EFFECT OF DIFFERENT SEMEN EXTENDERS ON FERTILITY OF CHICKEN SPERMATOZOA DURING SHORT TERM PRESERVATION

Thesis

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IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

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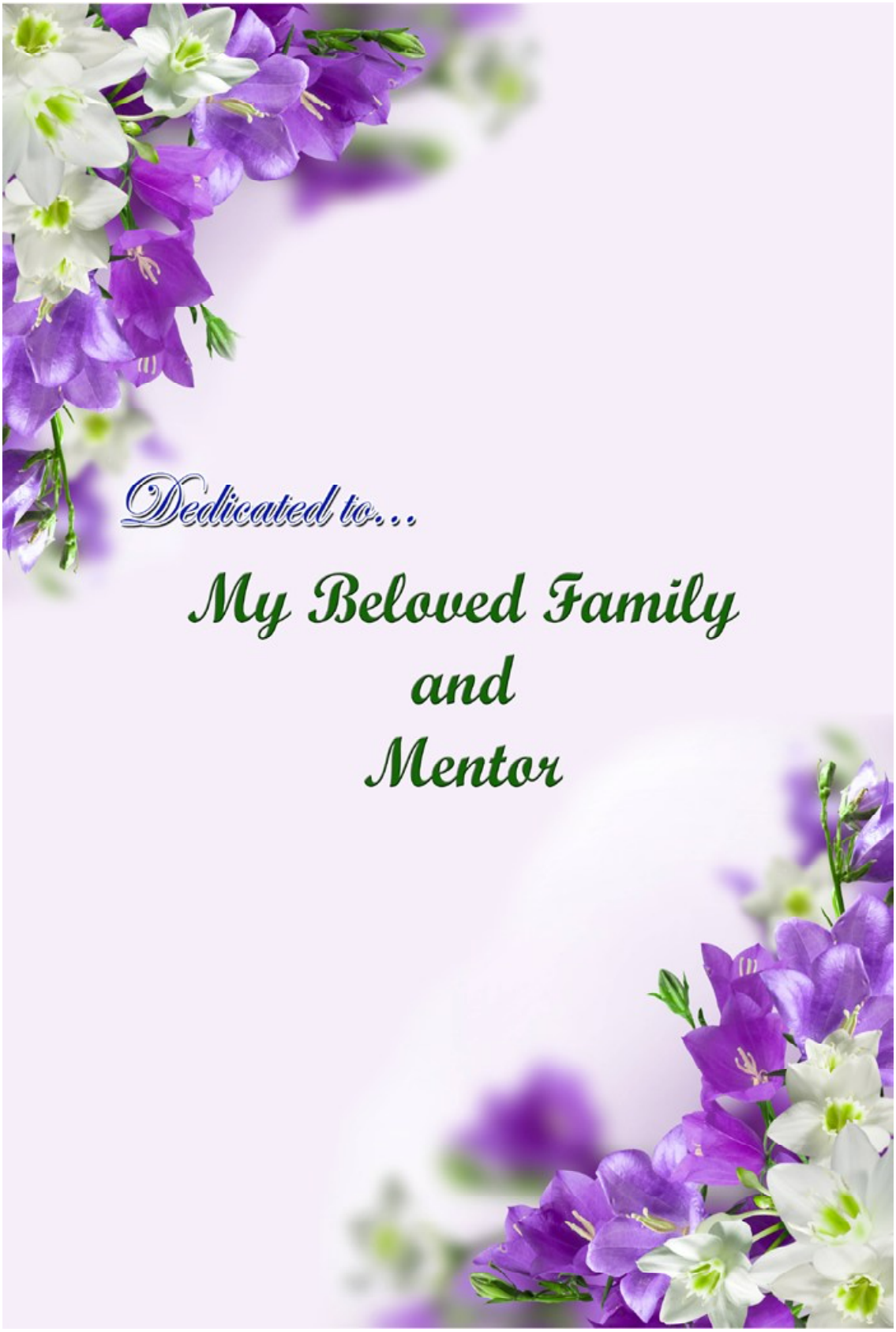
Dedicated to...
My Beloved Family
and
Mentor

भा.कृ.अनु.प.–केन्द्रीय पक्षी अनुसंधान संस्थान
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Ph.D.
Principal Scientist & Head

Dated: 24.06, **2017**

Certificate

Certified that the research work embodied in this thesis entitled **"Effect of different semen extenders on fertility of chicken spermatozoa during short term preservation"** *submitted by* **Dr. Beulah Pearlin, V., Roll No. M-5602,** *for the award of* **Master of Veterinary Science** *degree in* **Poultry Science** *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. Beulah Pearlin, V., Roll No. M-5602,** *has worked for more than 21 months in this Institute and has put in more than 150 days attendance under me from the date of registration for the degree of* **Master of Veterinary Science** *of the Deemed University, ICAR-Indian Veterinary Research Institute as required under the relevant ordinance.*

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Certificate

We the undersigned members of Advisory Committee of **Dr. Beulah Pearlin, V., Roll No. M-5602,** *a candidate for the degree of* **Master of Veterinary Science** *with the major discipline in* **Poultry Science,** *agree that the thesis entitled* **"Effect of different semen extenders on fertility of chicken spermatozoa during short term preservation"** *may be submitted in partial fulfillment 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, ICAR-Indian Veterinary Research Institute, Izatnagar.*

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U. Beulah Pearl

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(Beulah Pearlin .V)

ABBREVIATIONS

%	:	Per cent
µl	:	Microlitre(s)
°C	:	Degree Centigrade
ACP	:	Acid Phosphatase
AI	:	Artificial insemination
ALP	:	Alkaline Phosphatase
ALT	:	Alanine aminotranferase
AST	:	Aspartate aminotransferase
ATP	:	Adenosine triphosphate
ANOVA	:	Analysis of variance
BPSE	:	Beltsville poultry semen extender
CARI	:	Central Avian Research Institute
CHOD	:	Cholesterol oxidase
cm	:	Centimetre(s)
dl	:	decilitre(s)
et al.	:	et alli and others
gm	:	gram(s)
GDP	:	Gross domestic product
GOT	:	Glutamic Oxaloacetic Transaminase
GPO	:	Glycerol-3-phosphate oxidase
GPT	:	Glutamic Pyruvate Transaminase
Hr(s)	:	Hour(s)
IU	:	International Unit
Kg	:	Kilogram(s)
mg	:	milligram(s)
min	:	Minute(s)
ml	:	millilitre(s)
mm	:	millimetre(s)
Mm	:	millimolar
MOsmol	:	Milliosmole
$Na_3C_6H_5O_7$	:	Sodium citrate
NaCl	:	Sodium chloride
nm	:	nanometer(s)
OCPC	:	O- Cresolphthalein method
OD	:	Optical Density
PAP	:	Phenol+Aminophenazone

rpm	:	Revolution per minute
RRT	:	Resazurin Reduction Test
SE	:	Standard Error
SST	:	Sperm storage tubules
Std	:	Standard
U	:	Unit
viz.	:	namely
vs.	:	Versus
WLH	:	White Leghorn

LIST OF TABLES

LIST OF FIGURES

CONTENTS

Introduction

1 INTRODUCTION

The Poultry production in India has taken a quantum leap in the last four decades emerging from an unscientific farming practice to a strong agro-based commercial activity as a result of scientific and technological revolution. Currently the total poultry population in our country is 729.2 million numbers with the egg and meat production of around 78.48 billion and 3.05 million tonnes respectively. It accounts for about 1% of India's GDP and 11.70% of the GDP from the livestock sector (DADF Annual report, 2015-2016; Sasidhar and Suvedi, 2015). Though, India has made rapid strides in the poultry production, the current per capita availability of egg and meat per person per year is only 63 eggs and 3 kg whereas the nutritional advisory committee of ICMR recommends 180 eggs and 11 kg meat per person annually (DADF Annual report, 2015-2016; ICRA, 2016). This indicates a wide gap between demand and supply which need the improvement in poultry production to abridge this gap. At this juncture, suitable strategies that can enhance the reproductive efficiency of poultry birds like Artificial Insemination (AI) should be given the due importance to increase the poultry production.

It has been well established that AI in avian species expresses better fertility than natural mating (Saeki and Nagomi, 1964; Mohan *et al*., 2016). The advantages of AI increased the overall fertility and hatchability resulting in the reduction of cost of production per unit of day old chicks (Brillard, 2003). Artificial Insemination has been a critical component of reproduction in turkeys since the 1960s, and is used almost exclusively for commercial flock production. The differences in the size of the toms (large white strains approximately 33 kg) and hens (approximately 9 kg at the onset of lay) resulting in unsuccessful mating and consequent low fertility of the heavy broad-breasted strains after natural mating has forced the adoption of

AI in commercial poultry production (Lake, 1983; Donoghue and Wishart, 2000). For improvement of poultry production, the technique of AI is also getting momentum in other poultry species (Reddy, 1995).

For the success of AI in poultry, examination of the semen characteristics of different breeds of poultry (Haunshi *et al*., 2010; Mohan *et al*., 2011) and its preservation (Lake, 1960; Siudzinska and Lukaszewicz, 2008; Mohan *et al*., 2015) is the need of the day. Evaluation of physical and biochemical characteristics of poultry semen reflects the reproductive potential of cock and has been reported to be a major determinant of fertility and subsequent hatchability of eggs (Zahraddeen *et al*., 2005; Peters *et al*., 2004). Such types of studies are lacking in indigenous chicken breeds such as Nicobari and Ankleshwar. Nicobari fowl is an endangered and endemic breed of poultry of Andaman and Nicobar Islands and produces highest number of eggs among all the indigenous chicken breeds of India (Ahlawat and Chatterjee, 2002). Ankleshwar has derived its name from the area 'Ankleshwar' of Bharuch district of Gujarat and are being kept mainly by tribes under backyard poultry farming as a livelihood source of income. These birds are maintained without vaccination and medication, and have reasonable feed efficiency and excellent fertility (GAU Report, 2003). In spite of its unique characteristics, there is a lack of concern for the conservation and improvement of this breed under field conditions (Pandey *et al*., 2005). However, there is worldwide recognition of the need for the conservation of livestock diversity (FAO, 1995) and for characterization of breeds and populations. Hence, findings on physical and biochemical characteristics of semen of these indigenous breeds will be helpful in future research and breeding strategies for the conservation and improvement of native breeds.

In order for the poultry industry to take advantage of modern AI techniques, proper storage of poultry semen is necessary. To achieve better fertility by AI, fresh semen should be inseminated within 15-30 min after collection (Lake and Stewart, 1978). Within this short period it is difficult to inseminate the large number of birds. Further, the semen of good quality from valuable sires cannot be transported from one place to other. Therefore, these circumstances enforce the researchers to enhance the storage period of avian semen under *in vitro* conditions (Lake, 1960; Sexton and Fewlass, 1978; Tselutin *et al*., 1995; Lukaszewicz,

2002; Mohan *et al*., 2013). Besides, poultry semen is highly viscous and concentrated with low volume, containing 6 (chicken) to 12 (turkey) billion spermatozoa/ ml (Donoghue and Wishart, 2000). Hence, the extension of neat semen with proper diluents is required to utilize the low volume of semen from proven sires for inseminating large number of hens.

In past, several attempts have been made to develop the suitable diluents for poultry semen storage. The Central Avian Research Institute, a premier institute of poultry research in India has also developed 'CARI poultry semen diluent' (Mohan *et al*., 2017) for preservation of poultry semen for about 24 hr at low temperature (8^{o}C). Hence, comparing the effects of different semen extenders on fertility under similar conditions will be of great use in deriving the best semen extender for chicken. Semen can be extended by using high dilution rates or inseminating the lowest number of spermatozoa. Hence, in order to achieve the maximum gain through AI, knowledge of the semen quality, proper diluents and sperm dilution rates are vital (Purdy, 2006).

Based on the above discussion, the present study has been conducted assess the "Effect of different semen extenders on fertility of chicken spermatozoa during short term preservation" in order to derive the best semen extender with the following objectives:

Objectives

1. **To investigate the semen physical and biochemical characteristics of White Leghorn and indigenous (Nicobari and Ankleshwar) chicken.**
2. **To study the effect of different semen extenders on fertilizing ability of chicken spermatozoa during short term preservation.**
3. **To find the effect of different dilution rates on fertility using fresh semen.**

Review of Literature

2 REVIEW OF LITERATURE

Artificial insemination (AI) has received significant importance in the developing poultry industry to increase the commercial production. Among the great biotechnologies, AI holds upper hand for improving the reproductive efficiency of poultry and has got an enormous impact worldwide. The technique has several advantages and a few limitations as well (Lake, 1983; Mohan *et al*., 2011; Dhama *et al*., 2014). Even when under natural mating 80-85% of eggs are fertile, fertility can be increased by another 5-10% simply by adding AI to the propagation program (Gee *et al*., 2004). This technique avoids the spread of venereal diseases by natural mating and increase the dissemination of genetic material to a large number of birds. AI has the advantage that one cockerel can be used to inseminate 20 to 30 hens; while in natural mating one cockerel only services 8 to 10 hens per day (Mosenene, 2009). Besides, the unsuccessful mating and consequent low fertility of the heavy broad-breasted strains of turkey has resulted in the complete integration of AI in commercial production. This has stimulated a substantial scientific research effort in the chicken industry (Islam *et al.,* 2002).

In brief, Artificial Insemination in poultry is the process of collecting semen, evaluation of the semen, extending it with appropriate extenders, for either short (24 h or less) or long-term preservation and then manually placing the semen into the vagina of female (Mosenene, 2009).

2.1. Collection of semen

Semen collection is the first critical stage of AI and successful collection results in high quality semen being obtained with the maximum number of sperm being collected per ejaculation

(Mosenene, 2009). The first reported method of semen collection in birds was in 1913 by Ivanov, who recovered semen post-mortem from the vas deferens of roosters. Avian semen has also been recovered from the cloaca of the female post-coitus (Penquite *et al*., 1930; Jull and Quinn, 1931) and using collection devices attached to either male or female (Adamstone and Card, 1934; Parker *et al*., 1940). Other techniques include use of an artificial vagina (Tan, 1980) and electroejaculation (Nishiyama *et al*., 1976).

Burrows and Quinn (1937) were the two pioneers who developed a widely accepted and non-invasive abdominal massage method for collection of semen from roosters. The technique involves restraining the male and gently stroking the back of the bird from behind the wings towards the tail with firm rapid strokes. The male responds with tumescence erection of the phallus, at which time the handler gently squeezes the cloaca extracting semen through the external papillae of the ductus deferens (vas deferens) collecting the semen into a container. This method needs the assistance of one or two people. Cockerels need to be trained before semen can be collected for use in AI. Care should be taken to avoid any contamination of semen with the cloacal products and particularly with the transparent fluid (Blesbois and de Reviers, 1992).

2.2 Semen evaluation

The assessment of semen quality characteristics of poultry birds gives an excellent indicator of their reproductive potential and has been reported to be a major determinant of fertility and subsequent hatchability of eggs (Peters *et al*., 2004). Hence it would be beneficial to the commercial breeder if a rooster's semen could be evaluated before its use in the flock in order to select those males whose semen would improve fertility (Wilson *et al*., 1979). The semen characteristics of avian species are different to a greater degree from that of mammals owing to the diverse physiology and anatomy like the intra-abdominal placement of testes and absence of accessory sex glands. Moreover, the sperms of chicken are very fragile to high environmental temperature (Etches, 1996). Various workers have reported breed to breed variation in physical and biochemical characteristics of semen quality, storage of the semen and fertilizing ability of the spermatozoa (Gee, 1995; Mohan *et al*., 2011). Because of such inter

and intra-male variation in ejaculate quality, semen samples should be evaluated before processing for storage and AI.

The traditional semen evaluation procedures include determination of various characteristics such as semen volume, colour, concentration, motility, viability and morphology of spermatozoa (Getachew, 2016). Many of these assessments correlated with the fertilizing capacity of spermatozoa when fresh semen was evaluated (Wishart, 1995). Presently, semen quality assessment consists of two important steps such as physical and biochemical evaluation of semen.

2.2.1. Physical evaluation of semen

2.2.1.1 Semen colour

The colour of semen is generally an indicator of the density of the ejaculate. The semen of the domestic fowl varies from an opaque suspension of high sperm density or degrees of clear to milky white, with declining sperm numbers (Peters *et al*., 2008). Colour could also serve as an indicator of contamination by faeces or urine and thus become brown or green in colour (Lake, 1983). Sometimes flakes of blood may be present, which may be a result of excessive force during the collection process or injury. The colour of semen may depend on the species of bird used, but generally semen should be creamy which indicates a high sperm concentration (Cole and Cupps, 1977). The age of the roosters may be taken as the contributing factor for a considerable number of roosters yielding semen of inferior colour and consistency (Churchil *et al*., 2014).

2.2.1.2. Semen volume

Ejaculate volume is the total amount of semen produced and is quick to measure and provides information necessary to calculate dilution dose when performing an AI (Etches, 1996). The cockerel produces between 0.1 ml and 1.5 ml per ejaculation, with 0.6 ml being the average ejaculate volume recorded (Cole and Cupps, 1977). The average volume ejaculated using the abdominal massage technique is approximately 0.2 ml and contains on average $5000x10^6$ sperm/ml (Gordon, 2005). It is important to realize that semen volume and sperm concentration will determine the total number of sperm collected per ejaculation. This could

facilitate the determination of the number of insemination doses that can be prepared (Senger, 2003). Majority of the reports indicate that there is no significant relation between volume and fertility (Cooper and Rowell, 1958; Boone and Huston, 1963). However, Kamar (1960) reported positive relationship between volume and fertility. Anash *et al*. (1983) reported no difference in semen volume produced by males maintained either in cages or on the floor, but Carson *et al*. (1955) and Hafez (1995) observed that the ejaculates were significantly larger from males maintained in cages than from those in flocks. Different cockerels of the same species often produce different volumes of semen at different times (Anderson, 2001). In white leghorn chicken, Kundu and Panda (1990) and Elagib *et al*. (2012) reported lesser semen volume in summer season. Hence, the quantity of semen collected by the massage procedure is also dependent upon the breed, age, nutrition, season, frequency and technique of semen collection (Mosenene, 2009).

2.2.1.3. Sperm motility

The most widely used test of sperm quality from the initial stages of AI development until the present time has been the assessment of the proportion of normal, progressively moving sperm (Wheeler and Andrews, 1943; Maule, 1962; Salisbury *et al*., 1978). Sperm motility assessment is indicative of the viability of sperm and the quality of the semen sample. Evaluation of sperm motility is conducted with fresh and extended semen, and generally analyzed under the light microscope (x10 magnification). Sperm motility with appearance score is an important criteria to evaluate the breeding potential of males. Sperm motility has been reported to be significantly correlated with fertility of several investigations (Boone, 1968; Anash *et al*., 1980; Donoghue *et al*., 1998). Several authors have also found reduction in mass motility in hot seasons of the year (Boone and Huston, 1963; Chung *et al*., 1989; Elagib *et al*., 2012).

2.2.1.4. Sperm concentration

Sperm cell concentration is defined as the number of cells per ml ejaculate and normally predicts the number of breeding units that can be inseminated (Cole and Cupps, 1977). The density of the semen samples in the cockerel ranges from less than $800x10^3$ to over $6x10^6$ sperm/ml (Anderson, 2001). Orunmuyi *et al*. (2012) showed that concentration was within

the range of 3.40-9.70 billion/cc while Moya *et al*. (1996) obtained lesser than 4.3 billion sperm/ml in broiler cocks. It is therefore important to record the concentration of each ejaculate of the male. Kamar (1960) and De silva (1963) reported that sperm concentration is related to fertility. The ejaculates containing less than 500 million cells per ml have been associated with low fertility rates (Bearden *et al*., 2004). Seasonal variations in sperm concentration were also encountered in various chicken breeds with decline in summer season (Boone and Huston, 1963, Saeid and Al-Soudi, 1975). Hartmann and Gleichauf (1975) reported that in White Leghorn chicken, body weight had significant effect on ejaculate volume and sperm concentration with cocks from heavy lines having the greatest ejaculate volume and lowest sperm concentration.

2.2.1.5. Sperm viability

Sperm viability is a measure of the proportion of live, dead, and dying sperm cells from a semen sample (Etches, 1996). 'Viable' sperm simply implies that such sperm possess an intact plasmalemma and are assumed to be functional which is frequently determined using either a dead-cell or a live-cell stain alone or simultaneously (Bakst and Dymond, 2013; Lake and Stewart, 1978). If inseminated with poor quality semen, it reduces fertility, increases embryo mortality and forces the hen to rely on spermatozoa from previous inseminations (Thurston, 1995). Wilson *et al*. (1969) reported that fertility was lowest when the number of dead sperm exceeded 10% and there was significant increase in fertility when only 1% dead sperm were observed. Khalifa *et al*. (1983) observed significant correlation between fertility and percent live sperms in Plymouth Rock and Rhode Island Red. Kundu and Panda (1990) and Elagib (2012) found increase in dead sperm counts in summer season.

2.2.1.6. Morphological abnormality

Sperm morphology is the microscopic assessment of individual sperm cells and identifies problems with sperm such as missing acrosomes, bent necks or coiled tails that can render them unviable (Etches, 1996). Blesbois *et al*. (2007) described an eosin-nigrosin stain technique to assess the morphology of cockerel semen. The midpiece of cockerel sperm is considerably longer, compared to other species and this property makes poultry sperm to have more midpiece

bending than other species (Getachew, 2016). According to Kamar (1960) morphological abnormality of sperm are of three types i.e. Primary, Secondary and Tertiary. Primary abnormality is due to failure of spermatogenesis, while secondary abnormality is due to faulty transportation and tertiary abnormalities are those which cause damage to spermatozoa during or after ejaculation at the time of collection and processing (Bah *et al*., 2001). The deviation from normal morphology can be either at the head (absence of head, enlarged head, coiled head, acrosome damage etc.), mid piece (mid piece bending) or at the tail (absence of tail, broken, coiled or twisted tail) (Alkan *et al*., 2001). Only morphologically normal spermatozoa are capable of ascending through the vagina of the hen to the region where the sperm storage tubules are located (Bakst *et al*., 1994). Pytasz and Bzowska (1961) noticed that males had complete sterility when 20% of sperm were abnormal. Kamar (1960) observed that the percentage of different forms of sperm abnormality varied with season, with more occurrence of head abnormalities in winter and more tail deformities in summer. The abnormal sperms, a contributing factor for poor fertility has negative association with all the other characters like volume, appearance score, initial motility, live sperms and sperm count (Churchil *et al*., 2014).

2.2.2. Biochemical evaluation

2.2.2.1. Semen pH

The semen pH varies slightly between different breeds and bird species. The optimum semen pH ranges between 6.8 and 7.4 (Wilcox and Shaffner, 1957). In contrary, Donoghue and Wishart (2000) and Siudzinska and Lukaszewicz (2008), reported several trials that indicated that chicken sperm could tolerate a pH range of 6.0 to 8.0. This variation in semen pH may be due to many factors. The pH, especially that of ejaculated semen is dependent on several secretions involved. Poor quality semen generally contains large amounts of fluid from the accessory glands, which increases the semen pH. The pH of semen is likely to decrease as the time between collection and measurement increases, as the semen collection tubes are narrow in shape causing sperm to break down fructose in the semen to lactic acid under anaerobic conditions (Mosenene, 2009). Semen samples that contain many dead sperm may evolve ammonia, which will also increase the pH (Salisbury *et al.,* 1978).

A change in semen pH generally affects the sperm motility negatively and an increase in pH has been associated with poor buffering capacity. Sperm motility is generally high between

a pH of 7.0 and 7.4 (slightly alkaline) and also increases the fertilizing ability, compared to a pH of 6.4 (acidic), which is not suitable for semen preservation, as it may cause damage to the plasma membrane of the sperm cell (Latif *et al*., 2005).

2.2.2.2. Different enzyme profiles in seminal plasma

The activity of the number of the enzymes in seminal plasma (Wilcox, 1961; Hammond *et al*., 1965) and spermatozoa (Buckland, 1970) has been reported. Phosphatases (ACP and ALP) are involved in hydrolysis of carbohydrates and provide a source of energy for the spermatozoa (Hammond *et al*., 1965). Transaminases (GOT and GPT) are associated with the production of large amounts of glutamate which plays the chief role of anion in semen and is an index of spermatozoa membrane stabilising capacity (Lake and McIndoe, 1959; Yousef *et al*., 2003). Khan *et al*. (2012) observed a negative correlation among Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), ejaculate volume and spermatozoa concentration, while Al- Darraji (2000) found a positive correlation between transaminase activity in seminal plasma and percentage of dead and abnormal spermatozoa.

Hammond *et al*. (1965) reported that both acid and alkaline phosphatase were a group of nonspecific enzymes which hydrolysed many types of phosphatase esters and catalysed the dephosphorylation of ATP. Kumarova (1970) observed that semen with moderate (707 U) and high (800 U) alkaline phosphatase activity had fertilizing abilities of 96.7% and 93.7% respectively whereas the semen with moderate (2075 U) and high (2680 U) acid phosphatase activity had fertilizing abilities of 88.6 and 55.3% repectively.

Mohan *et al*. (2011) observed that irrespective of breed, semen enzyme activity was higher for ACP (Acid Phosphatase) than ALP (Alkaline Phosphatase) and the average activity of both GOT and GPT of the native breeds, Kadaknath and Aseel Peela was higher than in White Leghorn. This difference in transaminase activity between the native fowls and WL may be due to breed differences (Datta *et al*., 1980; Manoharan, 2000). The enzymatic values of GOT were greater than for GPT (Hammond *et al*.,1965; Datta *et al*., 1980; Mohan *et al*., 2011). In this context, it is of interest to note that the concentration of glutamic acid in the seminal plasma of cocks is exceptionally high and the high activity of GOT may be directed primarily towards the formation of this important amino acid Mohan *et al*. (2011).

2.2.2.3. Cholesterol and triglyceride profile

The cholesterol profile reflects the motility of chicken spermatozoa during storage (Mohan *et al*., 2007). Maule (1962) reported that adequate concentrations of cholesterol were needed for sperm protection and preservation. Mohan *et al*. (2011) reported that higher cholesterol concentration in semen might be correlated positively with sperm concentration because of its presence in the sperm membrane. The cholesterol/phospholipid ratio regulates sperm membrane fluidity. Therefore, variation in cholesterol content in the spermatozoa of different breeds may be associated with differences in membrane fluidity, which may explain why the freezability of semen varies from species to species. Biswas (2007) recorded higher mean values of cholesterol in the seminal plasma of White Leghorn than desi fowl and attributed it to the genetic difference between the breeds. Douard *et al*. (2000) observed significant amounts of cholesterol esters and triglycerides in seminal plasma in contrast to spermatozoa in turkey. Cerolini *et al*. (1997[b]) recorded free cholesterol, cholesterol esters and triglycerides in seminal plasma as 16.6, 8.8 and 3.0% of total lipids respectively in layer breeders. The relative content of free cholesterol and tryacylglycerols showed no change in spermatozoa during aging or in relation to fertility (Cerolini *et al*., 1997[a])

2.2.2.4. Total protein concentration

Hess and Thurston (1984) reported that refractive index of semen of turkey was highly correlated with plasma protein concentration. It was suggested that refractive index of semen and semen colour could be used to estimate the quality of semen with a significant correlation of 0.50 between GOT activity and plasma protein concentration and 0.70 between ACP activity and plasma protein. Mohan *et al*. (2011) reported higher seminal plasma protein in Kadaknath and Aseel Peela than White Leghorn. Kundu and Panda (1991) found increase in total protein concentration with increase in dead spermatozoa. Seminal plasma from turkeys with yellow semen syndrome was characterized by elevated protein (7 g/dl) concentration compared to normal semen (1.8 g/dl) (Thurston *et al*., 1982). It has been demonstrated in boars that seminal plasma basic proteins bind to the outer surface of spermatozoa membranes (Moore and Hibbitt, 1976). In addition, membrane-bound basic proteins may increase the permeability of biological membranes (Hibbitt and Benians, 1971).

2.2.2.5. Calcium concentration

The calcium concentration in the seminal plasma of chicken is in the range of 2.1-9.3 mg/dl as reported by several workers (Lake *et al.,* 1958; Lake and El Jack, 1964; Takeda, 1959). While Mann (1964) reported that calcium ions can depress the motility and metabolism of spermatozoa, Aghaei *et al.* (2010) found positive correlation between low/medium sperm motility groups and high sperm motility groups in broiler birds for calcium concentration. Kanyinji and Maeda (2010) remarked improved sperm motility by elevated calcium concentration in the chicken seminal plasma through dietary manipulation. In chicken, calcium restores sperm motility lost at normal avian body temperature of 41°C when added to a medium in which the spermatozoa are suspended (Thomson and Wishart, 1988). In contrast Graham *et al*. (1971) found negative correlation between calcium content in seminal plasma and fertilizing ability of spermatozoa.

2.2.2.6. Uric acid concentration

The uric acid level in seminal plasma and transparent fluid was found to be 7.03 mg% and 14.28 mg% respectively in broiler cocks (Mohan and Moudgal, 1996). Biswas (2007) reported uric acid concentration (mg%) in the seminal plasma of Kadaknath, Aseel peela and White Leghorn chicken as 26.46±0.49, 20.28±0.59 and 11.60±0.36 respectively. The variation in the uric acid concentration in the seminal plasma of different breeds was attributed to the contamination of semen with urates at the time of semen collection. Bell and Freeman (1971) noticed a wide range of (4.0-85.3 mg%) uric acid contamination of semen.

2.3. Semen dilution

Poultry semen is viscous, highly concentrated and is of low volume, containing 6 (chicken) to 12 (turkey) billion spermatozoa/ml (Vasicek *et al*., 2015). Further, after ejaculation a proportion of spermatozoa begins to die and remain it continue until diluted. Hence semen has to get diluted with suitable diluents in order to : increase semen volume with uniform distribution of sperm cells, increase in number of birds inseminated by per unit volume of semen and finally, it prolongs the sperm survival for both short and long term preservation of semen *in vitro* (Dhama *et al*., 2014). Dilution permits maximum utilization of semen from

superior males and can be used to extend semen during periods of low semen production. Thus, the efficiency of spermatozoal cells can be improved which ultimately results in lowering the insemination dose and at the same time maintains optimum fertility.

Lorenz (1964) reported that the fertilizing capacity of undiluted semen was rapidly lost at 1-2°C, but dilution allowed for storage at these temperatures. As a consequence, semen may be stored for considerably longer periods of time than undiluted semen stored under the same conditions. Clarke *et al*. (1984) found that dilution of chicken semen resulted in a significant decrease in the percentage of dead sperm. Hence, extenders are being commercialized to improve the general reproductive efficiency of the cockerels and lower the cost of AI (Bootwalla and Miles, 1992; Mosenene, 2009). Diluents are buffered salt solutions used to extend semen, maintain the viability of spermatozoa *in vitro*, and maximise the number of hens that can be inseminated. Semen diluents are based on the biochemical composition of chicken and turkey seminal plasma (Lake, 1995). Glutamic acid, the most prominent anionic constituent of avian seminal plasma, became a standard component of diluents (Lake and Mc Indoe, 1959).

According to Mann (1964) and Graham (1976) an ideal semen extender should have an optimum osmotic pressure (about 325 mOsmol/kg) and pH (about 7), contain chelators that could sequester heavy metals harmful to spermatozoal cells, bind calcium ions to prevent the acrosomal reaction, and contain appropriate antibiotics to inhibit bacterial growth. The extender should also be able to provide protection from the deleterious effects of cooling, especially under storage conditions. There are many diluents available for poultry semen, both published recipes and commercially available semen diluents such as Lake's, Tyrode, BPSE etc. (Lake, 1960; Van Wambeke, 1967; Sexton, 1977). The CARI poultry semen diluent for short term storage (24 hr) of poultry semen at low temperature has also been developed (Mohan *et al*., 2017).

2.4. Development of semen extenders for short term storage

Short term storage usually refers to holding the diluted semen from a few hours to a few days usually between 0°C and 5°C (Bootwalla, 1987). The storage of semen, even at low temperatures, often results in the fertilising potential of spermatozoa to decline with the passage

of time in a species specific manner. However, the use of liquid semen is still the method of choice for many avian species because the avian sperm have less cytoplasm as well as nuclear contents that badly affect sperm acrosomal integrity during the freeze thawing process (Blesbois *et al*., 2005). Liquid storage of semen is not a new concept since Van Wambeke (1967) showed *in vitro* short-term storage to be possible by maintaining diluted domestic fowl semen for up to 24 hr at 0°C without a major loss of fertility. Clarke *et al*. (1982) revealed that sperm motility was not affected by dilution at storage temperatures of 15°C and 5°C.

The most common practice for short term fowl semen storage requires the suspension of sperm in a suitable extender to maintain the sperm viability, *in vitro*. The development of semen diluents initially began with the use of NaCl (saline) solutions. Now complex diluents containing different osmotic regulators, energy sources and buffers are being used (Bootwalla and Miles, 1992). Lake (1960) became the first to describe a diluent which resembled the rooster's vas deferens fluid closely in respect to positive inorganic ions and glutamate but with added fructose. He reported the possibility of maintaining sufficient spermatozoa *in vitro* to produce satisfactory fertility and obtained up to 64 and 47% after 24 and 48 hr of storage, respectively. At this stage, partial success for the short term semen holding was achieved.

In 1977, Sexton developed a phosphate buffered semen extender, Beltsville Poultry Semen Extender (BPSE). He reported a fertility value of more than 88% when White Leghorn hens were inseminated weekly with 20 million spermatozaol cells suspended in the BPSE and stored at 5°C for 30 minutes. Fertility levels of more than 90% were achieved with diluted semen at the insemination dose of 100 million cells. He further reported that fewer sperm were needed in the insemination dose when diluted semen (1:2) was held at 5°C than at 25°C during a 30 minute storage time.

In 1995, Tselutin reported the fertility of freeze thawed chicken spermatozoa with Tselutin extender held for 20 minutes at the dilution ratio of 1:1 or 1:2 and found the percentage of fertile eggs of about 93% to 94% in White Leghorn hens. In the study of comparison of extenders for liquid storage of Indian Red Jungle Fowl spermatozoa, Rakha *et al*. (2016) found motility of about 75% and 29% at 0 and 24 hr respectively for EK extender. Similarly livability was around 79% and 20% at 0 and 24 hr respectively for EK extender. In the same

study, the motility was around 70% and 35% at 0 and 24 hr respectively and the percentage livability was around 77% and 25% at 0 and 24 hr respectively with Tselutin extender.

Siudzinska and Lukaszewicz (2008) tested the effect of Lake, EK and Tselutin extenders for their suitability to store semen in four chicken breeds. They concluded that in relation to fresh semen, the number of live, morphologically normal spermatozoa was higher in the EK extender than in Lake and Tselutin extenders after 24 hr of storage. The overall mean semen characteristics of chicken such as sperm motility, live sperm count, total hypo-osmotic swollen spermatozoa percentage was also better in EK extender than lake and skim milk extenders in Vanaraja chicken and indigenous chicken of Assam (Das *et al*., 2015) . It was concluded that EK extender could be used successfully for preservation of chicken semen at 4-5°C up to 72 hr.

Shinde *et al.* (2013) conducted a study on diluents such as CARI poultry semen diluent, BPSE, Lake and normal saline in Kadaknath chicken. They observed superior fertility with CARI poultry semen diluent compared to other diluents and concluded that CARI poultry semen diluent is better than other diluents for short term storage of Kadaknath chicken semen at refrigerated temperature. Mohan *et al.* (2015) conducted a study to identify the suitable diluent for short-term preservation of Guinea fowl (pearl variety) semen. It was interpreted that CARI poultry semen diluent expressed higher fertility at all the storage period (0 and 24 hr), than BPSE and Lake's semen diluents.

Factors affecting avian semen storage include dilution rate (Sexton, 1977), storage medium composition and associated osmotic and pH properties (Sexton and Fewlass, 1978; Lake and Wishart, 1984; Blanco *et al*., 2008), storage time and temperature (Clarke *et al*., 1982). Blesbois *et al*. (2005) stated that the interaction and ability of each extender to protect the integrity of sperm were highly specialised and species-specific. Hence species-specific studies are necessary for successful storage and the subsequent facilitation of assisted breeding (Blanco *et al*., 2000; Blesbois and Brillard, 2007).

2.5. Effect of dilution rate

An optimal dilution rate needs to be established when the duration of *in vitro* storage increases to reduce deleterious factors present in the seminal plasma, and also to complement

the natural protective and nutritional elements of seminal plasma (Blesbois and de Reviers, 1992; Blesbois and Brillard, 2007). Over-dilution should however be avoided, since elevated sperm respiration, morphological changes and loss of motility can occur (Clarke *et al*., 1982). Sexton (1981) stated that an insemination dose of 300 million sperm was needed for maximum fertility of semen samples diluted 1:5 and held at 5°C. Thus it appears that dilution rate is crucial for semen samples subsequently stored at low temperatures and that the limits for dilution appear to be extremely narrow.

Khongsen *et al*. (2015), studied the effect of fresh semen dilution rates of Dang cock (Thai native chicken) on fertility in commercial layers and suggested that the ratio of 1:1.5 could be used as appropriate ratio for artificial insemination. This ratio showed fertility of higher than 90% for 12 days. Though levels of semen dilution ranging from 1:1-1:5 have been advocated in various studies, dilution upto 1:10 produce high fertility and hatchability, provided the inseminated dose is 100 $x10^6$ spermatozoa (Bratte and Ibe, 1989).

Several investigators have attempted to determine the minimum number of sperm (20-30 million) required per dose of semen diluted in a variety of extenders (Wishart 1984; Bandyopadhyay, 2006). The results reported for minimum number of sperm ranged from 45 to 90 million sperm by Rowell and Cooper (1960) and Kim *et al.* (1974). Etches (1996) suggested 100 million cells per insemination for high fertility. The range of values could be attributed to certain experimental design factors such as the extender used, dilution rate and the semen holding conditions like time and temperature.

2.6 Fertility

The goal of AI is to produce a succession of fertilized eggs between successive inseminations. A significant feature of the reproductive physiology of the hen is her ability to prolong the sperm survival in oviduct after a single natural mating or AI. Sperm are able to survive in the oviduct for a longer period of time because of the specialized sperm storage tubules (SST) which receive, protect and nourish them. Following deposition in the oviduct, sperm are transported to SST by a combination of their intrinsic motility and cilia beat activity (Froman *et al*., 1997; Bakst, 2011).

After leaving the SST, spermatozoa are transported to the infundibulum, which is the site of fertilization and also serves as a secondary sperm storage site (Bakst, 1981). This capacity of SST to store and then gradually release spermatozoa assures continued fertilization of a succession of ova during the interval between inseminations. The so-called "fertile period" or "duration of fertility" defines the number of days during which a female lays fertile eggs after a single copulation or AI. The length of fertile period varies according to species such as domestic fowl (WLH)-12 days, duck-7 days, Japanese quail-6 days, domestic turkey-28 days and guinea fowl-7 days. The knowledge of fertile period is very helpful in achieving maximum fertility and hatchability in poultry (Proceedings of First National Workshop on AI in poultry, CARI, Izatnagar, 1982). This may be related to the number of SST which vary from species to species. For example, turkeys have been reported to have 20,000-30,000 SSTs, while chickens have been estimated to have only 5,000-13,500 SST (Birkhead and Moller, 1992). Additionally, after several generations of selection for high fertility, chicken hens possessed increased numbers of SST suggesting the number of SST may be positively correlated with fertility (Brillard *et al*., 1998). Cilia at the end of SST create a current away from the SST. Only sperm that have mobility high enough to prevent being swept away by this current can maintain their position in the SST and wait to be released at an opportune time to fertilize an ovum (Froman *et al*., 2006).

For the maximum fertility, it is important that an optimum number of sperm must enter the SST that needs good eversion of vagina and a deep insemination as close as possible to the SST. Avian spermatozoa are normally inseminated into the lower vagina from where, only 1-2% is able to reach and enter the SST at the uterovaginal junction. Sperm nearly take more than 24 hr to reach the upper part of the oviduct (Bakst, 1994). Fertility in domestic fowl hens is dependent on multiple factors. These include feed intake, photoperiod, temperature, environmental stress, and age (Brillard, 1993). Various workers have reported breed to breed variation in fertilizing ability of the spermatozoa (Mohan *et al*., 2011). Several aspects of semen quality have been correlated with fertilizing ability of male chickens such as sperm motility, sperm metabolism and percentage abnormal or dead sperm (McDaniel and Craig, 1959; Wilson *et al*., 1979). More recently, measures of sperm mobility (Donoghue, 1999; Froman *et al*., 1999) and sperm ATP content (Wishart and Palmer, 1986) also correlate strongly with fertility.

Materials and Methods

3 MATERIALS AND METHODS

The proposed research work entitled "**Effect of different semen extenders on fertility of chicken spermatozoa during short term preservation**" was carried out at the Division of Avian Physiology and Reproduction, Central Avian Research Institute, Izatnagar, Bareilly (UP). The study was carried out as per the guidelines and approval of Institute Animal Ethical Committee (IAEC) and Committee for the purpose of control and supervision of experiments on animals (CPCSEA). The experimental procedures and analytical techniques employed during the course of the study are briefly described in this section.

3.1 Experiment 1: To investigate the semen physical and biochemical characteristics of White Leghorn and indigenous (Nicobari and Ankleshwar) chicken

3.1.1 Experimental birds

Twenty healthy and adult males from the same hatch of each breed i.e. White Leghorn (exotic), Nicobari (indigenous) and Ankleshwar (indigenous) chicken were taken randomly and maintained in individual cages under uniform husbandry conditions. They were given normal breeder ration with maize, de-oiled rice bran (DORB), soyabean, oyster shell, marble chips, limestone, dicalcium phosphate (DCP), salt, DL-methionine, B-complex, vitamin and trace mineral premix. The birds were given water ad libitum and provided with constant light 14 hrs/ day. The experiment was conducted in two seasons i.e. winter and summer season.

3.1.2 Semen collection

Semen samples from the experimental birds were collected by abdominal massage method (Burrows and Quinn, 1937). Two persons were involved in the technique, one restraining

the male by the thighs in a crouching position and the second person gently stroking the lumbar region 3 to 4 times towards tail with palm of one hand. This process stimulates the copulatory organ of bird to become erect and protrude from the cloacal region. At this time gentle squeezing of the copulatory organ simultaneously with the thumb and forefinger of the same massage hand resulted in ejaculation of semen which was collected in wide mouth glass or plastic funnel with other hand. During the study period precautions were taken to avoid contamination of semen with fecal matter, urates and transparent fluid which deteriorate the semen quality.

3.1.3 Preparation of seminal plasma

Immediately after collection, semen volume was examined from individual birds. Subsequently, semen samples from each bird were immediately shifted on ice to the laboratory where the semen samples were subjected to various physical parameters and the rest of the samples were centrifuged at 4°C in a cooling centrifuge at 5000 rpm for 10 minutes. After centrifugation supernatant was collected as seminal plasma and stored in the freezer (-20°C) till further use.

3.1.4 Physical characteristics of semen

The physical characteristics of semen in White Leghorn, Nicobari and Ankleshwar chicken were evaluated in winter and summer season as follows:

3.1.4.1 Semen volume

In this study, the volume of ejaculated semen was measured according to the methods described by Shaffner and Andrew (1948) and Cooper and Rowell (1958). Accordingly, a tuberculin syringe of 1.0 ml capacity (graduated to measure 0.01ml sample) was used to quantify the semen volume.

3.1.4.2 Sperm motility

Immediately after collection, the sperm motility was assessed as described by Wheeler and Andrews (1943). A drop of semen (4-5 µl) was taken with the help of Pasteur pipette on a dry, clean glass slide and was spread uniformly by covering a glass slide. The slide was examined quickly under low power of light microscope. On the basis of the activity of swirls,

White Leghorn

Nicobari

Ankleshwar

Fig. 1: Different breeds of chicken under study

semen was graded and thus motility of spermatozoa was scored. The scoring system was as follows:

Motility (%)	Appearance under microscope
0	No motility
1-20	Slight undulating movement mostly weak and oscillatory few sperm
20-40	No waves or eddies, many sperm but many of inactive
40-60	Progressive motility, vigorous motion, slow eddies or waves
60-80	Progressive motility, waves and eddies of great rapidity and movement
80-100	Extremely rapid eddies and movement

3.1.4.3 Sperm Concentration

Sperm concentration was determined as per Brillard and McDaniel (1985) as follows:

Serial dilution

Semen samples were pooled to produce a standard curve with linear correlation between the sperm concentration determined by hemocytometer and the absorbance reading. Sperm samples were serially diluted with serial ratios of 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64. This was performed by mixing the sperm sample from each dilution step with the same volume of double distilled water and resulted in a set of six times serially dilution. To assure accuracy of the measurements of these diluted suspensions, the same pipette and tip were used for serial dilution. This dilution created a range of sperm concentrations, each of which was measured with spectrophotometer (Double beam UV-VIS spectrophotometer) for light absorbance 550 nm, and counted by use of hemocytometer (Marenfield Germany) for sperm concentration.

Absorbance measurement by use of spectrophotometer

The spectrophotometer used in this study for absorbance measurements was operated following the instructions provided by the manufacturer. Two ml of diluted semen was used in this study. Before loading samples in cuvette, the sperm suspension was mixed by tapping the

tubes 2 times each by a vortex mixer to provide homogeneity, and the absorbance was measured immediately to avoid precipitation of sperm. The double distilled water used for suspending the sperm samples, was used as a reference blank before measurement of samples. Between measurements, the cuvette was wiped clean with dry Kim-wipe. For each sample, the absorbance was measured three times, and an average of these absorbance values was used in data analysis.

Sperm counting with the hemocytometer

After serial dilution and measurement of absorbance, the concentration of each sample was determined by counting of sperm cells with 0.1 mm depth hemocytometer. A dilution step was applied before measurement to ensure the counting fell into the range of 30-100 sperms within each square (1/25 mm^2). A 10-µl sample was required for each hemocytometer count. Before loading samples into the counting chamber, the sperm suspension was mixed by tapping the tube 10 times, and for each sample the sperm number in the five squares (1/25 mm^2) located in the four corners and the middle was counted by use of a dark- field microscope (magnification x 200) after the sperm cells had completely settled within the counting chamber (about 10 min after loading the sample).

Establishment of standard curves between absorbance and sperm concentration

To establish a standard curve between absorbance and sperm concentration for each sample type, serial dilutions of semen samples measured at 550 nm for correlation analysis with the sperm concentration counted by hemocytometer. With the establishment of a linear relationship for each sample, the equation between absorbance and sperm concentration was deduced.

Validation of the deduced equations for predicting sperm concentration

To validate the deduced equations for estimating sperm concentration, semen samples were collected from 5 broiler birds. The samples were measured at 550 nm to calculate the sperm concentration with the equation deduced. Meanwhile, the samples were counted by use of hemocytometer for actual sperm concentration. The predicted concentrations from the equation were compared with the concentrations counted by hemocytometer.

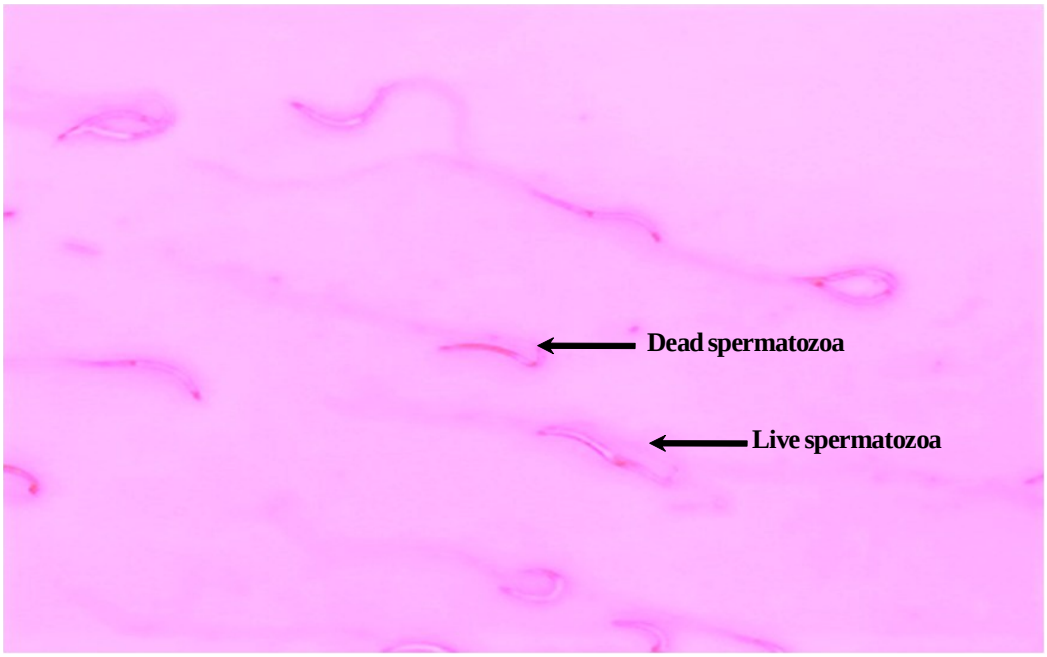

Fig. 2: Evaluation of sperm viability

Measurement of sperm concentration

Semen samples were diluted in 1:200 in DDW and mixed with vortex mixer. Then placed in cuvette and OD was measured from spectrophometer (550 nm) and compared with standard curve in excel.

3.1.4.4 Counting of the live and dead spermatozoa

The percentage of live and dead spermatozoa was examined as per the method described by Lake and Stewart (1978).

Step 1: A solution of nigrosin and eosin was prepared in the sodium citrate ($Na_3C_6H_5O_7.2H_2O$) solvent i.e. 0.16 gm eosin and 0.6 gm nigrosin per 100 ml of solvent. 10 ml of the solvent was taken in a test tube and added the nigrosin with constant stirring with a glass rod. After properly dissolving the nigrosin, the eosin was added and thoroughly mixed. Then it was filtered with filter paper.

Step 2: One drop of freshly collected and diluted semen was taken on a slide and 4-5 drops of stain were added to it, mixed thoroughly and allowed to stand for one minute at room temperature.

Step 3: Before smearing, once again the stained semen mixture was stirred gently and a small drop was taken on the edge of grease free, clean and dry glass slide and a smear was drawn and the slide was dried under blowing cool air.

Step 4: Examination of stained slide was done under the oil immersion objective (x100). Three hundred spermatozoa were examined and differentiated as live or dead by the following way

Dead: Stained and partially stained sperm

Live: Unstained sperm

3.1.4.5 Estimation of abnormal spermatozoa

The smears as prepared for live sperm estimation were used to study the per cent of abnormal spermatozoa (Lake and Stewart, 1978). A total of 100 spermatozoa were counted

under the oil-immersion objective of the microscope and classified as either normal or abnormal and the total % of abnormal spermatozoa was calculated. Various kinds of sperm abnormalities i.e. absence of head, coiled tail, twisted tail etc, were observed and grouped together.

3.1.5. Biochemical characteristics of semen

The biochemical parameters of seminal plasma in White Leghorn, Nicobari and Ankleshwar chicken were evaluated in winter and summer season as follows:

3.1.5.1 Estimation of Total protein

The total protein was estimated by Biuret method by using the kits procured from Coral Clinical Systems.

Principle: Proteins, in an alkaline medium, bind with the cupric ions present in the biuret reagent to form a blue-violet coloured complex. The intensity of the colour formed is directly proportional to the amount of proteins present in the sample.

Reagents

i. Biuret reagent

ii. Protein Standard (8 g/dl)

Procedure

	Blank	Std	Test
Biuret reagent	1.0 ml	1.0 ml	1.0 ml
Distilled water	0.02 ml	-	-
Protein Standard	-	0.02 ml	-
Sample	-	-	0.02 ml

Mixed well and incubated at 37°C for 10 minutes. The absorbance of standard and test was measured against the Blank at 550 nm using spectrophotometer.

$$\text{Total Proteins in g/dl} = \frac{\text{Abs. of Test}}{\text{Abs. of Std}} \times 8$$

3.1.5.2 Estimation of Triglyceride

Triglyceride was estimated using GPO/PAP method by using the kits procured from Coral Clinical Systems.

Principle

Lipoprotein lipase hydrolyses triglycerides to glycerol and free fatty acids. The glycerol formed with ATP in the presence of glycerol kinase forms glycerol-3-phosphate which is oxidised by the enzyme glycerol phosphate oxidase to form hydrogen peroxide. The hydrogen peroxide further reacts with phenolic compound and 4- aminoantipyrine by the catalytic action of peroxidase to form a red coloured quinoneimine dye complex. Intensity of the colour formed is directly proportional to the amount of triglycerides present in the sample.

Reagents

i. Enzyme Reagent 1

ii. Enzyme Reagent 2

iii. Triglycerides Standard (200 mg/dl)

Working reagent

Mix together 4 parts of Enzyme Reagent 1 and 1 part of Enzyme Reagent 2.

Procedure

	Blank	**Std**	**Test**
Working reagent	1.0 ml	1.0 ml	1.0 ml
Distilled water	0.01 ml	-	-
Triglycerides Standard	-	0.01 ml	-
Sample	-	-	0.01 ml

Mixed well and incubated at 37°C for 5 minutes. The absorbance of standard and test was measured against the Blank at 505 nm using spectrophotometer.

$$\text{Triglyceride in g/dl} = \frac{\text{Abs. of Test}}{\text{Abs. of Std}} 100$$

3.1.5.3 Estimation of Cholesterol

The cholesterol was estimated by CHOD/PAP method by using kits procured from Coral Clinical Systems.

Principle:

Cholesterol esterase hydrolyses esterified cholesterols to free cholesterol. The free cholesterol is oxidised to form hydrogen peroxide which further reacts with phenol and 4-aminoantipyrine by the catalytic action of peroxidase to form a red coloured quinoneimine dye complex. Intensity of the colour formed is proportional to the amount of cholesterol present in the sample.

Reagents

i. Enzyme Reagent 1

ii. Enzyme Reagent 2

iii. Cholesterol Standard (200 mg/dl)

Working reagent

Mix together 4 parts of Enzyme Reagent 1 and 1 part of Enzyme Reagent 2.

Procedure

	Blank	**Std**	**Test**
Working reagent	1.0 ml	1.0 ml	1.0 ml
Distilled water	0.01 ml	-	-
Cholesterol Standard	-	0.01 ml	-
Sample	-	-	0.01 ml

Mixed well and incubated at 37°C for 5 minutes. The absorbance of standard and test was measured against the Blank at 505 nm using spectrophotometer.

$$\text{Cholesterol in mg/dl} = \frac{\text{Abs. of Test}}{\text{Abs. of Std}} \times 200$$

3.1.5.4 Estimation of Glutamic pyruvate transaminase (GPT)

GPT was estimated by Reitman and Frankel's method by using kits procured from Coral Clinical Systems.

Principle

GPT converts L-alanine and L-ketoglutarate to pyruvate and glutamate. The pyruvate formed reacts with 2,4 dinitrophenyl hydrazine to produce a hydrazone derivative, which in an alkaline produces a brown coloured complex whose intensity is measured. A calibration curve is plotted using a pyruvate standard and the activity of GPT is read off this calibration curve.

Reagents

i. Substrate Reagent

ii. DNPH Reagent

iii. NaOH Reagent (4N)

iv. Pyruvate Standard (2Mm)

Working reagent

Dilute one ml of NaOH Reagent (4N) with 9 ml of distilled water.

Procedure

	Blank	**Test**
Substrate Reagent	0.50 ml	0.50 ml
	(Incubated at 37°C for 3 minutes)	
Sample	-	0.10 ml
	(Mixed well and incubated at 37°C for 30 minutes)	
DNPH Reagent	0.50 ml	0.50 ml
	(Mixed well and allowed to stand at room temp. for 20 minutes)	
Distilled water	0.10 ml	-
Working NaOH Reagent	5.00 ml	5.00 ml

Mixed well and allowed to stand at room temperature for 10 minutes. The absorbance of test was measured against blank at 505 nm and the activity of the test in U/ml was read from the calibration curve plotted using pyruvate standard.

3.1.5.5 Estimation of Glutamic oxaloacetic transaminase (GOT)

GOT was estimated by Reitman and Frankel's method by using kits procured from Coral Clinical Systems.

Principle

GOT converts L-aspartate and L-ktoglutarate to oxaloacetate and glutamate. The oxaloacetae formed reacts with 2,4 dinitrophenyl hydrazine to produce a hydrazone derivative, which in an alkaline produces a brown coloured complex whose intensity is measured. A calibration curve is plotted using a pyruvate standard and the activity of GPT is read off this calibration curve.

Reagents

i. Substrate Reagent

ii. DNPH Reagent

iii. NaOH Reagent (4N)

iv. Pyruvate Standard (2Mm)

Working reagent

Dilute one ml of NaOH Reagent (4N) with 9 ml of distilled water.

Procedure

	Blank	Test
Substrate Reagent	0.50 ml	0.50 ml
	(Incubated at 37°C for 3 minutes)	
Sample	-	0.10 ml
	(Mixed well and incubated at 37°C for 60 minutes)	
DNPH Reagent	0.50 ml	0.50 ml
	(Mixed well and allowed to stand at room temp. for 20 minutes)	
Distilled water	0.10 ml	-
Working NaOH Reagent	5.00 ml	5.00 ml

Mixed well and allowed to stand at room temperature for 10 minutes. The absorbance of test was measured against blank at 505 nm and the activity of the test in U/ml was read from the calibration curve plotted using pyruvate standard.

3.1.5.6 Estimation of Alkaline phosphatase (ALP)

Alkaline phosphatase (ALP) was estimated by modified Kind and King's method by using kits procured from Coral Clinical Systems.

Principle

ALP at an alkaline pH hydrolyses disodium phenylphosphate to form phenol, which reacts with 4-aminoantipyrine in the presence of potassium ferricyanide, as an oxidising agent, to form a red coloured complex. The intensity of the colour formed is directly proportional to the activity of ALP present in the sample.

Reagents

i. Buffer Reagent

ii. Substrate Reagent

iii. Colour Reagent

iv. Phenol Reagent

Procedure

	Blank	**Std**	**Control**	**Test**
Distilled water	1.05 ml	1.0 ml	1.0 ml	1.0 ml
Buffer Reagent	1.0 ml	1.0 ml	1.0 ml	1.0 ml
Substrate Reagent	0.10 ml	0.10 ml	0.10 ml	0.10 ml
(Mixed well and allowed to stand at 37°C for 3 minutes)				
Sample	-	-	-	0.05 ml
Phenol Standard	-	0.05 ml	-	-
(Mixed well and allowed to stand at 37°C for 15 minutes)				
Colour Reagent	1.0 ml	1.0 ml	1.0 ml	1.0 ml
Sample	-	-	0.05 ml	-

Mixed well after each addition and the absorbance of blank, standard, control and test was measured against distilled water 510 nm using spectrophotometer.

$$\text{Total ALP activity in K.A. Units} = \frac{\text{Abs. of Test - Abs. of Control}}{\text{Abs. of Std – Abs. of Blank}} \text{ X } 10$$

3.1.5.7 Estimation of Calcium

Calcium was estimated by OCPC method by using kits procured from coral clinical systems.

Principle

Calcium in an alkaline medium combines with o- Cresolphthalein Complexone to form a purple coloured complex. Intensity of the colour formed is directly proportional to the amount of calcium present in the sample.

Reagents

i. Buffer Reagent

ii. Colour Reagent

iii. Calcium Standard (10 mg/dl)

Procedure

	Blank	Std	Test
Buffer Reagent	0.5 ml	0.5 ml	0.5 ml
Colour Reagent	0.5 ml	0.5 ml	0.5 ml
Distilled water	0.02 ml	-	-
Calcium Standard	-	0.02 ml	-
Sample	-	-	0.02 ml

Mixed well and incubated at room temperature for 5 minutes. The absorbance of the standard and test was measured against blank at 570 nm using spectrophotometer.

$$\text{Calcium in mg/dl} = \frac{\text{Abs. of Test} \times 10}{\text{Abs. of Std}} \times 100$$

3.1.5.8 Estimation of Uric acid

Uric acid was estimated by Uricase/PAP method by using the kits obtained from Coral Clinical Systems.

Principle

Uricase converts uric acid to allantoin and hydrogen peroxide. The hydrogen peroxide formed further reacts with a phenolic compound and 4 aminoantipyrine by the catalytic action of peroxidase to form a red coloured quinoneimine dye complex. Intensity of the colour formed is directly proportional to the amount of uric acid present in the sample.

Reagents

i. Buffer reagent

ii. Enzyme reagent

iii. Uric acid standard (8 mg/dl)

Procedure

Addition sequence	Blank	Std	Test
Working reagent	1.0 ml	1.0 ml	1.0 ml
Distilled water	0.02 ml	-	-
Uric acid standard	-	0.02 ml	-
Sample	-	-	0.02 ml

Mixed well and incubated at 37^{O}C for 5 minutes. The absorbance of the standard and test was measured against blank at 520 nm using spectrophotometer.

$$\text{Uric acid (mg/dl)} = \frac{\text{Abs. of Test}}{\text{Abs. of Std}} \times 8$$

3.1.5.9 pH of semen

The neat semen pH was determined by digital pH-meter (Thermo Fisher Scientific, USA) fitted with a microelectrode.

3.1.5.10 Resazurin dye reduction test

The RRT test was carried out to assess semen quality. Semen samples were divided in two aliquot after being diluted (1:2) at 30^0C with 7.46% tri sodium citrate solution. Twenty µl of resazurin dye were added to 400 µl of extended semen sample. After mixing the sample were incubated 37^0C for one hour and then 2 ml n-butanol was added, vortexed and centrifuged for 10 min at 2000 rpm. The clear coloured upper layer of n-butanol was transferred into glass cuvette. Optical densities of the sample were measured at 580nm and 615nm against blank using spectrophotometer. The RRT ratio was calculated by dividing the absorption at 580 nm by the absorption at 615 nm according to Reddy and Bordekar (1999).

3.2 Experiment 2: To study the effect of different semen extenders on fertilizing ability of chicken spermatozoa during short term preservation

3.2.1. Experimental birds

Twenty healthy adult males from the same hatch of White Leghorn (WLH) chicken were taken randomly and maintained in individual cages under uniform husbandry conditions. To assess the fertility, 60 healthy adult females divided into 3 groups with 20 birds each from the same hatch of White Leghorn (WLH) were taken randomly and maintained in similar conditions. They were offered normal breeder ration and water *ad libitum* with a constant light 14 hrs/day.

3.2.2. Semen collection

Semen samples from the experimental birds were collected by abdominal massage method (Burrows and Quinn, 1937). Two persons were involved in the technique, one

Fig. 3: Semen collection and Artificial Insemination in White Leghorn

restraining the male by the thighs in a crouching position and the second person gently stroking the lumbar region 3 to 4 times towards tail with palm of one hand. This process stimulate the copulatory organ of bird to become erect and protrude from the cloacal region. At this time gentle squeezing of the copulatory organ simultaneously with the thumb and forefinger of the same massage hand resulted in ejaculation of semen which was collected in wide mouth glass or plastic funnel with other hand. During the study period precautions were taken to avoid contamination of semen with fecal matter, urates and transparent fluid which deteriorate the semen quality.

3.2.3 Semen dilution for short term preservation and motility assessment

The good quality of pooled semen samples of WLH birds were divided equally into three aliquots and were taken separately in 5ml round bottom glass tubes (length 7cm, diameter 1cm). Subsequently, semen samples of each aliquots were diluted in three different semen extenders such as CARI poultry semen diluent (Mohan *et al*., 2017), EK extender (Lukaszewicz, 2002) and Tselutin extender (Tselutin *et al*., 1995) respectively at a fixed dilution rate (1:2). In this manner 3 set of semen samples were prepared for AI at 0 hr (freshly ejaculated semen) and 24 hr. Diluted semen samples targeted for AI at 24 hr were stored at 8±1°C for CARI poultry semen diluents and 4±1°C for EK and Tselutin extender. Before AI, the diluted semen samples were examined for sperm motility at different storage period *viz.* 0, 24, 48, 72 and 96 hr. It was determined under low magnification (10x) of light microsope with cover slip and scored as described by Wheeler and Andrews (1943).

3.2.4. Artificial insemination and fertility assessment

For artificial insemination, vaginal eversion was made by gentle pressure on left side of the abdomen to cause eversion of oviduct (vagina) through cloaca (Burrows and Quinn, 1936). Subsequently AI was carried out by intravaginal insemination (80-100 sperm/hen) by using tuberculin syringe/AI gun (IMV, France) in all the 3 groups having 20 hens each with the respective diluted semen samples. The day next to AI was skipped and eggs were collected from 2-8 days. Fertility of eggs was examined by candling at 9th day of incubation. Some eggs were broken for fertility assessment in which fertility was not clear by candling. The percent

fertility was determined by the ratios of number of fertile eggs to the number of total eggs set in the incubator.

3.3. Experiment 3: To find the effect of different dilution rates on fertility using fresh semen

3.3.1 Experimental birds

Twenty healthy adult males from the same hatch of White Leghorn (WLH) chicken were taken randomly and maintained in individual cages under uniform husbandry conditions. To assess the fertility, 200 healthy adult females divided into 10 groups with 20 birds each from the same hatch of White Leghorn (WLH) were taken randomly and maintained in similar conditions. They were offered normal breeder ration and water *ad libitum* with a constant light 14 hrs/day.

3.3.2 Semen collection

Semen samples from the experimental birds were collected by abdominal massage method (Burrows and Quinn, 1937) as described in experiment 1.

3.3.3 Diluent for study

From the statistical observations of experiment 2, CARI poultry semen diluent (Mohan *et al*., 2017) exhibited the better fertility results compared to EK extender (Lukaszewicz, 2002) and Tselutin extender (Tselutin *et al*., 1995). In addition, CARI poultry semen diluent found to be more economical compared to other diluents. Hence, this diluent was chosen to study the effect of different dilution rates on fertility using fresh semen.

3.3.4 Dilution of semen at different dilution rates and motility assessment

The good quality pooled semen samples of WLH birds were diluted with CARI poultry semen diluent at different dilution rates of 1:2, 1:4, 1:6, 1:8, 1:10, 1:12, 1:14, 1:16, 1:18 and 1:20 with gradual decrease in the sperm concentration as shown in Table 1. Diluted samples were taken accordingly in separate 5 ml round bottom glass tubes (length 7cm, diameter 1cm) separately. In this manner 10 set of samples were prepared at different dilution rates for AI at 0 hrs (freshly ejaculated semen). Before AI, the diluted semen samples were examined for sperm motility at different storage period *viz.* 0, 24 , 48, 72 and 96 hr. It was determined

under low magnification (10x) of light microsope with cover slip and scored as described by Wheeler and Andrews (1943).

Table 1: Freshly ejaculated chicken semen diluted with CARI poultry semen diluent with different dilution rates and insemination dose/ AI

Dilution rate	Semen volume (ml)	Diluent (ml)	Total volume (ml)	Approx. Sperm concentration/AI ($x10^6$/0.05 ml)
1:2	1	2	3	89.10
1:4	0.60	2.40	3	53.46
1:6	0.43	2.57	3	38.19
1:8	0.33	2.67	3	29.70
1:10	0.27	2.73	3	24.30
1:12	0.23	2.77	3	20.50
1:14	0.20	2.80	3	17.82
1:16	0.18	2.82	3	15.72
1:18	0.16	2.84	3	14.07
1:20	0.14	2.86	3	12.71

3.3.5 Artificial insemination and fertility assessment

The calculated dose of semen samples were diluted with CARI poultry semen diluent accordingly and inseminated in the respective 10 groups with 20 birds each. AI was carried out by intra vaginal insemination same as described in experiment 1 by using tuberculin syringe/ AI gun (IMV, France) with 50 µl volume of inseminate using various dilution factors. The day next to AI was skipped and eggs were collected from 2-8 days. Fertility assessment was done same as described in experiment 1.

3.4. Statistical Analysis

Statistical analysis was done using statistical software package SPSS (version 20) for ANOVA and Duncan's multiple range tests (Duncan, 1955) for comparing the means for significant differences.

Results

4 RESULTS

Experiment 1: To investigate the semen physical and biochemical characteristics of White Leghorn and indigenous (Nicobari and Ankleshwar) chicken

4.1 Physical characteristics of semen

Chicken semen physical characteristics such as volume, motility, concentration, live spermatozoa, dead spermatozoa and morphological abnormal spermatozoa of White Leghorn (WLH), Nicobari and Ankleshwar were recorded in the winter and summer season and presented in the text as winter vs. summer.

4.1.1. Semen volume

The mean value of semen volume per ejaculate in WLH, Nicobari and Ankleshwar chicken recorded in winter and summer season is presented in Table 2. Semen volume of WLH (0.23±0.03 vs. 0.21±0.02 ml) was found to be significantly much lower than the indigenous breeds of Nicobari (0.55±0.01 vs. 0.54±0.01 ml) and Ankleshwar (0.44±0.01 vs. 0.41±0.02 ml) in both the seasons respectively. Between the native breeds, Nicobari seemed to have significantly higher semen volume than Ankleshwar chicken. The mean values in winter and summer season were not significantly different in all the breeds.

4.1.2. Sperm motility

The mean value of sperm motility percentage in WLH, Nicobari and Ankleshwar chicken recorded in winter vs. summer season is shown in Table 2. WLH showed significantly

higher sperm motility (88.03±0.97% vs. 84.92±1.16%) compared to Nicobari (81.78±0.96% vs. 78.31±1.38%) and Ankleshwar chicken (82.72±1.23% vs. 79.22±1.09%) in both winter and summer respectively. The mean values of sperm motility reduced in summer ($p<0.05$) in all the breeds.

4.1.3. Sperm concentration

The mean value of sperm concentration in WLH, Nicobari and Ankleshwar chicken recorded in winter vs. summer season is given in Table 2. The sperm concentration ($x10^9$cells/ml) in WLH (5.28±0.09 vs. 4.83±0.11) was recorded higher ($p<0.05$) followed by Ankleshwar (4.93±0.09 vs. 4.47±0.18) and Nicobari (4.75±0.13 vs. 4.31±0.14) in both winter and summer season respectively. Irrespective of the breed, sperm concentration was found to be significantly lower in summer season.

4.1.4. Percentage of live and dead sperm count

The mean values of live and dead sperm count in all three breeds in winter and summer season are indicated in Table 2. The percent live spermatozoa was found to be significantly higher in WLH (92.02±0.55% vs. 89.68±0.57%) than the native breeds in both the seasons respectively. Summer season exhibited lesser live sperm count in all three breeds. The percent dead sperm count was recorded more in Nicobari (7.10±0.78% vs. 9.05±0.38%) and Ankleshwar (6.51±0.48% vs. 8.44±0.46%) than in WLH (4.62±0.46% vs. 6.76±0.58%) in both winter and summer respectively. Dead sperms were recorded more in summer irrespective of the breeds. Among the native breeds Nicobari was recorded more dead sperms than Ankleshwar in both the seasons but without significant difference.

4.1.5 Percentage of abnormal spermatozoa

The mean values of abnormal spermatozoa in all three breeds in winter and summer season are shown in Table 2. The percent abnormal sperm count were recorded more in Nicobari (4.80±0.28% vs. 5.20±0.25%) and Ankleshwar (4.19±0.22% vs. 4.75±0.20%) semen than in WLH (3.36±0.33% vs. 3.56±0.16%) in both the seasons (winter vs. summer). The mean values of abnormal apermatozoa were not affected between seasons.

Table 2: Physical characteristics of semen in White Leghorn and indigenous (Nicobari and Ankleshwar) chicken (Mean±SE, n=6)

Parameter	White Leghorn			Nicobari			Ankleshwar		
	Winter	Summer	Average	Winter	Summer	Average	Winter	Summer	Average
Semen volume (ml)	0.23^{c}±0.03	0.21^{C}±0.02	0.22±0.02	0.55^{a}±0.01	0.54^{A}±0.01	0.54±0.01	0.44^{b}±0.01	0.41^{B}±0.02	0.42±0.01
Semen motility (%)	88.03*^{a}±0.97	84.92^{A}±1.16	86.47±0.78	81.78*^{b}±0.96	78.31^{B}±1.38	80.04±0.86	82.72*^{b}±1.23	79.22^{B}±1.09	80.97±0.84
Sperm concentration (x 10^{9}cells/ml)	5.28*^{a}±0.09	4.83^{A}±0.11	5.00±0.08	4.75*^{b}±0.13	4.31^{B}±0.14	4.56±0.10	4.93*^{b}±0.09	4.47^{AB}±0.18	4.74±0.10
Live spermatozoa (%)	92.02*^{a}±0.55	89.68^{A}±0.57	90.46±0.44	88.10*^{b}±0.65	85.75^{B}±0.34	86.53±0.34	89.30*^{b}±0.77	86.81^{B}±0.44	87.64±0.42
Dead spermatozoa (%)	4.62*^{b}±0.46	6.76^{B}±0.58	6.04±0.44	7.10*^{a}±0.78	9.05^{A}±0.38	8.40±0.38	6.51*^{ab}±0.48	8.44^{A}±0.46	7.7±0.40
Morphological abnormal spermatozoa (%)	3.36^{b}±0.33	3.56^{B}±0.16	3.50±0.15	4.80^{a}±0.28	5.20^{A}±0.25	5.07±0.19	4.19^{a}±0.22	4.75^{A}±0.20	4.56±0.15

*Mean values within breed in winter season compared with corresponding summer season ($p<0.05$) using Student 't' test
Mean values in rows in winter season bearing different superscripts (a, b, c) in various breeds differ significantly ($p<0.05$)
Mean values in rows in summer season bearing different superscripts (A, B, C) in various breeds differ significantly ($p<0.05$)

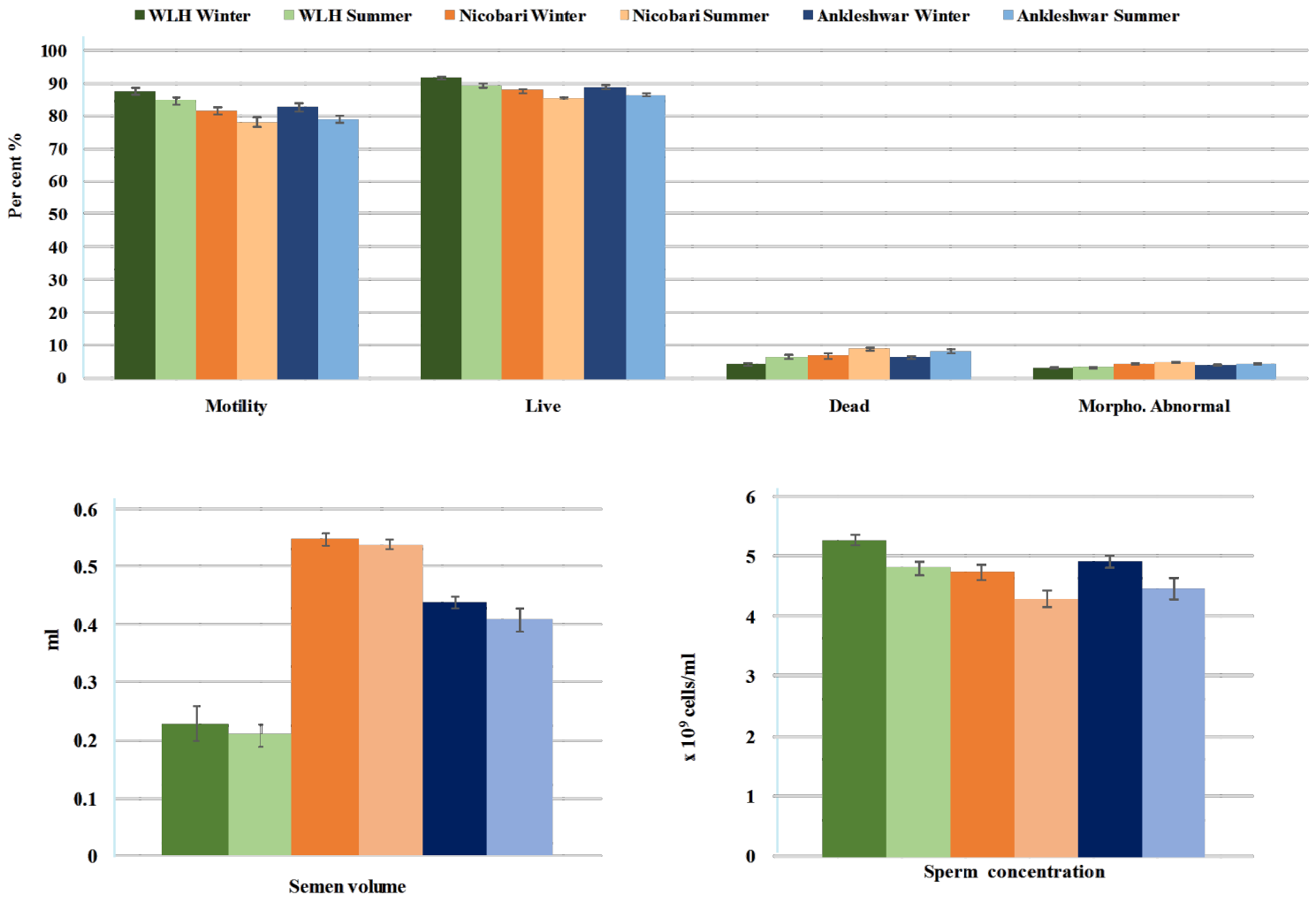

Fig. 4: Physical characteristics of semen in White Leghorn and indigenous (Nicobari and Ankleshwar) chicken

4.2 Biochemical characteristics of semen

Biochemical characteristics like total protein, triglyceride, cholesterol, GPT, GOT, ALP, calcium, uric acid, pH and RRT of WLH, Nicobari and Ankleshwar chicken were evaluated in winter and summer months and the data is presented in Table 3. Results of biochemical characteristics in two seasons (winter vs. summer) are as follows:

4.2.1 Total protein concentration

The observed mean value of total protein concentration (g/dl) in seminal plasma of all three breeds is shown in Table 3. The mean total protein concentration (g/dl) in WLH birds (2.15±0.02 vs. 2.17±0.02) was recorded significantly ($p<0.05$) lower than the Nicobari (2.25±0.03 vs. 2.28±0.03) and Ankleshwar (2.22±0.02 vs. 2.26±0.02) chicken. No significant variation was found between between seasons though the values tend to be higher in summer.

4.2.2. Triglyceride and cholesterol concentration

The observed mean values of triglyceride and cholesterol concentration in seminal plasma of all three breeds is given in Table 3. The triglyceride concentration (mg/dl) was found to be higher ($p<0.05$) in WLH (73.78±0.99 vs. 71.03±1.23) than the native Nicobari (66.28±1.62 vs. 62.47±1.67) and Ankleshwar (69.12±1.56 vs. 66.19±1.85) breeds in both the seasons. The cholesterol concentration (mg/dl) was recorded more in WLH (37.59±0.64 vs. 35.81±0.82) followed by Ankleshwar (34.94±1.18 vs. 33.92±1.13) and Nicobari (34.17±0.93 vs. 31.94±0.81) chicken in winter and summer respectively. Both triglyceride and cholesterol concentration did not differ significantly between seasons.

4.2.3. Enzyme profile (GPT, GOT, ALP) in seminal plasma

The mean values of the enzyme profile (GPT, GOT, ALP) in seminal plasma of all the three breeds is presented in Table 3. The average activity of GPT and GOT were recorded more ($p<0.05$) in native breeds compared to WLH birds. The mean values of GPT (U/ml) and GOT (U/ml) respectively in WLH (7.77±0.66 vs. 9.81±0.67; 174.39±1.42 vs. 179.44±1.88), Nicobari (11.38±0.64 vs. 14.05±0.68; 183.85±1.02 vs. 191.94±3.47) and Ankleshwar (12.48±0.88 vs. 14.94±0.82; 179.26±2.73 vs. 188.13±2.43) chicken were

observed more ($p<0.05$) in summer than the winter season. In contrast to other enzyme activities, the mean values of ALP (KA units) were higher in WLH (9.92±0.87 vs. 9.12±0.77) than Nicobari (5.45±0.40 vs. 4.30±0.30) and Ankleshwar (5.81±0.19 vs. 4.68±0.38) in both the seasons. The phosphatase activity tend to decrease in summer but did not differ significantly.

4.2.4. Calcium concentration

The observed mean values of calcium concentration in seminal plasma of all the three breeds is shown in Table 3. The concentration (mg/dl) was recorded 6.15±0.21, 5.81±0.19 and 5.49±0.18 in summer and 5.84±0.18, 5.46±0.17 and 5.18±0.21 in winter respectively in WLH, Nicobari and Ankleshwar breeds of chicken.

4.2.5. Uric acid concentration

The observed mean values of uric acid concentration in seminal plasma of all the three breeds are exhibited in Table 3. The mean concentration (mg/dl) was found 5.74±0.22, 6.35±0.77, 6.41±0.72 in winter and 6.67± 0.35, 7.14±0.81, 7.22±0.61 in summer respectively in WLH, Nicobari and Ankleshwar chicken.

4.2.6. pH of semen

The observed mean values of pH of semen of all the three breeds are shown in Table 3. In summer the pH was recorded 7.18±0.02, 7.23±0.03, 7.20±0.03 in winter and 7.17±0.02, 7.21±0.02, 7.22±0.03 in summer in WLH, Nicobari and Ankleshwar respectively.

4.2.7. Resazurin reduction test (RRT)

Resazurin reduction test is used for measurement of metabolic activity of spermatozoa. It is expressed by the ratio of OD at 580 and 615nm. The mean values of RRT in winter are 2.83±0.04, 2.61±0.05, 2.64±0.04 and 2.72±0.04, 2.48±0.05, 2.53±0.03 in summer in WLH, Nicobari and Ankleshwar chicken respectively.

Table 3: Biochemical characteristics of seminal plasma in White Leghorn and indigenous (Nicobari and Ankleshwar) chicken (Mean±SE, n=6)

Parameter	White Leghorn			Nicobari			Ankleshwar		
	Winter	Summer	Average	Winter	Summer	Average	Winter	Summer	Average
Total protein (g/dl)	$2.15^{a}\pm0.02$	$2.17^{A}\pm0.02$	2.16±0.01	$2.25^{b}\pm0.03$	$2.28^{B}\pm0.03$	2.27±0.02	$2.22^{b}\pm0.02$	$2.26^{B}\pm0.02$	2.24±0.02
Triglyceride (mg/dl)	$73.78^{a}\pm0.99$	$71.03^{A}\pm1.23$	72.41±0.81	$66.28^{b}\pm1.62$	$62.47^{B}\pm1.67$	64.38±1.18	$69.12^{b}\pm1.56$	$66.19^{B}\pm1.85$	67.66±1.22
Cholesterol (mg/dl)	$37.59^{a}\pm0.64$	$35.81^{A}\pm0.82$	36.64±0.54	$34.17^{b}\pm0.93$	$31.94^{B}\pm0.81$	33.06±0.63	$34.94^{ab}\pm1.18$	$33.92^{AB}\pm1.13$	34.44±0.81
GPT (U/ml)	$7.77^{*b}\pm0.66$	$9.81^{B}\pm0.67$	8.79±0.48	$11.38^{*a}\pm0.64$	$14.05^{A}\pm0.68$	12.72±0.49	$12.48^{*a}\pm0.88$	$14.94^{A}\pm0.82$	13.71±0.61
GOT(U/ml)	$174.39^{*b}\pm1.42$	$179.44^{B}\pm1.88$	177.33±1.27	$183.85^{*a}\pm1.02$	$191.94^{A}\pm3.47$	188.30±2.16	$179.26^{*ab}\pm2.73$	$188.13^{A}\pm2.43$	185.04±1.74
ALP (KA Units)	$9.92^{a}\pm0.87$	$9.12^{A}\pm0.77$	9.62±0.62	$5.45^{b}\pm0.40$	$4.30^{B}\pm0.30$	4.83±0.26	$5.81^{b}\pm0.19$	$4.68^{B}\pm0.38$	5.13±0.28
Calcium (mg/dl)	6.15±0.21	5.84±0.18	6.01±0.14	5.81±0.19	5.46±0.17	5.63±0.13	5.49±0.18	5.18±0.21	5.34±0.14
Uric acid (mg/dl)	5.74±0.22	6.67±0.35	6.21±0.21	6.35±0.77	7.14±0.81	6.74±0.55	6.41±0.72	7.22±0.61	6.82±0.47
pH**	7.18±0.02	7.17±0.02	7.18±0.02	7.23±0.03	7.21±0.02	7.22±0.02	7.20±0.03	7.22±0.03	7.21±0.02
RRT** (580/650 nm)	$2.83^{*a}\pm0.04$	$2.72^{A}\pm0.04$	2.78±0.03	$2.61^{*b}\pm0.05$	$2.48^{B}\pm0.05$	2.55±0.03	$2.64^{*b}\pm0.04$	$2.53^{B}\pm0.03$	2.58±0.03

*Mean values within breed in winter season compared with corresponding summer season ($p<0.05$) using Student 't' test
Mean values in rows in winter season bearing different superscripts (a, b) in various breeds differ significantly ($p<0.05$)
Mean values in rows in summer season bearing different superscripts (A, B) in various breeds differ significantly ($p<0.05$)
**pH and RRT was determined in semen

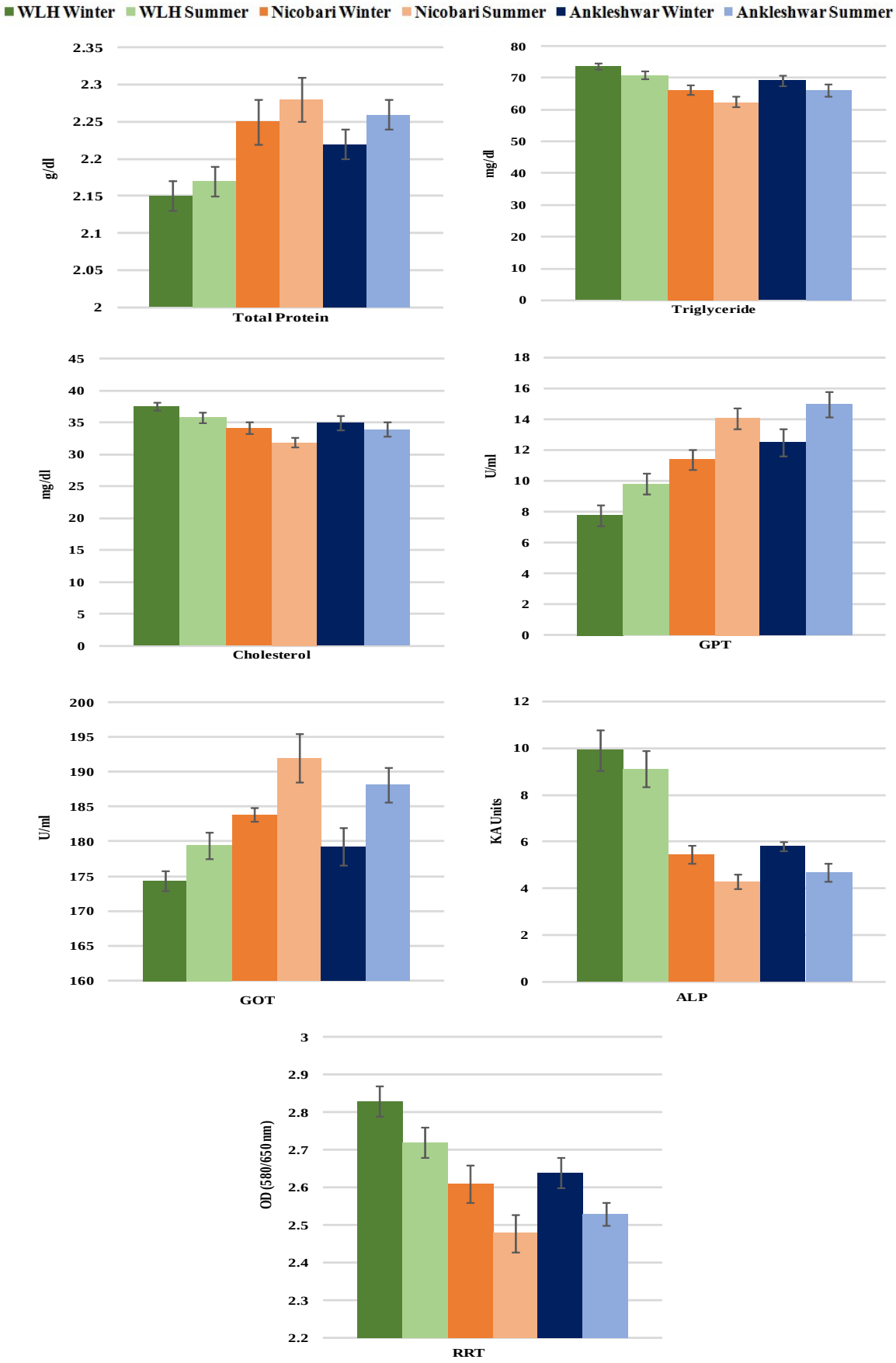

Fig. 5: Biochemical characteristics of seminal plasma in White Leghorn and indigenous (Nicobari and Ankleshwar) chicken

4.3. Experiment 2: To study the effect of different semen extenders on fertilizing ability of chicken spermatozoa during short term preservation

Under this study, effect of CARI poultry semen diluent was observed on the fertilizing ability of chicken spermatozoa and it was compared with the various commonly used diluents like EK extender and Tselutin diluent. These diluents were evaluated for their effect on sperm motility of White Leghorn chicken spermatozoa upon storage at 0, 24, 48, 72 and 96 hr and on fertilizing ability at 0 and 24 hr of storage at low temperature.

The mean values of sperm motility are presented in Table 4. The undiluted semen showed the sperm motility of 89.83±0.31% at 0 hr of storage. All the semen diluents expressed the sperm motility nearly in the similar range. Hence, there was no significant difference noticed among the different diluents along with the undiluted semen at this storage period. At 24 hr of storage, a significant ($p<0.05$) difference in sperm motility were found among all the diluents with the lowest motility in undiluted semen (10.17±0.65%). Subsequently, there was no sperm motility recorded in the undiluted semen at all the stages of storage period (48-96 hr). In the remaining three semen diluents, a drastic reduction in sperm motility was noticed after 48 hr of storage and onwards. At the end of storage period (96 hr), the semen sample diluted with CARI poultry semen diluent and EK extender showed 49.17±0.79% and 22.50±0.56% sperm motility respectively whereas no motility in sample diluted with Tselutin diluent.

The data on the effect of various semen diluents on the fertilizing ability at 0 hr (freshly ejaculated) and 24 hr stored White Leghorn chicken spermatozoa are shown in Table 5 and Table 6 repectively. In comparison to the total fertile period (2-8 days) at 0 hr of semen storage, higher fertility was obtained during 2-6 days after AI from CARI poultry semen diluent (93.21±1.11%) followed by EK extender (90.55±1.04%) and Tselutin extender (87.08±1.68%). Higher fertility (89.74±1.19%) was observed using CARI poultry semen diluent followed by EK extender (85.47±1.42%) and Tselutin extender (81.20±1.71%) in total fertile period. The fertility obtained at 0 hr storage for CARI poultry semen diluent and EK extender was nearly similar (Table 5).

Upon storage of diluted semen at 24 hr, superior fertility was shown by CARI poultry semen diluent (90.10±1.55%) from 2-6 days after AI followed by EK extender (83.20±2.18%) and Tselutin extender (76.13±1.41%). Similar fertility pattern was shown during 2-8 days of fertile period (Table 6).

4.4. Experiment 3: To find the effect of different dilution rates on fertility using fresh semen

Different dilution rates such as 1:2, 1:4, 1:6, 1:8, 1:10, 1:12, 1:14, 1:16, 1:18 and 1:20 were used to study their effect on fertility of white leghorn chicken spermatozoa using fresh semen. The sperm motility of chicken spermatozoa at different dilution rates was also evaluated at various storage periods (0, 24, 48, 72 and 96 hr). Since CARI poultry semen diluent was found to be more economical and expressed superior fertility as compared to other semen diluents in experiment 2, hence the same diluent was used in this experiment.

The mean values of sperm motility at different dilution rates are presented in Table 7. There was no significant difference in sperm motility at 0 hr storage period in all dilution rates. There was a gradual and subsequently significant reduction in sperm motility with the increase in dilution rates along with the storage period from 24 to 96 hrs. In 24 hr of storage period, there was a wide variation in the sperm motility recorded with a higher motility at 1:2 (89.50±0.43%) and 1:4 (88.17±0.25%) dilution rates which was comparable to control at 0 hr storage period. The lowest motility at this storage period was noticed at the dilution rate of 1:20 (9.50±0.62%). Similarly, at all the dilution rates, sperm motility reduced drastically with the enhanced storage period. There was no motility recorded at 1:14 and 1:10 dilution rates after 72 and 96 hr storage period respectively.

The fertility data on the effect of different dilution rates using fresh semen is presented in Table 8. Superior fertility was obtained from 2 to 6 days of fertile period after AI in the dilution rate of 1:2 (94.17±0.64%) followed by 1:4 (92.33±0.23%), 1:6 (90.95±0.31%), 1:8 (90.27±0.94%), 1:10 (87.23±1.35%), 1:12 (84.93±0.49%), 1:14 (82.09±0.43%), 1:16 (81.16±0.67%), 1:18 (79.13±1.83%) and 1:20 (77.33±0.49%). Similar pattern of fertility was obtained from 2-8 days of fertile period (Table 8).

Table 4: Effect of different semen diluents on sperm motility of white leghorn spermatozoa at different time intervals (Mean±SE, n=6)

Storage period (hr)	Undiluted semen	Diluted semen		
		CARI	EK	Tselutin
0	$89.83^{1}\pm0.31$	$90.17^{1}\pm0.54$	$90.33^{1}\pm0.67$	$89.33^{1}\pm0.49$
24	$10.17^{d2}\pm0.65$	$89.33^{a1}\pm0.56$	$85.50^{b2}\pm0.82$	$81.17^{c2}\pm0.31$
48	000^{d3}	$84.83^{a2}\pm0.60$	$75.25^{b3}\pm0.68$	$42.17^{c3}\pm0.40$
72	000^{d3}	$61.92^{a3}\pm0.49$	$36.17^{b4}\pm0.31$	$15.17^{c4}\pm0.31$
96	000^{d3}	$49.17^{a4}\pm0.79$	$22.50^{b5}\pm0.56$	000^{c5}

Mean values bearing different superscript (a, b, c, d) in rows differ significantly ($p<0.05$)
Mean values bearing different superscript (1, 2, 3, 4, 5) in column differ significantly ($p<0.05$)

Table 5: Effect of different semen diluents on fertilizing ability of 0 hr stored white leghorn spermatozoa (Mean±SE, n=6)

Fertile period (Days)	CARI	EK	Tselutin
2	97.46±0.84	98.38±0.82	95.75±1.49
3	95.28±2.63	95.75±2.32	93.25±2.59
4	90.75±3.58	90.50±2.15	86.63±4.64
5	93.88±3.07	86.63±3.53	87.13±3.23
6	88.68±3.10	81.50±4.34	72.63±2.90
Average: Day 2-6	**93.21^{a}±1.11**	**90.55^{ab}±1.04**	**87.08^{b}±1.68**
7	83.25±5.05	75.65±4.60	63.88±5.64
8	78.88±6.85	69.88±3.59	69.13±4.56
Average: Day 2-8	**89.74^{a}±1.19**	**85.47^{ab}±1.42**	**81.20^{b}±1.71**

Mean values bearing different superscript (a, b) in rows differ significantly ($p<0.05$)

Table 6: Effect of different semen diluents on fertilizing ability of 24 hr stored white leghorn spermatozoa (Mean±SE, n=6)

Fertile period (Days)	CARI	EK	Tselutin
2	98.38±1.10	91.88±3.36	82.50±3.66
3	92.88±3.07	84.00±4.53	87.25±4.56
4	93.25±4.12	92.13±2.33	79.63±4.11
5	83.13±5.83	77.88±6.59	75.75±7.02
6	82.88±3.01	70.13±4.46	55.50±4.91
Average: Day 2-6	**90.10^{a}±1.55**	**83.20^{b}±2.18**	**76.13^{c}±1.41**
7	75.88±4.72	71.13±4.47	55.75±6.31
8	69.38±4.48	56.63±4.81	54.50±4.44
Average: Day 2-8	**85.11^{a}±1.78**	**77.68^{b}±1.79**	**70.12^{c}±1.15**

Mean values bearing different superscript (a,b) in rows differ significantly ($p<0.05$)

Table 7: Effect of different dilution rates using CARI poultry semen diluent on sperm motility of white leghorn spermatozoa at different time intervals (Mean±SE, n=6)

Dilution rate	0 Hr	24Hr	48Hr	72Hr	96Hr
1:2	$90.25^{1}\pm0.31$	$89.50^{a1}\pm0.43$	$84.33^{a2}\pm0.33$	$65.83^{a3}\pm0.48$	$50.08^{a4}\pm0.55$
1:4	$90.17^{1}\pm0.28$	$88.17^{a2}\pm0.25$	$80.83^{b3}\pm0.75$	$60.83^{b4}\pm0.31$	$48.17^{b5}\pm0.48$
1:6	$89.75^{1}\pm0.29$	$74.92^{b2}\pm0.37$	$62.17^{c3}\pm0.70$	$56.08^{c4}\pm0.20$	$44.67^{c5}\pm0.21$
1:8	$90.00^{1}\pm0.37$	$73.95^{b2}\pm0.36$	$49.17^{d3}\pm0.40$	$41.00^{d4}\pm0.52$	$30.50^{d5}\pm0.34$
1:10	$89.62^{1}\pm0.32$	$41.67^{c2}\pm0.84$	$32.08^{e3}\pm0.20$	$25.50^{e4}\pm0.72$	000^{d5}
1:12	$89.78^{1}\pm0.31$	$33.17^{d2}\pm0.48$	$21.33^{f3}\pm0.42$	$10.08^{f4}\pm0.20$	000^{d5}
1:14	$89.53^{1}\pm0.15$	$24.33^{e2}\pm0.33$	$14.25^{g3}\pm0.31$	000^{g4}	000^{d5}
1:16	$89.50^{1}\pm0.41$	$23.34^{e2}\pm0.80$	$15.17^{g3}\pm0.54$	000^{g4}	000^{d5}
1:18	$89.56^{1}\pm0.33$	$15.00^{f2}\pm0.58$	$10.67^{h3}\pm0.49$	000^{g4}	000^{d5}
1:20	$89.33^{1}\pm0.21$	$9.50^{g2}\pm0.62$	$5.33^{i3}\pm0.56$	000^{g4}	000^{d5}

Mean values bearing different superscript (a, b, c, d, e, f, g, h, i) in column differ significantly ($p<0.05$)
Mean values bearing different superscript(1, 2, 3, 4, 5) in rows differ significantly ($p<0.05$)

Table 8: Effect of different dilution rates on fertilizing ability of 0 hr stored white leghorn spermatozoa (Mean±SE, n=6)

Fertile Period (Days)	Diluation rate									
	1:2	1:4	1:6	1:8	1:10	1:12	1:14	1:16	1:18	1:20
2	97.67±0.92	91.67±2.69	93.30±3.73	94.33±3.58	90.57±3.53	85.00±1.67	93.37±1.03	89.67±1.28	87.67±4.57	86.70±3.30
3	93.83±2.73	97.00±0.97	96.23±1.47	93.27±2.83	88.67±3.75	85.33±0.92	89.67±3.31	89.00±0.63	85.67±1.80	77.97±4.76
4	90.83±1.57	88.33±2.79	92.00±2.63	89.00±3.52	88.40±3.52	81.00±3.16	87.67±2.35	86.33±0.21	75.33±3.27	82.00±5.07
5	94.70±3.35	96.00±2.53	87.17±2.35	86.67±5.35	83.53±3.71	89.33±3.68	75.40±2.01	71.13±2.81	79.67±2.08	69.67±0.84
6	93.83±3.14	88.67±3.58	86.07±0.61	88.10±4.36	85.00±6.10	84.00±1.26	64.33±3.45	69.67±1.69	67.33±4.54	70.33±3.66
Average (Day 2-6)	**94.17^{a}±0.64**	**92.33^{ab}±0.23**	**90.95^{b}±0.31**	**90.27^{b}±0.94**	**87.23^{c}±1.35**	**84.93^{c}±0.49**	**82.09^{d}±0.43**	**81.16^{de}±0.67**	**79.13^{ef}±1.83**	**77.33^{f}±0.49**
7	89.83±2.65	84.67±5.32	83.42±2.19	84.00±6.03	87.67±1.48	80.00±7.30	79.33±1.48	73.67±4.77	56.67±1.28	63.00±3.79
8	80.17±2.04	77.00±4.12	73.33±1.65	69.27±4.98	79.33±6.58	75.00±2.92	65.00±0.73	67.33±2.74	59.33±2.97	56.00±5.67
Average (Day 2-8)	**91.55^{a}±0.82**	**89.05^{ab}±1.14**	**87.36^{b}±0.52**	**86.38^{b}±0.74**	**86.17^{b}±2.00**	**82.81^{c}±0.56**	**79.25^{d}±0.44**	**78.11^{d}±1.05**	**73.10^{e}±1.60**	**72.24^{e}±1.02**

Mean values bearing different superscript (a, b, c, d, e, f) in rows differ significantly ($p<0.05$)

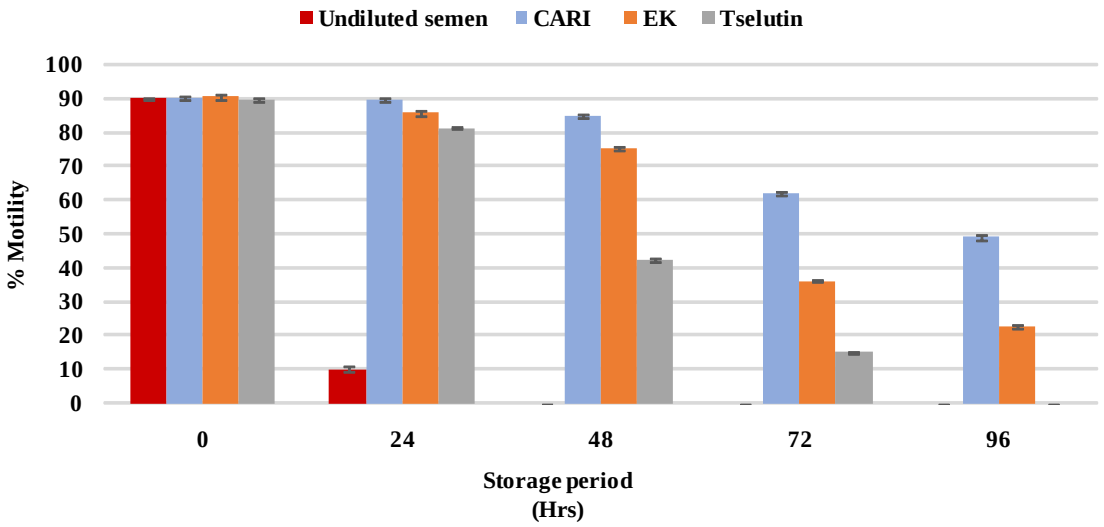

Fig. 6: Effect of different semen diluents on sperm motility of White Leghorn spermatozoa at different time intervals

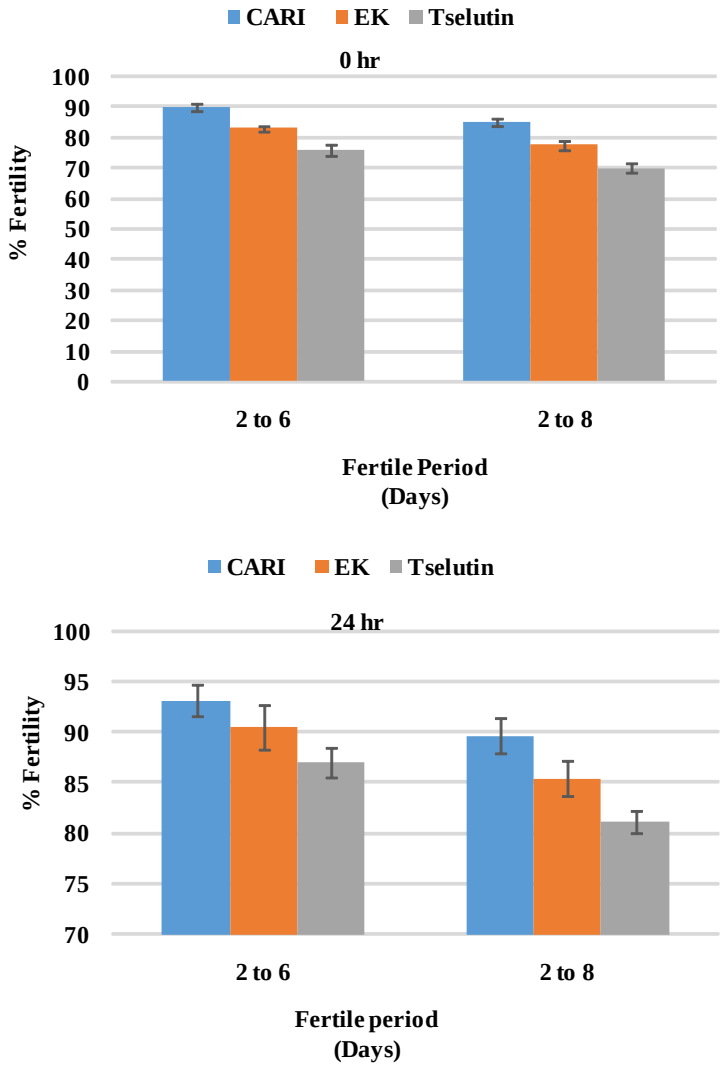

Fig. 7: Effect of different semen diluents on fertilizing ability of 0 and 24 hr stored White Leghorn spermatozoa

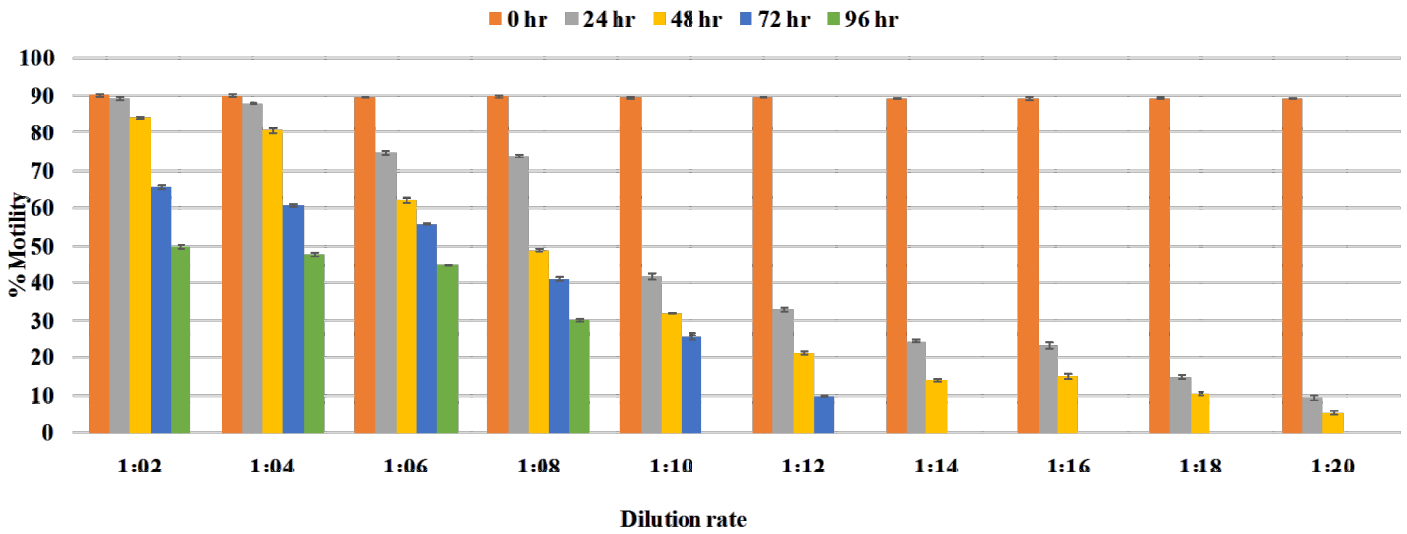

Fig. 8: Effect of different dilution rates using CARI poultry semen diluent on sperm motility of White Leghorn spermatozoa at different time intervals

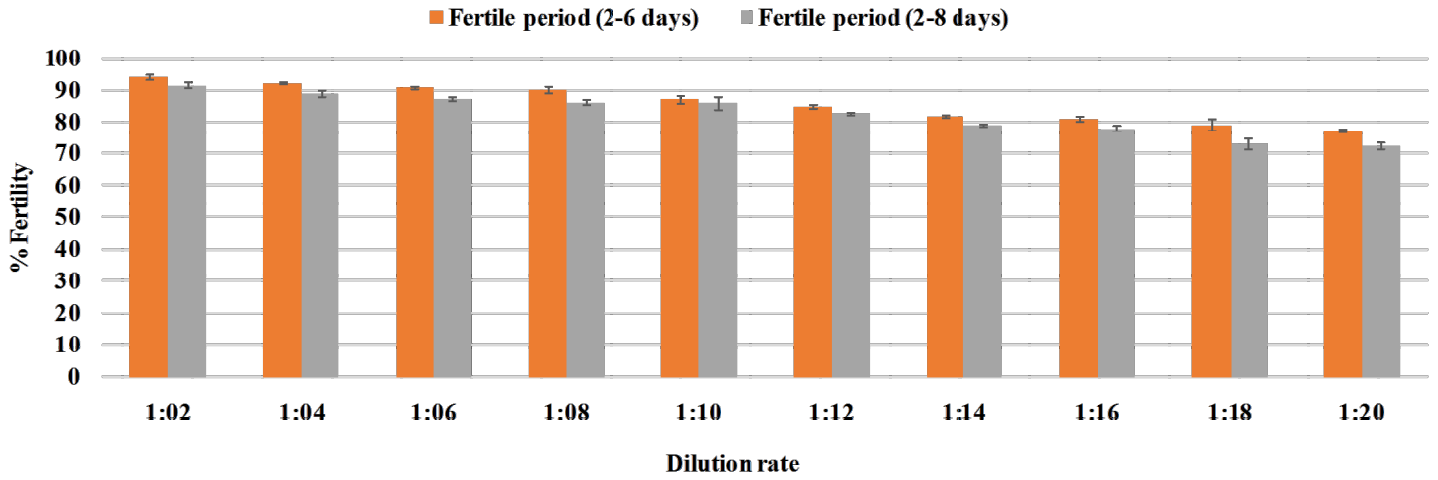

Fig. 9: Effect of different dilution rates on fertilizing ability of 0 hr stored White Leghorn spermatozoa

Discussion

5 DISCUSSION

5.1 Experiment 1: To investigate the semen physical and biochemical characteristics of White Leghorn and indigenous (Nicobari and Ankleshwar) chicken

For the success of AI in poultry, examination of the semen characteristics of different breeds of poultry and its preservation is the need of the day. Evaluation of physical and biochemical characteristics of poultry semen gives an excellent indicator of reproductive potential of cock (Zahraddeen *et al*., 2005). Such types of studies are limited in indigenous chicken breeds. Hence the present study was undertaken to evaluate the physical and biochemical characteristics of native breeds like Nicobari and Ankleshwar chicken. This data was compared with the most investigated exotic breed White Leghorn (WLH).

The semen volume of WLH was found to be 0.23±0.03 ml and 0.21±0.02 ml in winter and summer respectively, which is significantly much lower than the indigenous breeds of Nicobari (0.55±0.01 ml vs. 0.54±0.01 ml) and Ankleshwar (0.44±0.01 ml vs. 0.41±0.02 ml) in both the seasons. Between the native breeds, Nicobari breed has recorded more semen volume than Ankleshwar chicken. To the best of our knowledge, no data is available for Nicobari and Ankleshwar chicken to compare with WLH. The data obtained in this study on WLH is in agreement with Mohan *et al*. (2011) and Biswas (2007) who found significantly lesser semen volume in WLH compared to the native breed Aseel and Kadaknath chicken. In summer all the breeds exhibited lesser semen volume than winter though not significantly different. Our findings were comparable with Kundu and Panda (1990) and Elagib *et al*. (2012), who

reported comparatively lesser semen volume in summer than winter season. In contrast, some authors have also recorded increased semen volume in summer in different geographical conditions (Saeid and Al-Soudi, 1975). The wide variations in the normal volume of ejaculate reported in literatures can be attributed to the admixture of transparent fluid during massage method. In general, the hormonal fluctuations under stress conditions in different seasons might affect the semen production and volume (Kundu and Panda, 1990).

Initial sperm motility is a criterion of semen quality as higher the initial motility, the better is the quality and fertilizing capacity of semen. In our study, the sperm motility of WLH chicken was significantly higher ($p<0.05$) than Nicobari and Ankleshwar chicken in both the seasons. Mohan *et al*. (2011) showed higher motility in WLH chicken than native Aseel and Kadaknath chicken. Mukherjee and Bhattcharya (1949) found no significant variations between native breeds and Indian reared WLH while Peters *et al*. (2008) reported significant difference between WLH and other Indian breeds. Sperm motility found to decrease with summer season in all breeds and the results are comparable with Chung *et al*. (1989) and Elagib *et al*. (2012). The decrease in motility could be due to less metabolic activity of spermatozoa and increased dead and abnormal sperm counts upon heat stress during summer season. Variation in sperm concentration also reflects on the motility of spermatozoa.

The sperm concentration is another important attribute as it also determines the fertilizing ability of the semen. The sperm concentration was found to be higher in WLH than the indigenous breeds in both the seasons. Biswas (2007) found high sperm concentration in WLH than Aseel and Kadaknath native breeds. The sperm concentration in summer was significantly lesser in all the breeds. This is in accordance with Boone and Huston (1963) and Elagib *et al*. (2012) who recorded less sperm concentration at high temperature in White Plymouth Rock and White Leghorn birds respectively.

Live counts in the semen reflects the sperm viability and hence determines the fertilizing ability and survival of an embryo to hatching (Cooper and Rowell, 1958). In this investigation, the per cent live spermatozoa was higher ($p<0.05$) in WLH birds than the indigenous birds in both summer and winter seasons. Mohan *et al*. (2011) and Biswas (2007) observed more live counts in WLH than the native birds. In summer the live counts tend to decrease irrespective of the breeds.

The mean values of dead and abnormal spermatozoa were observed more in Nicobari and Ankleshwar birds than WLH. Nicobari found to have more dead and abnormal spermatozoa than Ankleshwar though not significant. Per cent dead and abnormal sperm have been found to be negatively correlated with fertility. In summer, the dead sperm counts were significantly higher in all the breeds. Hence, the more the stressful condition to the bird, the more the dead spermatozoa as reported by Bajpai (1963). The per cent abnormal spermatozoa were tend to increase in summer but not statistically significant in all the three breeds. The findings were comparable with Kundu and Panda (1990) and Elagib (2012).

The biochemical characteristics of semen of the different breeds of chicken are shown in Table 3. The concentration of total protein obtained by Biuret method seems to be higher than the earlier reports obtained by Lowry method (Mohan *et al*., 2011; Biswas, 2007). But our results are comparable with Amen and Al-Darraji (2011) who reported total protein concentration in broiler breeders by biuret method. The total protein concentration was found significantly higher in native breeds than WLH, which is in agreement with Mohan *et al*. (2011). No significant difference was seen between seasons. Kundu and Panda (1991) noticed increase in total protein concentration with increase in dead sperm counts which is in line with our study. Thurston *et al*. (1982) stated that blood proteins are poorly correlated with seminal plasma proteins. The triglyceride and cholesterol concentration were observed more in WLH than the native breeds and their concentrations were decreased in summer irrespective of the breeds. To our knowledge, data is not available in literature on triglyceride profile in seminal plasma of chicken to compare with ours. The cholesterol concentration may be correlated with sperm concentration. The results on cholesterol level in chicken seminal plasma were comparable with Biswas (2007). Douard *et al*. (2000) observed significant amounts of cholesterol esters and triglycerides in seminal plasma in contrast to spermatozoa.

The average enzyme activity of GPT and GOT in the seminal plasma of the native breeds was higher than exotic chicken (WLH) in both the seasons. This difference in enzyme activity among different breeds may be due to breed differences which is in accordance with the observations of Datta *et al*. (1980) and Manoharan (2000). The enzyme activities of GOT in our study is 15 - 20 times greater than GPT values. Hammond *et al*. (1965) and Datta *et al*.

(1980) reported 43-63 times higher activity of GOT and Mohan *et al*. (2011) observed 39-55 times higher activity of GOT than GPT. Hence, the concentration of glutamic acid is remarkably high in the seminal plasma of cocks in all observations. According to Lake and McIndoe (1959), this amino acid constitutes as much as 90% of the total amino acids in the seminal fluid and might have a special role in maintaining its osmotic balance and pH. The metabolic origin of glutamic acid in seminal plasma is however, uncertain. It might be envisaged that the high activity of GOT is primarily directed towards the formation of this important amino acid. The alkaline phophatase activity was found higher in WLH chicken and significantly lower ($p<0.05$) in native breeds. This may be due to breed difference as shown by Biswas (2007) and Shinde *et al.* (2012). Al-Darraji (2000) found a positive correlation between alkaline phosphate activity and sperm livability and concentration. In summer, the levels of transaminase enzymes were greater in all breeds than winter season. These enzymes leak from spermatozoa and other structures of male reproductive tract into the seminal plasma under stress conditions. The increase in GOT in seminal plasma is often considered as an index of sperm cell membrane instability and in turn reflect semen quality. The increase in seminal plasma GOT and GPT levels in summer season may be attributed to increased dead spermatozoa in this season and our findings are in agreement with Al-Darraji (2001) and Kundu and Panda (1991).

The calcium concentration in seminal plasma of three breeds showed no significant difference between breeds and season. However the concentration was recorded slightly higher in WLH birds than the native breeds. For calcium concentration in seminal plasma, Aghaei *et al*. (2010) found positive correlation between low/medium sperm motility groups and high sperm motility groups in broiler birds. The uric acid concentration was not significantly different between the breeds. This concentration could attribute to contamination of semen.

The hydrogen ion concentration of semen showed no significant difference with respect to breeds and seasons. The resazurin reduction test (RRT) showed greater ratio in WLH than the indigenous birds. The RRT depends on the ability of metabolically active cells to reduce the non-fluorescent dye resazurin (Alamar blue) to fluorescent resorufin and thus it can be used to monitor cell viability. Many reporters found significant correlation between RRT and fertility as

it is associated with the metabolic status of the active cells and concentration of motile sperms (Erb *et al*., 1952; Dart *et al*., 1994). In summer the values declined with significant difference ($p<0.05$), indicating poor semen quality in this season. Among the breeds, RRT level was found higher in WLH followed by Ankleshwar and Nicobari chicken. This suggested that WLH chicken spermatozoa are metabolically superior to other breeds.

5.2 Experiment 2: To study the effect of different semen extenders on fertilizing ability of chicken spermatozoa during short term preservation

Poultry semen is highly viscous and concentrated with low volume that requires the extension with suitable diluents, so that semen from proven sire can be stored and utilized for inseminating large number of hens. Further, it prolongs the sperm survival for both short and long term preservation of semen *in vitro*. Hence, the major objective of the diluent is to improve the reproductive efficiency of the cock and to reduce the cost of AI. In order for the poultry industry to take advantage of modern AI techniques, proper storage of poultry semen is necessary. Short term storage usually refers to holding the diluted semen from a few hours to a few days usually between 0°C and 5°C (Bootwalla, 1987). The effect of short term storage on the fertilizing ability of spermatozoa of diluted domestic fowl semen was first reported by Schindler *et al*. (1955).

There are several diluents available for poultry semen, both published recipes and commercially available products containing different osmotic regulators, energy sources and buffers (Bootwalla and Miles, 1992). These are currently being used frequently wherever the technique of AI is employed seriously, especially in broiler and turkey industry. Several investigators have compared the composition of various diluents and summarized fertility data across studies (Bakst, 1990; Bootwata and Miles, 1992). These studies indicated that there is no proper diluent for poultry semen. Keeping this in view, the effect of different semen extenders on fertilizing ability of chicken (WLH) spermatozoa during short term preservation was studied extensively using CARI poultry semen diluent (Mohan *et al*., 2017), EK extender (Lukaszewicz, 2002) and Tselutin extender (Tselutin *et al*., 1995) as per the technical

programme. During semen storage, characteristics of these diluents were evaluated based on the sperm motility and fertilizing ability of spermatozoa. The mean values of sperm motility recorded in white leghorn chicken is presented in Table 4. There was no significant difference among the different diluents and undiluted semen at 0 hr of storage. Clarke *et al*. (1982) also observed no difference in the motility of spermatozoa from undiluted and diluted semen of chicken and turkey. Many researchers found no significant difference in the initial sperm motility of both Indian reared white leghorn and as well as in native chicken (Mukherjee and Bhattcharya, 1949; Nayak *et al*., 1990; Shinde *et al*., 2012). At 24 hr storage period, drastic reduction in sperm motility was expressed by undiluted semen and subsequently no motility was recorded from 48 to 96 hr. It is known that sperm motility and the fertilizing ability of undiluted neat fowl semen stored in vitro usually decrease within 1 hr of collection (Carter *et al*., 1957). Therefore, to store fowl semen, the type of diluent and storage temperature are very important to avoid a reduction in sperm quality (Dumpala *et al*., 2006). In 24 hr storage period, CARI poultry semen diluent showed motility comparable to 0 hr storage period while other diluents reduced significantly. From 48 to 96 hr storage period, there was significant reduction in sperm motility of all the semen diluents. Overall CARI poultry semen diluent showing higher motility compared to other diluents (Table 4). Kammerer *et al*., (1972) found that the number of progressively motile sperm per ejaculate was the most consistent and reliable trait correlated with fertility. Several investigators found positive correlation between sperm motility and fertility (McDaniel and Craig, 1962; Monsi *et al*., 1975). Accordingly in our study, CARI poultry semen diluent showed remarkable fertility than EK and Tselutin at 24 hr storage period (Table 6).

The effect of different semen diluents on fertilizing ability of 0 hr stored chicken spermatozoa showed superior fertility during 2-6 days of fertile period with CARI poultry semen diluent (93.21±1.11%) which is comparable with EK extender (90.55±1.04%), while Tselutin extender showed comparatively lower fertility (87.08±1.68%). In case of 24 hr stored chicken semen, maximum fertility (90.10±1.55%) expressed by CARI poultry semen diluent and minimum (76.13±1.41%) by Tselutin extender (fertile period 2-6 days). Same pattern was followed in the remaining fertile period (Table 6). Similar findings were recorded with CARI poultry semen diluent when it was compared to BPSE and Lake's diluent in chicken

(Shinde *et al*., 2013; Mohan *et al*., 2017) and guinea fowl (Mohan *et al*., 2015). Subsequently, EK extender exhibited superiority over Tselutin extender at both the intervals (0 vs. 24 hr) (Table 6). The findings are in agreement with Siudzinska and Lukaszewicz (2008) who reported EK extender was better than Tselutin extender with respect to fresh semen characteristics such as number of live and morphologically normal spermatozoa. Das *et al*. (2015) showed EK extender to be superior to Lake extender and skim milk extender in overall mean semen characteristics upto 72 hr.

From this study, it can be concluded that CARI poultry semen diluent expressed superior fertility as compared to other diluents during 24 hr storage of chicken spermatozoa.

5.3. Experiment 3: To find the effect of different dilution rates on fertility using fresh semen

The main objective of AI is to achieve maximum desirable fertility following a single insemination for a period of one week or more. Generally, 100 million spermatozoa are used per insemination in AI of chicken (Etches, 1996). The present experiment was conducted to investigate if higher dilution rates of chicken semen using CARI poultry semen diluent could result optimum desired fertility in chicken. The efficient use of semen diluents for AI with low insemination dose of spermatozoa from the superior sires will help in culling the inferior breeder cocks saving huge expenditure on feed and maintenance in commercial poultry enterprises. Besides, the use of minimum optimum insemination dose could cover simultaneously large number of hens compared to the conventional method. Hence, the present study was undertaken to determine the lowest possible dilution rate of fresh semen of white legorn chicken using CARI poultry semen diluent and also sperm motility of the different different diluted semen samples were evaluated at 0, 24, 48, 72 and 96 hr of storage period.

The mean values of sperm motility at different dilution rates are presented in Table 7. There was no significant difference in sperm motility at 0 hr storage period in all dilution rates. There was a gradual and significant reduction in sperm motility with the increase in dilution rates from 24 to 96 hrs of storage period. In 24 hr of storage period, there was a wide variation in the sperm motility recorded with a higher motility in 1:2 (89.50±0.43%) dilution rate and the lowest in 1:20 (9.50±0.62%). Similarly, in 48 hr storage period, large variations

were recorded with 84.33±0.33% sperm motility in 1:2 dilution rate to 5.33±0.56% in 1:20 dilution rate. There was no motility recorded in 1:14 and 1:10 dilution rates at 72 and 96 hr storage period respectively. The results were comparable with Clarke *et al*. (1982) as with higher dilution rate, elevated sperm respiration, morphological changes and loss of motility can occur.

However, with respect to fertility study of fresh diluted semen, superior fertility of 94.17±0.64%, 92.33±0.23%, 90.95±0.31%, 90.27±0.94% was obtained in the dilution rates of 1:2, 1:4, 1:6 and 1:8 respectively in 2-6 days after AI. The dilution rates of 1:10 to 1:16 expressed fertility in the range of 81.16±0.67 to 87.23±1.35 while 1:18 and 1:20 showed 79.13±1.83% and 77.33±0.49% respectively. The possible reason for the decline in fertility with increase in dilution rate could be due to reduction in sperm concentration with increase in dilution rates as in agreement with Wilcox (1958). Several investigators have attempted to determine the minimum number of sperm required per dose of semen diluted in a variety of extenders. The results reported for minimum number of sperm ranged from 45 to 90 million **(**Rowell and Cooper, 1960; Kim *et al.*, 1974). Van Wambeke (1996) remarked that the optimum required number of spermatozoa per insemination is 200-250 million per hen and Etches (1996) suggested 100 million cells per insemination for high fertility which is currently in use at the dilution rate of 1:2. Dilution of semen with CARI poultry semen diluent at the rate of 1:2, 1:4, 1:6 and 1:8 with approximately 89, 53, 38 and 29 million sperm concentrations respectively showed optimum desired fertility of 90% and more and 1:10 dilution (24 million sperm/hen) revealed upto 87.23±1.35% fertility. The results were also comparable with Bandyopadhyay *et al*. (2006) who exhibited higher fertility levels with minimum sperm concentrations. This study revealed nearly 77% fertility during the fertile period of day 2-6 by inseminating 12 million sperm/hen (1:20 dilution). This is the very high dilution rate which expressed very good fertility. To the best of our knowledge, we do not have data in literature to compare with ours at this dilution rate. Hence, the present study also gives scope for obtaining optimum fertility levels in chicken at higher dilution rates than the conventional methods.

Summary and Conclusion

6 SUMMARY AND CONCLUSIONS

It has been well established that artificial insemination (AI) expresses better fertility than natural mating in avian species. As chicken semen is highly viscous and concentrated with low volume, the extension of neat semen with suitable diluents is required. There are many diluents available for short term storage of poultry semen with published recipes and commercially available products. These are currently being used frequently wherever the technique of AI is employed seriously. Several investigators have compared the composition of various diluents and summarized fertility data across studies. These studies indicated that there is no standard diluent for poultry semen. Keeping this in view, the present study entitled "Effect of different semen extenders on fertility of chicken spermatozoa during short term preservation" was carried out to derive the best suitable semen extender for chicken (White Leghorn) spermatozoa that gives maximum fertility by comparing different semen extenders under short term preservation. For the success of AI in poultry, examination of the semen characteristics of different breeds of poultry is the need of the day. Such works are much limited especially in indigenous chicken. Hence, the study targeted to evaluate the physical and biochemical characteristics of indigenous Nicobari and Ankleshwar chicken in comparison to White Leghorn birds. Further, the effect of different dilution rates on fertility using fresh semen was conducted to find the maximum possible dilution rate to maintain optimum fertility. The results of this study are summarized as follows.

The first experiment was conducted to investigate the physical and biochemical characteristics of White Leghorn (WLH) and indigenous (Nicobari and Ankleshwar) chicken semen. Semen samples were evaluated for the physical (volume, motility, sperm concentration,

live-dead and abnormal spermatozoa) and biochemical (pH, total protein, triglyceride, cholesterol, GPT, GOT, ALP, calcium, uric acid, RRT) characteristics in two different seasons such as winter and summer.

Mean values of semen volume were found to be significantly higher ($p<0.05$) in Nicobari followed by Ankleshwar chicken while WLH birds ejaculated the least semen volume in both the seasons. In summer all the breeds exhibited lesser semen volume than winter. The sperm motility, concentration and live counts were recorded significantly higher ($p<0.05$) in WLH chicken than the native breeds in both the seasons. The mean values of dead and abnormal spermatozoa were observed more ($p<0.05$) in Nicobari and Ankleshwar chicken followed by WLH in both the seasons. The semen quality was found to decline in summer with significant reduction in sperm motility, concentration and live counts while the dead and abnormal sperm counts tend to increase. Based on the semen characteristics between the native breeds, the semen quality of Ankleshwar chicken was found to be better than Nicobari breed.

The total protein concentration of seminal plasma was higher in native breeds than WLH, while the triglyceride and cholesterol concentrations were recorded more in WLH than the native breeds. Their concentration did not differ significantly between seasons in all the breeds. The mean values of GPT and GOT activity in seminal plasma was comparatively higher in native breeds than the exotic breed (WLH). Both GOT and GPT enzymes tend to increase ($p<0.05$) in summer. The ALP activity was found higher ($p<0.05$) in WLH than indigenous breeds and the concentration tend to decrease in summer without significant difference. The calcium, uric acid and hydrogen ion concentration in the seminal plasma showed no significant difference among the breeds as well as between seasons. The resazurin reduction test (RRT) showed greater ratio in white Leghorn followed by Ankleshwar and Nicobari chicken. The ratio significantly ($p<0.05$) declined in summer irrespective of the breeds.

The second experiment was carried out to study the effect of different semen extenders on fertilizing ability of chicken (WLH) spermatozoa during short term preservation. Various diluents such as CARI poultry semen diluent, EK extender and Tselutin diluent were chosen for the study. These diluents were tested for sperm motility (0, 24, 48, 72 and 96 hr) and

fertilizing ability of chicken spermatozoa at different storage intervals (0 and 24 hr). No significant difference was observed in sperm motility among the different diluents and undiluted semen at 0 hr (freshly ejaculated) storage period. At 24 hr storage, a significant ($p<0.05$) reduction was found in sperm motility of undiluted semen and further no motility was recorded from 48-96 hr. The CARI poultry semen diluent at 24 hr showed motility comparable to 0 hr storage, while the other diluents revealed significant reduction in motility. From 48-96 hr storage period, there was significant reduction in sperm motility in all the semen diluents.

The effect of different semen diluents on fertilizing ability of 0 hr stored chicken spermatozoa showed superior fertility during 2-6 days of fertile period with CARI poultry semen diluent, which is comparable with EK extender, while Tselutin extender showed lower fertility. In case of 24 hr stored chicken semen, maximum fertility was expressed by CARI poultry semen diluent and minimum by Tselutin extender during the fertile period of 2-6 days. Same pattern was followed in the remaining fertile period. EK extender exhibited superiority over Tselutin extender at both the intervals (0 vs. 24 hr). Overall, CARI poultry semen diluent expressed superior fertility as compared to other diluents during 24 hr storage of chicken spermatozoa.

The third experiment was conducted to find the effect of different dilution rates (1:2, 1:4, 1:6, 1:8, 1:10, 1:12, 1:14, 1:16, 1:18 and 1:20) on fertility using fresh chicken semen. CARI poultry semen diluent was chosen for the study, since this diluent was economical and expressed superior fertility as compared to other diluents as investigated in experiment 2. Sperm motility was observed at different storage periods *viz.* 0, 24, 48, 72 and 96 hr. There was no significant difference in sperm motility at 0 hr storage period in all the dilution rates. There was a gradual and subsequently significant reduction in the sperm motility with the increase in dilution rates along with enhanced storage period. There was no motility recorded in 1:14 and 1:10 dilution rates at 72 and 96 hr storage period respectively. With respect to fertility assessment of fresh diluted semen, superior fertility in the range of 90.27±0.94% to 94.17±0.64% was obtained in the dilution rates of 1:2, 1:4, 1:6 and 1:8 during 2-6 days of fertile period after AI. The dilution rates of 1:10 to 1:16 expressed fertility in the range of 81.16±0.67% to 87.23±1.35% while 1:18 and 1:20 dilution rates showed 79.13±1.83% and 77.33±0.49% respectively.

Conclusion

1. White Leghorn birds donated good quality of semen compared to indigenous Nicobari and Ankleshwar chicken as revealed from the physical and biochemical characteristics of semen.

2. Significant variations ($p<0.05$) were found between winter and summer seasons in physical (sperm motility, concentration, live spermatozoa, dead spermatozoa) and biochemical (GPT, GOT, RRT) characteristics of semen in all the breeds.

3. Irrespective of the breeds, semen quality was found superior in winter season than summer.

4. CARI poultry semen diluent expressed higher motility as compared to other diluents at different storage intervals (0, 24, 48, 72 and 96 hr)

5. At 0 hr (freshly ejaculated) storage of semen, superior fertility was expressed by CARI poultry semen diluent, which was comparable with EK extender while Tselutin extender showed lower fertility.

6. At 24 hr storage of semen, superior fertility was observed in CARI poultry semen diluent as compared to other diluents.

7. Using fresh semen with CARI poultry semen diluent, superior fertility was recorded in the dilution rates of 1:2, 1:4, 1:6 and 1:8 as compared to higher dilution rates (1:10-1:20).

Mini Abstract

7 MINI ABSTRACT

Attempts were made to determine the semen quality characteristics of indigenous (Nicobari and Ankleshwar) breeds in comparison to White Leghorn (WLH) in winter and summer season. The semen volume was observed lower while the semen motility, concentration and live counts were recorded higher in WLH than the native breeds. Dead and abnormal sperm counts were recorded more in Nicobari and Ankleshwar chicken. The physical characteristics of semen tend to deteriorate in summer irrespective of the breeds. The total protein concentration was observed lesser while triglyceride and cholesterol concentration were recorded more in WLH than the native breeds. The transaminase activity was found higher and alkaline phosphatase activity was recorded lower in native chicken than WLH birds. The mean values of Resazurin Reduction Test (RRT) were found higher in WLH than Nicobari and Ankleshwar chicken. In summer, the transaminase activity in seminal plasma increased while the RRT ratio declined irrespective of the breeds. Overall, WLH showed superior semen quality followed by Ankleshwar and Nicobari. Various semen diluents (CARI, EK, Tselutin) were used in this study in which, CARI poultry semen diluent expressed higher motility as compared to others at different storage intervals (0, 24, 48, 72 and 96 hr). Effect of various diluents on fertility of freshly ejaculated (0 hr) chicken spermatozoa during 2-6 and 2-8 days of fertile period showed superior fertility in CARI poultry semen diluent, which is comparable with EK extender, while Tselutin extender showed lower fertility. In case of 24 hr stored chicken semen, maximum fertility was expressed by CARI poultry semen diluent as compared to other diluents. While studying the effect of different dilution rates on motility of chicken spermatozoa, all the dilution rates (1:2-1:20) expressed nearly similar high motility at 0 hr storage period. Subsequently, there was a gradual reduction in the sperm motility with the increase in dilution rates and storage period from 24-96 hr. No motility was observed in 1:14 and 1:10 dilution rates at 72 and 96 hr storage period respectively. However, with respect to fertility assessment of freshly diluted semen, superior fertility was obtained in the dilution rates of 1:2, 1:4, 1:6 and 1:8 during 2-6 days of fertile period after AI using CARI poultry semen diluent. From this study, it can be concluded that irrespective of season, WLH expressed better semen quality. However, it was deteriorated in summer months in all the breeds. As compared to others, CARI diluent showed superior fertility.

लघु सारांश

8 लघु सारांश

प्रस्तुत अध्ययन में ग्रीष्म एवं शीत ऋतु में स्वदेशी कुक्कुट नस्लों (निकोबारी एवं अंकलेश्वर) तथा सफेद लेगहार्न की वीर्य गुणवत्ता की तुलना का प्रयास किया गया है। देशी नस्लों की तुलना में सफेद लेगहार्न मुर्गों में वीर्य की मात्रा कम पायी गई जबकि वीर्य गतिशीलता, सधनता एवं जीवित शुक्राणुओं की संख्या सफेद लेगहार्न मुर्गों में ज्यादा पाई गई। मृत एवं असमान्य शुक्राणुओं की संख्या निकोबारी एवं अंकलेश्वर में ज्यादा पाई गई। ग्रीष्म ऋतु में सभी नस्लों के वीर्य की भौतिक विशेषताओं में गिरावट पायी गई। सफेद लेगहार्न की तुलना में स्वदेशी कुक्कुट नस्लों में ट्रांसअमीनेस का स्तर अधिक एवं अलकलाइन फॉस्फेटेस स्तर कम पाया गया। निकोबारी और अंकलेश्वर नस्लों की तुलना में सफेद लेगहार्न मुर्गों में रिजाजुरीन रिडक्सन टेस्ट का औसत मान ज्यादा देखा गया। ग्रीष्म ऋतु में सभी नस्लों में ट्रांसअमीनेस स्तर बढ़ा मिला एवं रिजाजुरीन रिडक्सन का अनुपात घटा हुआ पाया गया। कुल मिलाकर सफेद लेगहार्न के वीर्य की गुणवत्ता अंकलेश्वर तथा निकोबारी से उच्चतर पाई गई। इस अध्ययन के दौरान विभिन्न वीर्य डाइल्युएंट प्रयोग किए गए (इके, कैरी, सेल्युटिन) जिनमें कैरी कुक्कुट वीर्य डाइल्युएंट में शुक्राणुओं की गतिशीलता विभिन्न संग्रहण अंतरालो (0, 24, 48, 72 एवं 96 घण्टे) में अधिक पाई गई। 2–6 और 2–8 दिनों के अंतराल पर जीवित उपजाऊ शुक्राणुओं पर विभिन्न वीर्य डाइल्युएंट के प्रभाव को देखा गया। कैरी कुक्कुट वीर्य डाइल्युएंट का प्रभाव इके एक्सटेण्डर से बेहतर पाया गया जबकि सेल्यूटिन एक्सटेण्डर का प्रभाव कम था। वीर्य को विभिन्न वीर्य डाइल्युएंट के साथ 24 घण्टों तक संग्रहण करके देखा गया जिसमें दूसरों की तुलना में कैरी वीर्य डाइल्युएंट में बेहतर प्रजनन क्षमता दशाई गयी। विभिन्न वीर्य डाइल्युसन करके शुक्राणुओं की गतिशीलता का अध्ययन किया गया। वीर्य के सभी डाइल्युसन की समान शुक्राणु गतिशीलता दिखाई दी। जैसे–जैसे संग्रहण का समय (24 से 96 घण्टे) और डाइल्युसन की दरों को बढ़ाया गया, वैसे–वैसे शुक्राणुओं की गतिशीलता में धीरे–धीरे कमी दिखाई दी। 1:14 डाइल्युसन दर पर 72 घण्टों तक और 1:10 डाइल्युसन दर पर 96 घण्टों तक संग्रहित करने पर शुक्राणुओं में गतिशीलता नहीं पाई गयी। कैरी कुक्कुट वीर्य डायल्युएंट का उपयोग करके वीर्य की उपजाऊ विशेषता को देखा गया जोकि 2–6 दिनों में 1:2, 1:4, 1:6 और 1:8 डायल्युसन दरों में बेहतर पाई गयी। उपरोक्त शोध से यह निष्कर्ष निकाला जा सकता है कि प्रत्येक ऋतु में सफेद लेगहार्न मुर्गों का वीर्य देशी नस्लों के मुर्गों से अच्छा है। यद्यपि वीर्य की गुणवत्ता सभी नस्लों में ग्रीष्म ऋतु में खराब हो जाती है। विभिन्न प्रकार के डाइल्युटरों का अध्ययन करने पर पता चला की कैरी कुक्कुट वीर्य डाइल्युटर सर्वेश्रेष्ठ है।

References

9 REFERENCES

Adamstone, F. B. and Card, L. E. 1934. A study of the spermatozoon of the fowl with particular reference to osmophilic bodies in the sperm head. J. Morphol., **56**(2): 325-337.

Aghaei, A., Tabatabaei, S. and Nazari, M. 2010. The correlation between mineral concentration of seminal plasma and spermatozoa motility in Rooster. J. Anim. Vet. Adv., **9**(10): 1476-1478.

Ahlawat, S. P. S. and Chatterjee, R. N. 2002. Conservation of indigenous poultry germplasm of A & N Islands. Proceedings of National Workshop on Characterization and Conservation of Indigenous Poultry Germplasm. CARI, Port Blair, India. 26-27 February, pp. 9-16.

Al-Darraji, H. J. 2000. Effect of vitamin E on semen quality and fertilizing ability of roosters. Dirasat, Agric. Sci., **27**: 360-365.

Al-Darraji, H. J. 2001. Effects of holding temperature and time on acrosomal abnormalities of fowl sperm. In. J. Anim. Sci., **7**: 32-34.

Alkan, S., Baran, A., Ozdas, O. B. and Evecen, M. 2001. Morphological defects in turkey semen. Tur. J. Vet. Anim. Sci., **26**: 1087-1092.

Amen, M. H. M. and Al-Daraji, H. J. 2011. Effect of dietary Zinc supplementation on some seminal plasma characteristics of broiler breeder males. J. Poult. Sci., **10**(10): 814-818.

Anash, G. A., Buckland, R. B., Chan, C. W. and Touchburn, S. P. 1983. Effects of floor and cage housing of male turkeys on semen production and fertility of fresh and stored semen. Poult. Sci., **62**: 1899-1901.

Anash, G. A., Buckland, R. B., Crober D. C. and Softon, A. E. 1980. Artificial insemination individually caged broiler breeders. II. The relationship of juvenile male traits with subsequent reproductive traits. Poult. Sci., **59**: 1912-1917.

Anderson, J. 2001. The semen of animals and its use for artificial insemination. Green world publishers, India.

Bah, G. S., Chaudhari, S. U. R. and Al-Amin, J. D. 2001. Semen characteristics of local breeder cocks in the Sahel region of Nigeria. Revue Elev. Med. Vet. Pays trop., **54(2)**: 153-158.

Bajpai, P. K. 1963. The effect of photoperiodicity on semen characteristics of poultry. Poult. Sci., **42**: 462-465.

Bakst, M. R. 1981. Sperm recovery from oviducts of turkeys at known intervals after insemination and oviposition. J. Reprod. Fertil., **62**: 159-164.

Bakst, M. R. 1990. Preservation of avian cells. In: Crawford, R.D. Poultry breeding, Elsevier, Amsterdam: 91-108.

Bakst, M. R. 1994. Oviducal sperm storage in poultry. In: Proc. 9th European Poultry Conference, Glasgow.

Bakst, M. R. 2011. Physiology and Endocrinology Symposium: Role of the oviduct in maintaining sustained fertility in hens. J. Anim. Sci., **89**(5): 1323-1329.

Bakst, M. R. and Dymond, J. S. 2013. Artificial Insemination in Poultry, Success in Artificial Insemination. In: INTECH, Quality of Semen and Diagnostics Employed (ed. Lemma, A.), Chap.10. pp. 175-195.

Bakst, M. R., Wishart, G. and Brillard, J. P. 1994. Oviductal sperm selection, transport, and storage in poultry. Poult. Sci. Rev., **5**: 117-143.

Bandyopadhyay, U. K., Chaudhuri, D., Johari, D. C. and Agarwal, S.K. 2006. Determination of minimum insemination dose of spermatozoa for optimum fertility and profitability in white leghorn chicken using artificial insemination. Indian J. Poult. Sci., **41**(1): 95-97.

Bearden, H. J., Fuquay, J. W. and Willard, S. T. 2004. Applied Animal Reproduction. (6th ed). Pearson Education, Inc. New Jersey. pp. 109-127.

Bell, D. J. and Freeman, B. M. 1971. Physiology and biochemistry of the domestic fowl. Volume 3, Academic Press, London.

Birkhead, T. R. and Moller, A. P. 1992. Numbers and size of sperm storage tubules and the duration of sperm storage in birds: A comparative study. Biol. J. Linnean. Soc., **45**(4): 363-372.

Biswas, A. 2007. Age dependent variation in physio-biochemical characteristics of reproductive system in different breeds of desi fowl. Ph.D. thesis, Indian Veterinary Research Institute.

Blanco, J. M., Gee, G., Wildt, D. E. and Donoghue, A. M., 2000. Species variation in osmotic, cryoprotectant, and cooling rate tolerance in poultry, eagle, and peregrine falcon spermatozoa. Biol. Reprod., **63**: 1164-1171.

Blanco, J. M., Long, J. A., Gee, G., Donoghue, A. M. and Wildt, D. E., 2008. Osmotic tolerance of avian spermatozoa: Influence of time, temperature, cryoprotectant and membrane ion pump function on sperm viability. Cryobiology. **56**: 8-14.

Blesbois, E. and Brillard, J. P. 2007. Specific features of *in vivo* and *in vitro* sperm storage in birds. Animal. **1***:* 1472-1481.

Blesbois, E. and de Reviers, M. 1992. Effect of different fractions of seminal plasma on the fertilising ability of fowl spermatozoa stored *in vitro*. J. Reprod. Fertil., **95**: 263-268.

Blesbois, E., Grasseau, I. and Seigneurin, F., 2005. Membrane fluidity and the ability to survive cryopreservation in domestic bird spermatozoa. Reproduction. **129**: 371-378.

Blesbois, E., Seigneurin, F., Grasseau, I., Limouzin, C., Besnard, J. B., Gourichon, D., Coquuerelle, G., Rault, P. and Tixier-Boichard, M. 2007. Semen cryopreservation for ex situ management of genetic diversity in chicken: creation of the French avian cryobanks. Poult. Sci., **86**: 555-564.

Boone M. A. and Huston. T. M. 1963. Effects of high temperature on semen production and fertility in the domestic fowl. Poult. Sci., **42**: 670.

Boone, M. A. 1968. Family differences in semen quality in one strain of White Plymouth Rocks. Poult. Sci., **47**: 1049-1051.

Bootwala, S. M. and Miles, R. D. 1992. Development of extenders for domestic fowl semen. World's Poult. Sci. J. **48**: 121-128.

Bootwalla, S. M. 1987. Factors affecting semen quality and its effect on the fertilizing ability of broiler breeder spermatozoa using a fluorometric assay. Ph.D. Thesis, Oregon State University. USA.

Bratte, L. and Ibe, S. N. 1989. Optimal insemination interval, semen dose and dilution for optimum fertility in chickens under humid tropical conditions. Anim. Reprod. Sci., **20**: 179-185.

Brillard, J. P. 1993. Sperm storage and transport following natural mating and artificial insemination. Poult. Sci., **72**: 923-928.

Brillard, J. P. 2003. Practical aspects of fertility in poultry. World's Poult. Sci. J., **59**: 441-446.

Brillard, J. P. and McDaniel, G. R. 1985. The reliability and efficiency of various methods for estimating spermatozoa concentration. Poult. Sci., **64**: 155-158.

Brillard, J. P., Beaumont, C. and Scheller, M. F. 1998. Physiological responses of hens divergently selected on the number of chicks obtained from a single insemination. J. Reprod. Fertil., **114**(1): 111-117.

Buckland, R. B. 1970. Some enzyme activities of chicken spermatozoa. Poult. Sci., **49**(6): 1638-1641.

Burrows, W. H. and Quinn, J. P. 1936. Artificial insemination in fowls. J Hered., **27**(1): 31-38.

Burrows, W. H. and Quinn, J. P. 1937. The collection of spermatozoa from the domestic fowl and turkey. Poult. Sci., **24**: 19-24.

Carson, J. D., Lorenz, F. W. and Asmundson. V. S. *1955*. Semen production in the turkey male. 1. Seasonal variation. Poult Sci., **34**: 336-343.

Carter, R. D., McCartney, M.G., Chamberlin, V.D. and Wyne, J.W. 1957. The effect of storage time and temperature on fertilizing ability of turkey semen. Poult. Sci., **36**: 618-621.

Cerolini, S., Kelso., K.A., Noble, R.C., Speake, B.K., Pizzi, F. and Cavalchini, L.G. 1997[a].Relationship between spermatozoan lipid composition and fertility during aging of chickens. Boil. Reprod., **57**: 976-980.

Cerolini, S., Surai, P., Maldjian, A., Gliozzi, T. and Noble, R. 1997[b]. Lipid composition of semen in different fowl breeders. Poult Avian Biol Rev., **8**: 141-148.

Chung, H. S., Hwang, H. C. and Tai, C. 1989. Seasonal effect on semen characteristics of leghorn lines. J. Taiwan Livest. Res., **22**: 59-77.

Churchil, R. R., Praveena, P. E. and Sharma, D. 2014. Semen quality parameters, their inter-relationship and post-washing sperm attributes of Rhode Island Red roosters, Vet. World. **7**(12): 1117-1122.

Clarke, R. N., Bakst, M. R. and Ottinger, M. A., 1984. Morphological changes in chicken and turkey spermatozoa incubated under various conditions. Poult. Sci., **63**: 801-805.

Clarke, R. N., Sexton, T. J. and Ottinger, M. A. 1982. Effect of holding temperature and storage time on respiratory rate, motility, and fertility of chicken and turkey semen. Poult. Sci., **61**: 1912-1917.

Cole, H. H. and Cupps, P. T. 1977**.** Reproduction in domestic animals. (3rd ed). Academic press. New York. pp. 195.

Cooper, D. M. and Rowell, J. G. 1958. Relations between fertility, embryonic survival and some semen characteristics in the chicken. Poult. Sci., **37**: 699.

Dart, M. G., Mesta, J., Cremshaw, C. and Ericsson, S. A. 1994. Modified resazurin test for determining the fertility potential of bovine spermatozoa. Arch. Androl., **33**: 71

Das, K. K., Ahmed, K. and Deka, N. 2015. Effect of different extenders on the quality of poultry semen during preservation at 4-5°C. Int. J. Appl. Pure. Sci. Agric., **1**(7): 105-107.

Datta, I. C., Prabhu, G. A. and Khan, A. G. 1980. Influence of genotype and season upon phosphomonoesterase and transaminase activity in seminal plasma of fowl (*Gallus domesticus*). Indian. J. Exp. Biol., **18**(10): 1195-1198.

De Silva, P. L. G. 1963. Evidence for absence of an effect of temperature shock on fertilizing ability of fowl spermatozoa. J. Reprod. Fertil., **6**: 371-374.

Department of Animal Husbandry Dairying & Fisheries (DADF), Annual report 2015-2016.

Dhama, K., Singh, R. P., Karthik, K., Chakraborty, S., Tiwari, R., Wani, M. Y. and Mohan, J. 2014. Artificial insemination in poultry and possible transmission of infectious pathogens: A review. Asian J. Anim. Vet. Adv., **9**: 211-228.

Donoghue, A. M. 1999. Prospective approaches to avoid flock fertility: Predictive assessment of sperm function traits in poultry. Poult. Sci., **78**: 437-443.

Donoghue, A. M. and Wishart, G. J. 2000. Storage of poultry semen. Anim. Reprod. Sci., **62**: 213-232.

Donoghue, A. M., Holsberger, D. R., Evenson, D. P. and Froman, D. P. 1998. Semen donor selection by *in vitro* sperm mobility increases fertility and semen storage in the turkey hen. J. Androl., **19**: 295-301.

Douard, V., Hermier, D. and Blesbois, E. 2000. Changes in turkey semen lipids during liquid *in vitro* storage. Biol. Reprod., **63**: 1450-1456.

Dumpala, P. R., Parker, H. M. and McDaniel, C. D. 2006. The effect of semen storage temperature and diluent type on the sperm quality index of broiler breeder semen. Int. J. Poult. Sci., **5**(9): 838-845.

Duncan, B. 1955. Multiple range and multiple F tests. Biometrics. **11**: 1-12.

Elagib, H. A. A., Musharaf, N. A., Makawi, S. A. and Mohamed, H. E. 2012. The effects of age and season on semen characteristics of white leghorn cocks under sudan conditions. Int. J. Poult. Sci., **11**(1): 47-49.

Erb, R. A., Ehlers, M. A. and Flerchinger, F. H. 1952. Modified resazurin reduction test for estimating fertilizing capacity of bull semen. J. Dairy Sci., **35**(10): 881-888.

Etches, R. J. 1996. Reproduction in Poultry. CAB International, University Press, Cambridge, UK.

FAO. 1995. Global project for the maintenance of domestic animal genetic diversity (MoDAD)-Draft project formulation report, FAO, Rome, Italy.

Froman, D. P., Feltmann, A. J. and McLean, D. J. 1997. Increased fecundity resulting from semen donor selection based upon in vitro sperm motility. Poult. Sci., **77**(1): 73-77.

Froman, D. P., Wardell, J. C. and Feltmann, A. J. 2006. Sperm mobility: deduction of a model explaining phenotypic variation in roosters (*Gallus domesticus*). Biol. Reprod., **74**: 487-491.

Froman, D. P., Feltmann, A. J., Rhoads, M. L. and Kirby, J. D. 1999. Sperm mobility: A primary determinant of fertility in the domestic fowl (*Gallus domesticus*). Biol. Reprod., **61**: 400-405.

GAU Report. 2003. Annual Progress Report, 2003-04, Network Project on Survey of Poultry Genetic Resources: Ankleshwar poultry, by College of Veterinary Science and Animal Husbandry, Gujarat Agriculture University, Anand, India.

Gee, G. F. 1995. Artificial insemination and cryopreservation of semen from nondomestic birds. In: Bakst, M. R. and Wishart, G. J. (eds), Proc. First Int. Symp. Artificial Insemination of Poultry. Poult. Sci. Asso., Savoy, Illinois. pp. 262-279.

Gee, G. F., Bertschinger, H., Donoghue, A. M., Blanco, J. M. and Soley, J. 2004. Reproduction in non-domestic birds: Physiology, semen collection, artificial insemination and cryopreservation. Avian Poult. Biol. Rev., **15**: 47-101.

Getachew, T. 2016. A review article of artificial insemination in poultry. World's Vet. J., **6**(1): 25-33.

Gordon, I. 2005. Reproductive technologies in farm animals. CABI Publishing, UK. Chap: 1, pp.16-28.

Graham, E. F. 1976. Fundamentals of the preservation of spermatozoa in the integrity of frozen spermatozoa. Proc. of a Round table Conference. Nat. Acad. Sci., 4-44.

Graham, E. F., Schmehl, M. L., Brown, K. I., Crabo, B. G. and Ertel, G. 1971. Some chemical studies of the female reproductive tract and seminal plasma of the male turkey and their relationship to fertility. Poult. Sci., 50(4): 1170-81.

Hafez, E. S. E. 1995. Reproduction in farm animals. (6th ed), Lea and Febiger, Philadelphia, USA.

Hammond, M., Boone, M. A. and Barnett, B. D. 1965. Study of the glucose, electrolytes, enzymes and nitrogen components of fowl in seminal plasma. J. Reprod. Fertil., **10**: 21-28.

Hartmann, W. and Gleichauf, R. 1975. Effect of line on semen production in White Leghorn cocks and its relationships with fertility. Poult. Abst., **1**: 212.

Haunshi, S., Doley, S. and Kadirvel, G. 2010. Comparative studies on egg, meat and semen qualities of native and improved chicken varieties developed for backyard poultry production. Trop. Anim. Health Prod., **42**: 1013-1019.

Hess, R. A. and Thurston, R. J. 1984. Protein, cholesterol, acid phosphatase and aspartate aminotransaminase in the seminal plasma of turkeys (*Meleagris gallopavo*) producing normal white or abnormal yellow semen. Biol. Reprod., **31**(2): 239-243.

Hibbitt, K. G. and Benians, M. 1971. Some effects *in vivo* of the teat canal and effects *in vitro* of cation proteins on staphylococci. J. Gen. Microbiol., **68**: 123-128.

ICRA Research Services. 2016. Indian Poultry Industry, Continued high poultry feed costs combined with non remunerative broiler realizations put pressure on sector profitability. (Available at http://www.icra.in/Files/ticker/SH-2016-1-ICRA-Poultry.pdf)

Islam, M. S., Howlider, M. A. R., Kabir, F. and Alam, J. 2002. Comparative assessment of fertility and hatchability of Barred Plymouth Rock, White leghorn, Rhode Island Red and White Rock Hen. Int. J. Poult. Sci., **1**: 85-90.

Ivanov, E. 1913. Experience Sur la Fecondation Artificielle des Oiseaux. Compt. Rend. Biol., **75**: 371-374.

Jull, M. A. and Quinn, J. P. 1931. The inheritance of body weight in the domestic fowl. J. Hered., **22**: 283-291.

Kamar, G. A. R. 1960. The influence of semen characteristics on hatching results of chicken eggs. Poult. Sci., **39**: 188-193.

Kammerer, D.M., Moreng, R.E., Muller, H.D. and Hobbs, H.W. 1972. Turkey semen evaluation for fertility prediction. Poult. Sci., **51**: 77-82.

Kanyinji, F. and Maeda, T. 2010. Additional dietary calcium fed to Barred Plymouth Rock roosters reduces blood cholesterol, elevates seminal calcium and enhances sperm motility, thermo-tolerance and cryosurvivability. Anim. Reprod. Sci., **120**: 158-165.

Khalifa, M. A., Shebaita, M. K., Kamar, G. A. R. and Sarhan, A. A. 1983. Studies on some economical traits in Fayomi and its crosses along with semen evaluation. Anim Breed Abstr., **52**: 7566.

Khan, R. U., Rahman, Z. U., Javed, I. and Muhammad, F. 2012. Effects of vitamins, probiotics, and protein level on semen traits and some seminal plasma macro and microminerals of male broiler breeders after zinc-induced molting. Boil. Trace. Elem. Res., **148**: 44-52.

Khongsen, M., Niyomdecha, A. and Boongaew, P. 2015. Effect of fresh semen dilution rates of Dang cock (Thai native chicken) on percentage of fertile egg in commercial layers. Khon. Kaen. Agr. J., **43**(2): 212-215.

Kim, J. K., Shin, W. J., Suh, G. S., Sul, D. S. and Lee, J. K. 1974. Effect of dilution rate of semen and insemination interval on fertility in the domestic fowl. Res. Rep. Off. Rural Develop., Suwon, Korea, p. 77-81.

Kumarova, V. V. 1970. Biochemical indices and fertility ability of cock semen. Anim. Abstr., **39**: 5296.

Kundu, A. and Panda, J.N. 1990. Variation in physical characteristics of semen of white leghorn under hot and humid environment. Indian J. Poult. Sci. Vol. **25**(3): 195-203.

Kundu, A. and Panda, J.N. 1991. Variations in biochemical characteristics of semen of white leghorn cocks adapted to tropical environment. Indian J. Poult. Sci. Vol. **26**(1): 26-29.

Lake, P. E. 1960. Studies on the dilution and storage of fowl semen. J. Reprod. Fertil., 1: 30-35.

Lake, P. E. 1983. Factors affecting the fertility level in poultry, with special reference to artificial insemination. Agricultural Research Council. **39**(2): 106-117.

Lake, P. E. 1995. Historical perspective of artificial insemination technology. In: Bakst, M. R. and Wishart, G. J. (eds), Proc. First Int. Symp. Artificial Insemination of Poultry. Poult. Sci. Asso., Savoy, Illinois. pp. 1-20.

Lake, P. E. and El Jack, M. H. 1964. Further observations on the chemical composition of the seminal plasma of the domestic cock. Proc. V. Intern. Congr. Anim. Reprod. and A.I., Trento, Italy, II: 359-363.

Lake, P. E. and McIndoe, W. M. 1959. The glutamic acid and creatine content of cock seminal plasma. Biochem. J., **71**: 303-306.

Lake, P. E. and Stewart, J. M. 1978. Artificial insemination in poultry. Bulletin 213. Her Majesty's Stationary office, Ministry of Agriculture, Fisheries and Food, London.

Lake, P. E. and Wishart, G. J. 1984. Comparative physiology of turkey and fowl semen. In: Cunningham, F. J., Lake, P. E. and Hewitt, D. (eds). Reprod. Biol. Poult., Longman group, Harlow. pp. 151-160.

Lake, P. E., Butler, E. J., McCallum and MacIntyre, I. J. 1958. A chemical analysis of the seminal and blood plasma of the cock. Quart. J. Expt. Physiol. 43: 309-313.

Latif, A., Ijaz, A., Aleem, M. and Mahmud, A, 2005. Effect of osmotic pressure and pH on the short-term storage and fertility of broiler breeder sperm. Pak. Vet. J., **25**: 179-183.

Lorenz, F. W. 1964. Recent research on fertility and artificial insemination of domestic birds. 5th Int. Congr. Anim. Reprod., Trento, Italy, **4**: 7-32.

Lukaszewicz, E. 2002. An effective method for freezing White Italian gander semen. Theriogenology. **58**:19-27.

Mann, T. 1964. The biochemistry of semen and of the male reproductive tract. John Wiley & Sons, Inc., New York.

Manoharan, S. 2000. Studies on chicken semen dilutors and its role in short term preservation of spermatozoa. M.V.Sc. Thesis, Deemed University, IVRI, Izatnagar, India.

Maule, J. P. 1962. The semen of animals and artificial insemination. Common Wealth Agricultural Bureau, England.

McDaniel, G. R. and Craig, J. V. 1959. Behaviour traits, semen measurements and fertility of White Leghorn males. Poult. Sci., **38**: 1005-1014.

McDaniel, G. R. and Craig, J. V. 1962. Predicting mal fertilizing capacity in high and low fertility strains of chicken. Poult. Sci., **41**: 866-869.

Mohan, J. and Moudgal, R. P. 1996. Biochemical characteristics of transparent fluid and its effect on in vitro storage of cock spermatozoa. Indian J. Poult. Sci., **31**(2): 96-100.

Mohan, J., Khanday, J. M., Singh, R. P. and Tyagi, J. S. 2013. Effect of storage on the physico-biochemical characteristics and fertility of Guinea Fowl semen. Adv. Anim. Vet. Sci., **1**: 65-68.

Mohan, J., Moudgal, R. P., Sastry, K. V. H. and Tyagi, J. S. 2007. Effects of garlic feeding on certain biochemical constituents and sperm motility in chicken. Indian J. Poult. Sci., **42**: 215-217.

Mohan, J., Sastry, K. V. H. and Kataria, J. M. 2017. A process for the preparation of "CARI poultry semen diluent" in patent office, New Delhi. Patent application No. 201711007119 dated: 28/2/2017.

Mohan, J., Sharma, S. K., Kolluri, G., Singh, R. P., Tyagi, J. S. and Kataria, J. M. 2016. Semen quality characteristics and seasonality in different varieties of male Guinea Fowl. Adv. Anim. Vet. Sci., **4**(6): 320-326.

Mohan, J., Sharma, S. K., Kolluri, G., Tyagi, J. S. and Kataria, J. M. 2015. Selection of Diluent for short term preservation of guinea fowl semen. Asian J. Anim. Vet. Adv., **10**(8): 360-364.

Mohan, J., Singh, R. P., Sastry, K. V. H., Moudgal, R. P., Biswas, A. and Shit, N. 2011. Influence of chicken native breeds on some physical and biochemical characteristics and short-term storage of semen. Br. Poult. Sci., **52**: 395-400.

Monsi, A., Enos, H.L., Moreng, R.E. and Pickett, B.W. 1975. Low sperm concentration as a method of evaluating fertility among toms. Poult. Sci., **54**: 1797.

Moore, H. D. and Hibbitt, K. G. 1976. The binding of labelled basic proteins by boar spermatozoa. J. Reprod. Fertil., **46**: 71-81 .

Mosenene, T. B. M. 2009. Characterisation and cryopreservation of semen of four South African chicken breeds. M.Sc. (Agric), Thesis. University of the Free State, Bloemfontein, South Africa.

Moya, A., Gonzalez, A. and Capote, M. 1996. Selection of White Leghorn cocks for artificial insemination 1: Sire semen evaluation. Poult. Sci., **75**: 1112-1119.

Mukherjee, D. P. and Bhattacharya, P. 1949. Semen studies and artificial insemination in poultry. Indian J. Vet. Sci., **19**: 79-85.

Nayak, N. R., Misra, S. C. and Misra, M. S. 1990. Studies on various seminal attributes of different breeds of broiler birds. Orissa. Vet. J., **16**: 11-16.

Nishiyama, H., Nakashima, N. and Fujihara, N. 1976. Studies on the accessory reproductive organs in the drake, 1. Addition to semen on the fluid from the ejaculatory groove region. Poult. Sci., **55**(1): 234-242.

Orunmuyi, M., Ankkwa, C. L. and Nwagu, B. I. 2012. Semen quality characteristics and effect of mating ratio on reproductive performance of Hubbard broiler breeders. J. Agr. Sci., **5**(1): 154-159

Pandey, A. K., Dinesh, K., Sharma, R., Sharma, U., Vijh, R. K. and Ahlawat, S. P. S. 2005. Population structure and genetic bottleneck analysis of Ankleshwar poultry breed by microsatellite markers. Asian-Aust. J. Anim. Sci., **18**: 915-921.

Parker, J. E., McKenzie, F. F. and Kempster, H. L. 1940. Observations on the Sexual Behavior of New Hampshire males. Poult. Sci., **19**: 191-197.

Penquite, R., Craft, W. A. and Thompson, R. B. 1930. Variation in activity and production of spermatozoa by White Leghorn males. Poult. Sci., **9**: 247-256.

Peters, S. O., Omidiji, E. A., Ikeobi, C. O. N., Ozoje, M. O. and. Adebambo, O. A. 2004. Effect of naked neck and hatchability in local chicken. In: Self Sufficiency of Animal Protein in Nigeria. Proc. of the 9th Annual Conference of Anim. Sci. Assoc., Nig., Ebonyi State Univ., Abakaliki, Nig. 13-16 September, pp. 262-264.

Peters, S. O., Shoyebo, O. D., Ilori, B. M., Ozoje, M. O., Ikeobi, C. O. N. and Adebambo, O. A. 2008. Semen quality traits of seven strain of chickens raised in humid tropics. Int. J. Poult. Sci., **7**: 949-953.

Proceedings of First National Workshop on AI in poultry, CARI, Izatnagar, 1982.

Purdy, P.H. 2006. A review on goat sperm cryopreservation. Small Ruminant Res., **63**: 215-225.

Pytasz, M. and Bzowska, B. 1961. The effect of collection frequency on the amount density and chemical composition of cock semen. Weterynaria. **10**:79-80.

Rakha, B. A., Ansari, M. S., Hussain, I., Anwar, M., Akhter, S. and Blesbois, E. 2016. Comparison of extenders for liquid storage of Indian Red Jungle Fowl (*Gallus gallus murghi*) spermatozoa. Avian Biol. Res., **9**: 207-212.

Reddy, K. V. and Bordekar, A. D. 1999. Resazurin reduction test. Ind. J. Exp. Biol., **37**: 782

Reddy, R. P. 1995. Artificial insemination of broilers: Economic and management implications. In: Bakst, M. R. and Wishart, G. J. (eds), Proc. First Int. Symp. Artificial Insemination of Poultry. Poult. Sci. Asso., Savoy, Illinois. pp. 73-89.

Rowell, J. G. and Cooper, D. M. 1960. Some effects of diluting cock semen. Poult. Sci., **39**: 1381-1389.

Saeid., J. M. and Al-Soudi, K. A. 1975. Seasonal variation in semen characteristics of white leghorn, new Hampshire and indigenous chicken in Iraq. Br. Poult. Sci., **16**(2): 97-102.

Saeki, Y. and Nogami, Y. 1964. Seasonal differences in fertility and hatchability of eggs produced by the natural mating and artificial insemination methods of chickens. Jap. J. Anim. Reprod., **10**: 37-43.

Salisbury, G. W., Van Demark, N. L. and Lodge, J. R. 1978. Extenders and extension of unfrozen semen. In: Salisbury, G. W., (ed). Physiology of reproduction and artificial insemination of cattle, 2nd ed. San Francisco: W.H. Freeman and Company, pp. 442-493.

Sasidhar, P. V. K. and Suvedi, M. 2015. Integrated contract broiler farming: An evaluation case study in India. Modernizing extension and advisory services. (Available at www.meas.illinois.edu)

Schindler, H., Weinstein, S., Moses, E. and Gabriel, I. 1955. The effect of various diluents and storage time on the fertilizing capacity of cock semen. Poult. Sci., **34**: 1113-1117.

Senger, P. L. 2003. Pathways to pregnancy and parturition. (2nd ed). Pullman, Washington, USA.

Sexton, T. J. 1977. A new poultry semen extender: 1. Effect of extension on the fertility of chicken semen. Poult. Sci. **56**: 1443-1444.

Sexton, T. J. 1981. Sperm number required for maximum fertility of chicken semen processed for freezing. Reprod. Nutr. Dev., **21**: 1043-1048.

Sexton, T. J. and Fewlass, T., 1978. A new poultry semen extender. 2. Effect of the diluent components on the fertilizing ability of chicken semen stored at 5°C. Poult. Sci., **57**: 277- 284.

Shaffner, C. S. and Andrews, F. N. 1948. The influence of thiouracil on semen quality in the fowl. Poult. Sci., **27**: 91-102.

Shinde, A. S., Mohan, J., Sastry, K. V. H., Singh, R. P., Chouhan, L. and Tyagi, J. S. 2013. CARI diluent for short-term preservation of Kadaknath chicken semen. Indian J. Poult. Sci., **48**(2): 240-243.

Shinde, A. S., Mohan, J., Singh, R. P., Agarwal, R., Tyagi, J. S. and Sastry, K. V. H. 2012. Physico-biochemical characteristics of kadaknath and broiler chicken semen under storage condition. Indian J. Poult. Sci., **47**(3): 336-339.

Siudzinska, A. and Lukaszewicz, E. 2008. Effect of semen extenders and storage time on sperm morphology of four chicken breeds. J. Appl. Poult. Res., **17**: 101-108.

Takeda, A. 1959. Studies on the cock semen. 1. Na, K and Ca levels on the seminal plasma. Res. Rep. Fac. Text. Sericult Shinshu Univ., **9**: 55-59.

Tan, N. S. 1980. The frequency of collection and semen production in Muscovy ducks. Br. Poult. Sci., **21**: 265-272.

Thomson, M. F. and Wishart, G. J. 1988. Elucidation of the mechanism responsible for the temperature-dependant reversible inactivation of the motility of fowl spermatozoa. Br. Poult. Sci., **30**: 687-692.

Thurston, R. 1995. Storage of poultry semen above freezing for twenty-four to forty-eight hours. In: Bakst, M. R. and Wishart, G. J. (eds), Proc. First Int. Symp. Artificial Insemination of Poultry. Poult. Sci. Asso., Savoy, Illinois. pp 107-122.

Thurston, R. J., Hess, R. A., Froman, D. P. and Biellier, H. V. 1982. Elevated seminal plasma protein : a characteristic of yellow turkey semen. Poult. Sci., **61**: 1905-1911.

Tselutin, K., Narubina, L., Mavrodina, T. and Tur, B. 1995. Cryopreservation of poultry semen. Br. Poult. Sci., **36**: 805-811.

Van Wambeke, F. 1967. Storage of fowl semen: 1. Preliminary results with new extenders. J. Reprod. Fertil., **13**: 571-575.

Van Wambeke, F. 1996. Proc. XX World's Poultry Congress, New Delhi, India. **1**: 531-538.

Vasicek, J., Kuzelova, L., Kulikova, B. and Chrenek, P. 2015. Effect of diluent and storage time on sperm characteristics of rooster insemination doses. Avian Biol. Res., **8**(1): 41-46.

Wheeler, N. C. and Andrews, F. N. 1943. The influence of season on semen production in the domestic fowl. Poult. Sci., **22**: 361-367.

Wilcox, F. H. 1958. The effect of dilution and concentration of chicken semen on fertility. Poult. Sci., **37**(6): 1357-1362.

Wilcox, F. H. 1961. Phosphatases in chicken semen. J. Reprod. Fertil., **2**: 148-151.

Wilcox, F. H. and Shaffner, C. S. 1957. Effect of differences in salt and hydrogen ion concentration on the fertilizing ability of avian sperm. J. Appl. Physiol., **11**: 429-434.

Wilson, H. R., Piesco, N. P., Miller, E. R., and Nesbeth, W. G., 1979. Prediction of the fertility potential of broiler breeder males. World's Poultry Sci. J., **35**: 95-118.

Wilson, H. R., Warnick, A. C. and Gutierez, J. H. 1969. Differentiation of live from dead spermatozoa in cock semen. Poult. Sci., **48**: 714.

Wishart, G. H. 1984. Effects of lipid peroxide formation in fowl semen on sperm motility, ATP content and fertilizing ability. J. Reprod. Fert. **71**: 113-118.

Wishart, G. J. 1995. New approaches to evaluating male and female fertility. In: Bakst, M. R. and Wishart, G. J. (eds), Proc. First Int. Symp. Artificial Insemination of Poultry. Poult. Sci. Asso., Savoy, Illinois. pp. 207-223.

Wishart, G. J. and Palmer, F. H. 1986. Correlation of the fertilising ability of semen from individual male fowls with sperm motility and ATP content. Br. Poult. Sci., **11**: 97-102.

Yousef, M. I., Abdallah, G. A. and Kamel, K. I. 2003. Effect of ascorbic acid and vitamin E supplementation on semen quality and biochemical parameters of male rabbits. Anim. Reprod. Sci., **76**: 99-111.

Zahraddeen, D., Butswat, I. S. R., Kalla, D. J. U., Sir, S. M. and Bukar, M. T. 2005. Effect of frequency of ejaculation on semen characteristics in two breeds of turkeys (*Meleagris gallopavo*) raised in a tropical environment. Int. J. Poult. Sci., **4**: 217-221.

Appendix

APPENDIX

1. EK Extender

Potassium citrate	0.140 gm
Sodium dihydrogen Phosphate	0.210 gm
Disodium hydrogen phosphate	0.980 gm
Glucose	0.700 gm
Sodium glutamate	1.400 gm
D- fructose	0.200 gm
Inositol	0.700 gm
Polyvinylpyrrolidone	0.100 gm
Protamin sulphate	0.020 gm
Distilled water	100 ml

2. Tselutin Extender

D- fructose	0.800 gm
Protamin sulphate	0.032 gm
Sodium glutamate	1.920 gm
Potassium acetate	0.500 gm
Polyvinylpyrrolidone	0.300 gm
Distilled water	100 ml

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