राणु समारोह को विनियमित करने में महत्वपूर्ण भूमिका निभाते हैं। हालांकि, गोजातीय शुक्राणु की अनुकूल क्षमता और कार्यात्मक क्षमता पर परासरणीयता एवं अम्लताका प्रभाव अल्प है। शुक्राणु को दो अलग 6.8 और 7.4 अम्लता सहित 300, 355 और 420 mOsm माध्यमों के अधीन किया गया। अधिक परासरणीयता तनाव के संपर्क में आने से शुक्राणु की गतिशीलता, वीएसएल, वीसीएल एवं बीएपी सहित वेग और सिर क्षेत्र काफी प्रभावित हुए थे। ऑस्मोएडेप्टेशन क्षमता के कारण बैलो बीच पुनप्राप्ति की तीव्रता और अवधि भिन्न थी। आरुस्मोएडेप्टेशन (2h) के कारण प्राप्त वेग नियंत्रण माध्यम से काफी अधिक था। इसी तरह, समान परासरणीयता माध्यम की तुलना में गर्भाशय और डिंबवाहिनी की अधिक परासरणीयता में शुक्राणु के कापासिटेशन प्रतिशत काफी अधिक थे। परासरणीयता और अम्लता के प्रभाव कापासिटेशन को विनियमित करने में महत्वपूर्ण था। अधिक परासरणीयता ने शुक्राणु की संरचनात्मक और कार्यात्मक झिल्ली एकीकरण एवं माइटोकॉन्ड्रिय के कार्य को काफी संरक्षित किया। अध्ययन में विकसित डिंबवाहिनी ऑस्मो–संवेदनशीलता सूचकांक (ओवीआई) बैल की गुणवत्ता वीर्य उत्पादन क्षमता की अनुमान करने में कुशल था हालांकि इसे बड़ी संख्या की पशुओं में पुष्टि करने की आवश्यकता है। ओस्मो–उत्तरदायी जीन *ADAM1B*, *NFAT5* तथा *SLC9C1* की अभिव्यक्ति का स्तर, अच्छे ओस्मो–अनुकूलन क्षमता वाले शुक्राणु में अधिक थे। एक घण्टे के भीतर यूरिया प्रेरित संरचनात्मक क्षति देखी गई, जिसके बाद 2–4 घण्टे में गतिशीलता और माइटोकॉन्ड्रिय झिल्ली क्षमता में गिरावट पाई गई। ये प्रभाव यूरिया परिवाहक के माध्यम से संभव हो सकते हैं, क्योंकि शुक्राणु में *UT-B* की अभिव्यक्ति देखी गई थी। इस अध्ययन से पता चला है कि वीर्य के नमूनों में परासरणी और अम्लता अनुकूलन क्षमता भिन्न भिन्न होती हैं। डिंबवाहिनी ओस्मो– संवेदनशीलता सूचकांक का उपयोग बैल की गुणवत्ता वीर्य उत्पादन क्षमता का अनुमान लगाने के लिए किया जा सकता है। यूरिया माध्यम से शुक्राणु कार्यात्मक विशेषताओं और माइटोकॉन्ड्रिय कार्यशक्ति की क्षती गर्भाधान प्रक्रिया या प्रारंभिक भ्रूण उत्तरजीविता को प्रभावित करने से बांझपन का कारण बन सकती है।

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Appendix

APPENDIX-I

Preparation of sperm transport and washing media:

a) Tris egg yolk extender

A measured amount of tris, citric acid and fructose were added one after the other to 500 mL of ultra-pure water in a beaker and mixed thoroughly. Then the volume was made up to 1 L. The prepared solution was sterilized by autoclaving at 121°C for 15 min at 15 psi. Antibiotics Benzyl penicillin and streptomycin were added to the media at the concentration of 1000 IU/mL and 1mg/ mL, respectively and stored at 4°C.

Preparation of egg yolk

On the day of sample collection, a fresh chicken egg was sterilized by wiping the egg shell with 70 % ethyl alcohol. The albumin was drained out without disturbing the egg yolk. After draining the albumin, the egg yolk was transferred to a clean sterile filtered paper. The egg yolk was gently rotated 2-3 times to remove the remaining albumin. Then the yolk was broken carefully with a sterile needle and transferred to a measuring cylinder. The egg yolk (20 % ; V/V)was added to the tris extender and stirred in a magnetic stirrer for uniform dispersion of egg yolk.

Composition: Chemical	Catalog no & source	Concentration (g/L)	Concentration (mM)
Tris	GRM262, Himedia	30.28	250
Citrate	C2404, Sigma	17	88.5
Fructose	RM1355, Himedia	12.5	69.38
Egg yolk	Fresh chicken egg	20 % (V/V)	
Benzyl penicillin	15070063,ThermoFisher Scientific	1000 IU/ mL	
Streptomycin	15070063,ThermoFisher Scientific	1 mg / mL	

Expected osmolality – 407 mOsm/kg ; Measured osmolality – 329 mOsm/kg

b) Tyrode balanced salt solution

The following ingredients were added except sodium bicarbonate to 900 mL of ultra-pure water and mixed thoroughly before adding sodium bicarbonate. Then,1 gm of sodium bicarbonate was added and mixed well. Finally, the volume was made up to 950mL. The pH of the prepared solution was checked (pH 8.2) and was adjusted to the desired pH (6.8) with 1N HCL. The final volume was made up to one liter. The osmolality was checked. The solution was filtered through 0.22 μm syringe filter and stored in a sterile container at 4°C.

Composition: Chemical	Catalog no & source	Concentration (g/L)	Concentration (mM)
Calcium chloride dihydrate	MB034, Himedia	265	1.80
Magnesium chloride	RM1281,Himedia	49.9	0.241
Potassium chloride	P5405, Sigma	200	2.68
Sodium chloride	S5886, Sigma	8000	136.89
Sodium hydrogen phosphate	106349, Merck	50	0.36
D-glucose	MB037, Himedia	1000	5.55
Sodium bicarbonate	S5761,Sigma	1000	11.9

Expected osmolality – 314 mOsm/kg; Measured osmolality – 294 mOsm/kg

c) Modified low bicarbonate Tyrode solution (mLBT)

The following ingredients were added to 900 mL of Ultrapure water and mixed thoroughly before adding sodium bicarbonate. Then, 168 mg of sodium bicarbonate (2 mM) was added and mixed, the volume was made upto 950 mL and the pH of the prepared solution was adjusted to 6.75. The final volume was made up to 1L. The osmolality was checked and the solution was filtered through 0.22 μm syringe filter and stored in a sterile container at 4°C.

Composition: Chemical	Catalog no & source	Concentration (g/L)	Concentration (mM)
Calcium chloride dihydrate	MB034, Himedia	265	1.80
Magnesium chloride	RM1281,Himedia	49.9	0.241
Potassium chloride	P5405, Sigma	200	2.68
Sodium chloride	S5886, Sigma	8000	136.89
Sodium hydrogen phosphate	106349, Merck	50	0.36
D-glucose	MB037, Himedia	1000	5.55
Sodium bicarbonate	S5761,Sigma	168	2

Expected osmolality – 304 mOsm/kg; Measured osmolality – 288 mOsm/kg

d) Modified tris egg yolk extender (75%)

As the composition of the tris egg yolk extender was hyperosmotic, the composition was altered to make it isosmotic.

Altered Composition:

Composition: Chemical	**Catalog no & source**	**Concentration (g/L)**	**Concentration (mM)**
Tris	GRM262,Himedia	23.145	191.05
Citrate	C2404, Sigma	12.56	65.375
Fructose	RM1355,Himedia	9.375	52.03
Egg yolk	Fresh chicken egg	20 % (V/V)	
Benzyl penicillin	15070063,ThermoFisher Scientific	1000 IU/ mL	
Streptomycin	15070063,ThermoFisher Scientific	1 mg / mL	

Expected osmolality – 308 mOsm/kg; Measured osmolality – 246, 249 mOsm/kg

The observed osmolality was lesser than the expected osmolality every time and so the tris extender was hypothesized to be non-ideal solution. In ideal solutions, the solute molecules dissociate in predicted fashion and solute particles do not re-associate with each other once dissolved. The difference between the ideal and non-ideal (real) solution is that the true measured osmolality of the solution differs from that of the ideal predicted osmolality. In real solutions, there is a statistical probability that some cations and anions of dissolved electrolytes re-associate.

Osmotic coefficient of tris extender, ô = True osmolality/ Predicted osmolality

= 308/246 = 1.25

In order to find the interaction between the components of tris solution, the following experiment was carried out.

Association between various components of tris extender

SI no	Component	Concentration (g/L)	Concentration (mM)	Expected Osmolality (mOsm)	Measured Osmolality (mOsm)
1	Tris	30.86	250	250	254
2	Fructose	12.5	69.38	69.38	72
3	Citrate	16.75	88.5	88.5	91
4	Tris	30.86	250	319.38	323
	Fructose	12.5	69.38		
5	Tris	30.86	250	**338.5**	**262**
	Citrate	16.75	88.5		
6	Fructose	12.5	69.38	157.88	164
	Citrate	16.75		88.5	

It was found that tris and citrate were undergoing association and the osmotic coefficient was found to be 1.29. Considering the osmotic co-efficient the composition was altered to make the solution isosmotic and the following composition was selected.

e) Modified Tris egg yolk extender (87%)

Composition

Ingredient	Source & Catalog no	Concentration (g/L)	Concentration (mM)
Tris	GRM262, Himedia	26.84	221.56
Citrate	C2404, Sigma	14.57	75.84
Fructose	RM1355, Himedia	10.875	60.36
Egg yolk	Fresh chicken egg	20 % (V/V)	
Benzyl penicillin	15070063,ThermoFisher Scientific	1000 IU/ mL	
Streptomycin	15070063,ThermoFisher Scientific	1 mg / mL	

Calculated osmolality –357 mOsm/kg

Expected osmolality –296 mOsm/kg

Measured osmolality – 286 mOsm/kg

APPENDIX-II

Preparation of reagents used in assays

Phosphate buffered saline

Sodium chloride	8 g
Potassium chloride	0.2 g
Sodium hydrogen phosphate	1.44 g
Potassium hydrogen phosphate	0.24 g

The above components were mixed well in 800 mL of double distilled water, adjusted pH to 7.4 and final volume made to 1 L. The solution was autoclaved (20 min for121 °C) and stored at 4 °C.

Structural membrane integrity

1. 3% Sodium citrate buffer : 3 g Sodium citrate buffer in 100mL of double distilled water
2. 5% Eosin stain: 5 g of Eosin (water soluble) in 100mL of sodium citrate buffer.
3. 10% Nigrosin stain: 10 g of Nigrosin (water soluble) in 100mL of sodium citrate buffer.

   Equal volume of Eosin and Nigrosin were mixed together and used freshly.

Functional membrane integrity

1. 300 mOsm media

Tri Sodium Citrate	14.7 g
Fructose	27 g

   Distilled water to make up to 1000 mL

2. 150 mOsm media

Tri Sodium Citrate	7.3 g
Fructose	13.5 g

   Distilled water to make up to 1000 mL

3. Bufferd formal saline (BFS)

Di Sodium Hydrogen phosphate	6.194g (43mM)
Potassium Dihydrogen phosphate	2.54g (18.37mM)
Sodium chloride	5.40g (92.50mM)
Formalin (35%)	125mL

   Distilled water to make up to -1000 mL

4. 0.3% Glutaraldehyde

   3 mL of 25% Glutaraldehyde in 100mL of distilled water

5. Giemsa stain

Stock preparation: 1g of Giemsa in 60 mL of Glycerol + 66mL of absolute methanol. About 1g of Giemsa (water soluble) was weighed and ground thoroughly by adding glycerol step by step up to 60 mL using mortar and pestle. The prepared solution was stored over night. Then 66mL of methanol was added and stirred in a stirrer over night in the amber bottle. The stain was then stored in dark at 4 °C for curing. The stain was mixed thoroughly everyday before use.

Working solution:

Stock Giemsa stain	4 mL
BFS	6 mL
Distilled water	90 mL

Giemsa working solution was prepared freshly as and when required.

Chromatin distribution

1. 10% Neutral buffered formalin solution

Sodium phosphate Monobasic	4 g
Di Sodium phosphate Dibasic	6.5 g
Formaldehyde (37%)	100 mL
Distilled water	900 mL

2. Sulpfite water (prepare freshly)

10% (w/v) potassium metabisulfite	5mL
1N Hydrochloric acid	5mL
Distilled water	90 mL

3. 10% potassium metabisulfite 10g in 100 mL distilled water
4. 1N HCl – 8.2 mL of stock solution was slowly added to 25 mL of distilled water and the final volume was made up to 100mL.
5. 5N HCl - 41.059 mL of concentrated HCl make the volume to 100mL.

Acrosome reaction

1. FITC- PSA stain (stock) : 1mL of PBS (pH 7.4) was added to 1mg of powder.
2. PI Stock: 1mL of PBS (pH 7.4) was added to 5 mg of powder.
3. FITC-PSA-PI Working standard:

FITC - PSA stock	10 µL
PI Stock	2 µL

PBS (pH 7.4) = 1988 µL to make up the volume to 2mL

Capacitation status

1. 1 N NaOH : 4 g of sodium hydroxide was dissolved in 100 mL
2. Chlortetracycline stock : 3.864 mg of Chlortetracycline (Sigma, USA) was dissolved in 2 mL of 1 N NaOH solution and stored in dark at – 20 °C.
3. Tris HCl buffer :

Tris HCl	24.22 mg

NaCl	75.97 mg
Cysteine	6.06 mg

The above components were dissolved in 8 mL of distilled water. The pH was then adjusted to 7.8 with 1 N NaOH

4. Chlortetracycline working solution :

   20 µL of Chlortetracycline stock solution was mixed in 80 µL with TRIS HCL buffer by vortexing in amber tube. The working solution was prepared freshly

Mitochondrial membrane potential

1. JC stock : 1 mL of DMSO was added to 5mg of JC 1 powder.
2. JC working solution: 10 µL of JC stock added to 90 µL of PBS (pH 7.4). JC working solution was prepared freshly and kept in dark at – 20 °C until use. 1 µL of the working solution in 50 µL of PBS with 2 million sperm.

Agarose gel electrophoresis

1. TBE buffer

Tris	108 g
Boric acid	55 g
1 M EDTA	20 mL

The above components were dissolved in 1 L of double distilled water, and pH was adjusted to 8 with 1 N NaOH.

VITAE

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Educational qualifications

Degree	**University/ College**	**Year**	**O.G.P.A.**
B.V.Sc. & A.H.	VCRI,TNI (TANUVAS)	2017	8.71
M.V.Sc (VGO)	ICAR-IVRI	2020	8.58

Awards and recognition

- Rao Bahadur Dr.R. SwaminathaAyyar Medal (2017)
- Thiru R. Nachimuthu Memorial Award (2017)
- DHIA Prof. M.E. Doraiswamy Endowment Award (2017)
- AranganurThiru A. Rajamanickam, Teacher Endowment Medal, (2017)
- Rao Sahib Dr. T. Vinayaka Mudaliar Medal, (2017)
- TANUVAS VICE CHANCELLOR AWARD for overall best presentation at TANUVAS 9th CLINICAL CASE CONFERENCE
- Certificate of merit for undergoing externship training in veterinary clinical medicine and community medicine at University of Georgia, USA
- First prize under Farm animal reproduction in post graduate category at TANUVAS 11th CLINICAL CASE CONFERENCE
- S.N. Luktuke award for the best extempore presentation, ISSAR (2019)