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EFFECT OF DIETARY QUERCETIN SUPPLEMENTATION ON RABBIT GAMETES DURING SUMMER HEAT STRESS

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(Ph.D Thesis)

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ABBREVIATIONS

GnRH	Gonadotropin-releasing hormone
LH	Luteinizing hormone
FSH	Follicle-stimulating hormone
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
ATP	Adenosine triphosphate
ROS	Reactive oxygen species
HPG	Hypothalamic-pituitary-gonadal
CL	Corpus luteum
ICSI	Intracytoplasmic sperm injection
IVF	*In Vitro* fertilization
SOD	Superoxide dismutase
CAT	Catalase
GSH	Glutathione
MDA	Malondialdehyde
THI	Temperature humidity index
CASA	Computer-assisted sperm analysis

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ÖZET

RASYONA İLAVE EDİLEN QUERCETİN'İN SICAK STRESİ ALTINDAKİ TAVŞANLARDA GAMET KALİTESİ ÜZERİNE ETKİSİ

NASEER Z. Adnan Menderes Üniversitesi Sağlık Bilimleri Enstitüsü Reprodüksiyon ve Suni Tohumlama Programı Doktora Tezi, Aydın, 2016.

Bu çalışma sıcak stresi etkisi altındaki tavşanlarda quercetin'in erkek ve dişi gamet kalitesi üzerine olan etkilerinin araştırılması amacıyla planlandı. Tavşanlar (erkek ve dişi) ya temel rasyonla (HS) ya da quercetin (Que-HS; 30mg/kg/day) ilave edilmiş temel rasyonla beslendiler. Erkek tavşanlarda reaksiyon süresi, sperma hacmi, ozmolalite, yoğunluk, motilite, anormal spermatozoon oranı, canlı, akrozom bütünlüğü ve mitokondrial potansiyel değerleri belirlendi. Deneyin sonunda tavşanlardan testis dokusu örnekleri toplandı ve germinatif epitel hücrelerinde apoptosis oranları belirlendi. Dişi tavşanlarda oositler toplandı ve oositlerin (COC) morfolojik derecelendirilmesi (A, B ve C) yapılarak boyutları tespit edildi. A ve B kalite oositler seçilerek standart maturasyon medyumlarına aktarıldılar. Deneyin ikinci aşamasında ovaryum örnekleri toplanarak folliküler popülasyonun saptanması ve apoptosis belirlenmesi amacıyla histolojik kesitler yapıldı. Deneyin 48 ve 56. günlerinde yapılan karşılaştırmalarda sperma hacmi, spermatozoon yoğunluğu ve total motilite değerlerinin Que-HS grupta daha yüksek ($p<0.05$) olduğu gözlendi. 48 ve 56. günlerdeki karşılaştırmalarda spermatozoonlara ait bazı kinetik parametrelerin (VCL, VSL, VAP, LIN, WOB, ALH ve BCF) yanı sıra canlı, akrozom bütünlüğü tam spermatozoon oranları ve mitokondrial potansiyel oranlarında Que-HS ve HS grupları arasında farklılıklar ($p<0.05$) görülmüştür. Que-HS grubunda 30, 48 ve 56. günlerde alınan sperma örneklerinde anormal spermatozoon oranları kontrol grubundan daha düşük ($p<0.05$) bulunmuştur. Que-HS grupta testislerdeki germinatif epitellerde apoptotik hücre oranları önemli ölçüde fazla bulunmuştur ($p<0.05$). Deney 2 de Que-HS gruptaki kesitlerde follikül, A kalite oosit sayısı ve elde edilen oosit sayıları diğer gruba oranla daha yüksek bulunmuştur. Her iki grupta toplanan oositlerde kümülüs hücre expansiyonu ve çekirdek boyaması sonuçları dikkate alındığında maturasyon oranları benzer olarak ($p>0.05$) bulundu. Que-HS tavşanlarda primordial ve antral follikül sayılarını önemli ölçüde ($p<0.05$) arttırdı. Primer ve antral folliküllerde Que-HS grupta daha yüksek oranda ($p<0.05$) apoptotik hücre gözlendi. Sonuç olarak, sıcak stresi altındaki tavşanlarda rasyona quercetin ilavesinin sıcak stresi altında gamet kalitesinin korunmasına yardım ettiği kanısına varılmıştır.

Anahtar Kelimeler: sıcak stresi, quercetin, spermatozoa, oosit, tavşan

ABSTRACT

EFFECT OF DIETARY QUERCETIN SUPPLEMENTATION ON RABBIT GAMETES DURING SUMMER HEAT STRESS

NASEER Z. Adnan Menderes University, Institute Of Health Sciences, Reproduction and Artificial Insemination, Doctorate Program Aydın, 2016.

Two experiments have been conducted to determine the effect of quercetin on semen quality, follicular development, oocyte quality and maturation rate, testicular and ovarian cells apoptosis in heat induced male rabbits. The heat stress (HS) male and female rabbits were either fed basal diet (HS) or basal diet supplemented with quercetin (Que-HS; 30mg/kg/day). In male group, weekly collected semen samples were evaluated for volume, osmolarity, sperm concentration, motility, viability, acrosome reaction, mitochondrial potential and morphology. At the end of trial, testes were collected to observe the apoptosis in germ cells. In female group, oocytes were collected through laparotomy, grading was performed and submitted to *in vitro* maturation. At the end of the experiment, ovaries were retrieved to estimate the follicle population and apoptosis rate in developing follicles. The results of experiment 1 showed that the higher ($p<0.05$) semen volume, sperm concentration and sperm motility was observed at day 48 and 56 of trial in Que-HS group. The sperm kinetic parameters (VCL, VSL, VAP, LIN, WOB, ALH and BCF), sperm viability, acrosome integrity and sperm functional mitochondrial potential differed significantly ($p<0.05$) at day 48 and 56 in Que-HS group. A significantly ($p<0.05$) low number of head, mid-piece, tail and total sperm abnormalities were observed following day 30, 48 and 56 in Que-HS group. Similarly, a significantly low ($p<0.05$) number of apoptotic germ cells were observed in seminiferous tubules in Que-HS group. The results of experiment 2 described that follicle and retrieved oocyte number was significantly higher in quercetin group. The proportion of A-grade oocyte greater ($p<0.05$) in Que-HS group, whereas, the maturation rate was similar ($p>0.05$) across the groups. The quercetin significantly improved ($p<0.05$) the number of primordial and antral follicles heat stress (HS) in rabbits. Low proportion ($p<0.05$) of apoptotic cells were observed in primary and antral follicles in Que-HS group. It is concluded that quercetin supplementation could be helpful to improve the follicular development, oocyte competence and follicle apoptosis in HS female rabbits; moreover, it improves the sperm quality and protects the testes induced damages.

Keywords: Heat stress, quercetin, sperm, oocyte, rabbit

1. INTRODUCTION

1.1. Summer Heat Stress

The mammals maintain constant body temperature with a certain range under diverse climatic conditions. They perform best in the comfort zone and any deviation from the normal comfort zone reduces their productive efficiency. The heat stress not only affects the physiological functions but also influences the productivity of the heat exposed animals. In the domestic animals, the response to environmental variations depends upon their genetics and physiological adaptations to the environment (Hansen, 2009). The phases of life from the growth to reproduction which involves a complex set of mechanism, influenced by the genetics and environmental conditions. The variations in ambient temperature especially rise in temperature elicit a set of serial fluctuations in biological functions which impairs the production and reproduction in animals (Marai et al., 2002).

The basic thermoregulatory system in mammalian's body maintains the body temperature by radiating the heat out of body when it exceeds the ambient temperature. At that moment, heat is dissipated through the evaporation process. If ambient conditions overshoot the body temperature, the heat influxes the body and the animal suffer from heat stress. Hence, it is a key element to estimate the thermal environment where animals are housed. Using the temperature-humidity index (THI), the surroundings conditions could be estimated in view of the air temperature and humidity; therefore, THI provides great help to sustain the animal's efficiency under normal and stress conditions (Collier et al., 2006).

Heat stress (HS) can be defined as that a state of external temperature where animals strive to sustain the efficiency above set-point of body temperature. The cattle and rabbits are residing in a comfort zone having a temperature between 5 to 20°C and 12 to 20°C, respectively. A rise in temperature above the comfort zone in cattle and rabbits show the high metabolic rate of combustion of body reservoir which further influence feed intake. An increase in temperature above the set-point zone in mammals results in dehydration due to fluctuations in fluid membrane mosaic, distortion in protein structure in response to a high rate of sweating and panting. Reduced growth rates, altered metabolism, high mortality rates and altered body composition with high adipose tissue and low skeletal muscle mass have been primarily characterized in HS animals (Rhoads et al., 2013). High temperatures in the tropical and sub-

tropical areas are also a limiting factor for animal protein production in beef producing animals. Other than morphological changes in HS animals, HS influences animals at cellular level remarkably such as restricted DNA formation, interrupted RNA copying, processing and translation, disruptions in cell cycle process; amalgamation and disordered proteins synthesis or higher degradation of proteins by proteasomal and lysosomal mechanism. In addition, the disorganization in cytoskeletal components, changes in metabolic rate which subsequently lower down the cellular ATP level, and altered membrane permeability with higher intracellular movement of Na, H, and Ca molecules are reported (Sonna et al., 2002).

In spite of HS effects on productive parameters, reduced reproductive efficiency of domestic animals has also been outlined. Disrupted reproductive function during HS is brought about by higher flow of blood from the body to the peripheral surface results in increased sensible heat dissipation and low feed consumption (Hansen, 2009). HS affect the reproductive efficiency in both male and female animals such as altered spermatogenesis process, reduced oocyte development and maturation, diminished embryonic progression with a low growth rate in fetal and placental size. In dairy cows, the delayed initiation and establishment of lactation process has also been reported (Silanikove et al., 2009). There is involvement of different pathological mechanisms with HS which affects the gametes and early embryo. The superfluous production of ROS is the main major component triggering all these reactions in HS exposed animal body. However, the genetic adaptation of some animal species to HS had the capacity to combat the HS by regulating the body temperature by eliciting the cellular resistance to high temperature.

1.2. Effect of Summer Heat Stress on Reproductive Performance of Animals

Environmentally induced heat stress influences the animal body by disturbing the balance between in- and outflow of thermal energy which adversely affect the health and production performance of nearly all mammalian species. Heat stress is the aggregate effect of exterior factors to a homeothermic animal that propels to alter the body temperature from the normal value (Yousef, 1984). The stress state in the body leads to change in the animal physiological and productive performance. Similarly, the hyperthermia due to heat stress directly influences the reproductive potential in the animals (Hansen and Arechiga, 1999). Heat stress as a devastating factor for fertility in the cattle and its severity become double during the hottest summer months. HS greatly influence the fertility in cows by changing the

physiological condition or affecting the hypothalamic–pituitary–gonadal (HPG) axis and it depends on the severity and extent of high temperature (Al-Katanani et al., 1999; Khodaei et al., 2011). The detrimental influences of high surrounding temperature on reproductive performance in different animals like in cattle (Reviewed by De Rensis and Scaramuzzi, 2003; Jordan, 2003; De Rensis et al., 2015), swine (Love et al., 1995; Einarsson et al., 2008), rabbits (Tůma et al., 2010; Marai et al., 2002), buffalo (reviewed by Marai and Habeeb, 2010; Chaudhari et al., 2012), goat (Mellado et al., 2006; Mellado and Meza-Herrera, 2002) and sheep (reviewed by Marai et al., 2007), have been reviewed in detail. The changes like short estrus duration, irregular estrus cycle, abnormalities in ovulation process, reduced fertilization occurrence, and high embryonic and fetal mortality during early gestation are the major constraints related to HS in domestic animals. The occurrence of these abnormalities is directly related to the dysfunction of endocrine system by HS (Khodaei et al., 2011).

The chronic HS exerts the long-term effects on fertility, especially in dairy cows. The lower fertility rate in autumn season in HS cows is a good example of this phenomenon. The immediate influence of HS over the hypothalamic-hypophyseal-ovarian axis in female animals that lead to uterine milieu disruption, irresponsiveness of follicles and CL to specific hormones and finally interruptions in the development of embryos (Ullah et al., 1996; Wolfenson et al., 2000). Short duration with less intense estrus phase is observed in HS cows in the summer months compared to other seasons. Reduced motor activities, high incidence of silent estrus and ovulation, reduced number of mounts during hotter months are major salient which result in poor heat detection with less number of inseminations or a high proportion of inseminations with meagre pregnancy rate. This phenomenon is more prominent in the dairy cows in lactation (Omran and Al-Saffar, 2011).

The mechanisms involved with alterations in circulating reproductive hormones levels under HS are not well understood. There are different opinions about the release and inhibition pattern of these hormones when animals are exposed to the HS. Regulation of hypo-gonadal axis is based on the release and inhibition of GnRH, FSH and LH. It is noticed that HS affect the frequency and amplitude of LH tonic releasing pattern in cows, whereas, the releasing pattern of LH surge was affected more prominently in HS heifer compared to adult cows. The dominant follicle grows in an environment with low LH release and this follicle further secret the reduced or inadequate estradiol which results in poor estrus expression with no pregnancy. Moreover, the lower plasma inhibin concentrations in HS cows is also a featuring hormone that affects follicular dynamics by up or down regulating the secretion of FSH. High corticosteroid

release during summer stress, has been considered as a factor predominantly influencing the secretions of GnRH and LH secretion. Intermittent release of LH affects the dominance of selected follicle that further alters the plasma estradiol level in HS dairy cows. In contrast, there are different notions of plasma concentration of progesterone in HS cows. Some studies reported that heat stress has no noticeable effect on plasma progesterone levels. The variations in plasma progesterone levels might be linked to uncontrolled factors like dry matter intake, the rate of luteal production and hepatic metabolism. The low concentration of plasma progesterone in HS cows resulted in compromised fertility (Jonsson et al., 1997). Abnormal oocyte maturation, early embryonic death and failure of implantation are the events which is contributed by primarily by reduced plasma progesterone concentrations and resulted into low fertility (Trout et al., 1998).

Growth incidence of small and large follicles population and deviation rate of the large follicle are greater under HS due to altered hormonal secretions (Guzeloglu et al., 2001). Reduced follicular diameter, low number of follicles to attain the dominance stage, delayed deviation of subordinate follicles and delayed attainment of Graafian follicular stage are main constraints during the developmental phase "from emergence to selection dominant follicles" are greatly affected by HS (Badinga et al., 1993; Howel et al., 1994; Roth et al., 2000; Wilson et al., 1998). The uneven follicle development has been related to the gonadotrophin release and inhibits the process. Impairment of granulosa cells is responded irregular release of estradiol or inhibin which subsequently affects the release or inhibition of FSH as feedback mechanism (Roth et al., 2000). The reduced granulosa and thecal cells functionality and low expression of FSH or LH receptor to the respective cells have also been reported (Roth et al., 2001; Bridges et al., 2005). The reasons for the lower functionality of thecal and granulosa cells under HS is still unclear. A detailed information from the differentiation to the functional activity of thecal and granulosa cells during HS is needed to elaborate the possible HS influences (reviewed by Paula-Lopes et al., 2012).

Lower production of progesterone during summer HS is also a great dilemma for the successful establishment of pregnancy. Reduced performance of luteal cells during HS results into low plasma progesterone and greatly increase the embryonic losses due to altered endometrial morphology. In addition, progesterone level is also affected by the some other factors during HS, like adrenal progesterone secretion, the high metabolic activity of liver, lactation stage, temperature-humidity index (THI) degree, type of HS, age and feeding practices. HS impairs the ovarian structures (follicle and CL) morphology and physiology i.e. retarded follicle/CL growth and hormonal release from the follicle and/ CL. All these

collectively influence the circulating endocrine milieu. Impairment of ovarian functions at cellular and molecular level is a big nude area and the understanding of influential factors can help to adopt the new mechanism to combat the heat stress. There is scanty information about heat stress effects on oocyte maturation and developmental competence about cellular and molecular alterations. The impairment of meiosis resumption, disruption of cytoplasmic organelles, changes in maternal transcripts, and loss of mitochondrial function are important known alterations in heat-stressed oocytes during maturation phase. The phenomenon of reduction in heat-stressed oocyte competence and heat induced apoptosis or variation in redox state has been highlighted. However, there is dire need to understand the mechanisms how heat stress impairs oocyte competence at the cellular level that is highly important to develop new methodologies for coping with this problem (Johnson et al., 2013).

1.3. Effect of Summer Heat Stress on Sperm Quality

In most of the mammals, the testes are protected by scrotum sac exterior to the body cavity. There is a complex mechanizing system that ensures the slightly lower testicular temperature than core body temperature. The mechanism of temperature regulation is mainly controlled by heat exchange counter current ways between cold and warm blood circulating the testes and muscular activity of tunica dartos and cremaster muscle in response to temperature alteration (Werdelin and Nilsonne, 1999; Bedford, 2004). There are some opinions about the scrotum evolution that it occurred due to the maintenance of low temperature during spermatogenesis, storing the sperm or to reduce the mutation changes in DNA. However, HS even in the presence of above mentioned protective measures, affects the testicular texture, testicular weight, sperm output, sperm quality and sperm morphology. These alterations are not only occurring with the ambient rise in temperature but also associated with the elevated body temperature, scrotal insulation, and/or cryptorchidism. During the spermatogenesis process, the spermatocyte and spermatids stages are more susceptible to the HS and spermatogonial cells readily undergo to apoptosis and DNA damages with the progression of oxidative stress (Setchell 1998). Moreover, the histological picture of testis under HS also represented that pachytene spermatocytes and early spermatids having higher vulnerability to HS (Perez-Crespo et al., 2005; Paul et al., 2009).

The sperm variables are not seen abruptly in HS exposed testis because affected cells are ejaculated by completing the whole spermatogenesis cycle. The seminal changes are

directly linked with duration of the HS exposure and spermatogenesis cycle of each species. The HS alteration in semen parameters is observed until the whole cycle after HS cessation. In addition, individual response to the HS also variable. The presence of damaged sperm after 2 to 3 weeks of HS showed the sensitivity of sperm present in epididymis region. It has been believed and proved after repeated experimentation that HS changes are totally reversible (Karabinus et al., 1997).

On another hand, HS influences the Sertoli and Leydig cells that have been manifested in the form of reduced androgen binding secretions, lower expression of intermediate filaments and an inappropriate assortment of adult Sertoli cells (Zhang et al., 2006). Clearance of apoptotic germ cells during the spermatogenesis cycle is physiological phenomenon to maintain the viable germ cells quality and a similar mechanism is adopted in testicular level to remove the HS damaged germ cells by apoptosis (Ishii et al., 2005). It has been observed that DNA deterioration triggering the apoptosis process in germ cells under HS situations and damaged cells are removed by apoptosis. The incidence of apoptosis is more prominent at the stages of spermatocytes and round spermatids of spermatogenesis (Durairajanayagam et al., 2015). Moreover, the overexpression of Bax and Fas are pro-apoptotic and apoptotic factors which indicate a higher incidence of apoptosis during HS (Yin et al., 1997) and manifestation of nitric oxide synthase is an indicator of apoptosis process in HS exposed testis (Ishikawa et al., 2005). The reduced sperm production is also associated with a decline in testosterone concentration in HS animals. Similarly to the alteration in LH secretion in females, LH secretion in male is also severely affected by HS, which reduces the spermatogenic cell lineage in the testis. Although HS directly affects the spermatogonial differentiation phase, cellular deterioration become double by variation in testosterone release during HS exposure (Setchell, 2006). A male exposed to HS produces the viable sperm but the mutations and DNA damages in sperm results in a high proportion of abnormal offspring (Setchell, 1998). Similarly, it has been observed that mating unexposed HS females with HS male had lighter masses of fetus or placenta compared to the control (Paul et al., 2008). Lower embryo development has been observed by sperm from HS males for IVF procedures (Yaeram et al., 2006).

1.4. Effect of Summer Heat Stress on Oocyte Quality

Mammalian oocytes arrest at the prophase stage after the first meiosis and resume the meiotic competence and fertilization capacity with follicle progression. Alike to the male

gametes, heat stress alters the developmental and functional integrity of the oocyte. Although oocyte is encased inside the follicles, HS induce the changes like changes in estradiol concentrations, reduce the expression of LH receptors, aromatase activity and subsequently delay the ovulation, which results in the disruption of oocyte quality (Ozawa et al., 2005). The aforesaid factors collectively execute the aging process in oocytes in developing follicles. It is known that HS can affect the follicular dynamics (Roth et al., 2000), steroid hormones release (Roth et al., 2001; Ozawa et al., 2005) and the expression of genes linked with HS (Argov et al., 2005). There are numerous reports about *in vitro* or *in vivo* HS effects on oocytes. It has been reported that HS influences the oocyte maturation; fertilization rate and proportion of fertilized oocyte most likely develop into an abnormal embryo. An *in vivo* report revealed that exposure of animals to HS during preovulatory phase reduces the oocyte quality and damage to oocyte is by the involvement of ROS production (Roth et al., 2008). Similar results have been observed during *in vitro* experiments and improvement in oocyte fertilization rate has been achieved by supplementing antioxidants in medium (Lawrence et al., 2004).

Moreover, chemically activated or *in-vitro* fertilized HS oocytes have also shown a reduction in embryonic development (Zeron et al., 2001; Al-Katanani et al., 2002). HS did not influence the GV breakdown but it hindered the further progression to MII stage and subsequent blastocyst development (Payton et al., 2011; Aroyo et al., 2007). HS impedes the cumulus-cell hyaluronic acid production and affects the further meiotic resumption process (Lenz et al., 1983). HS also negatively affects the nuclear and cytoplasmic maturation processes by reducing the activity of translocation of cortical granules to the oolemma, cytoskeleton rearrangement, and spindle formation in maturing oocytes (Gendelman and Roth, 2012a). In addition, the changes in fatty acids, heat shock proteins, and antioxidants levels are the related cellular injuries in HS oocytes (Zeron et al., 2001). Similar to sperm response to HS, oocyte also undergoes the apoptotic process. The apoptosis is also observed in maturing oocyte when animals undergo the HS and about 15 to 30 % higher rate of apoptosis has been reported compared to control (Roth and Hansen 2004).

1.5. Effect of Summer Heat Stress during Embryonic Development

The synchrony between the embryo and uterine environment is an important aspect of the early implantation and the subsequent successful establishment of pregnancy in mammalian. Any deviation in the uterine environment leads to the early embryonic death

(Barnes, 2000). In this aspect HS is a big involved factor that lowers the fertility by affecting the embryos at early developmental stages or changing the uterine milieu. It has been elaborated by various studies using lab and farm animals as an experimental model. The early embryo has a great susceptibility to ambient HS; however, it remains unaffected in later growth stages. Early embryos (1 to 2 cells stage) are more susceptible compared to 8–16 cells stage, morula or blastocysts (Hansen, 2007).

The exposure of female to heat around insemination or preimplantation embryo phase adversely affects the establishment of pregnancy. Exposure of females to heat increase the incidence of ROS production in the oviduct, uterus and in developing embryos. As the embryonic development progress, the intracellular antioxidants level also increase which in turn helps the embryos to combat the ROS produced by HS (Sakatani et al., 2004). A decrease in antioxidant enzymes especially in reduced glutathione in recovered embryos is an evident HS-induced oxidative stress (Ozawa et al., 2002; Matsuzuka et al., 2005).

Reduced apoptosis process with an increase heat shock protein 70 (HSP70) level in embryos has been observed under HS. HSP70 helps to maintain the cellular protein and organelles in developing embryos against HS (Gao et al., 2014). In addition, caspase inhibitor factor reduces the level of apoptosis and help the HS affected embryo developmental progression (Brad et al., 2007). Low fetal and placental weights and variation in placental hormones concentrations are the indications of HS and HS greatly affects the fetal growth during 2nd and last trimester. The greater expressions of some factors like vascular endothelial growth and placental growth factors enhance placental angiogenesis and further the vascular resistance in placenta (Reynolds et al., 2004). Another anomaly is the lower expression of GLUT8 genes (Limesand et al., 2004) in cotyledonary placenta under HS which further influence the glucose transportation across the placenta (Thureen et al., 1992). The higher incidence of teratologic anomalies is also one of the aspects of HS (Graham et al., 1998). To cope with the HS, different studies has been designed and revealed that adoption of embryo transfer techniques during HS phase can protect the embryo against detrimental effects of HS (Rutledge, 2001; Hansen, 2007).

1.6. Flavonoids and Their Impact during Stress Conditions

Several plant-derived foods contain the polyphenolic compounds such as flavonoids. The inclusions of vegetables and fruits in daily food having a higher level of flavonoids that

provide beneficial effects on health. The flavonoids do act as antioxidants in body cells and their free radical scavenging activities rely on the location of hydroxyl groups and other features of chemical configuration. The flavonoids have been considered as a substitute of other antioxidants and it has the capacity to replace the other antioxidants such as vitamin E during cellular activities (Parabathina et al., 2010). Due to the significant versatile health benefits, free radical scavenging and metal chelating properties, flavonoids are getting popularity as a non-immunogenic medicinal tool (Capcarova et al., 2013). Several studies have highlighted the role of flavonoids *in vitro* such as protection against different diseases. The flavonoids supplementation protect the DNA, maintain the minimal level of low-density lipoproteins, reduce the enzymatic activity and aggregate the platelet. In addition, more focused area of flavonoids study is *in vivo* metabolism which can clearly elaborate the flavonoids activities following ingestion (Peluso and Palmery, 2015). The flavonoids possess the different properties which are as follows:

Antioxidant Activity: Flavonoids have contained several biochemical properties; however, the capability to work as antioxidants is well-embarked property amongst every group of flavonoids. The antioxidant action of flavonoids is brought about by suppressing the ROS production by inhibiting the ROS linked enzymes or through chelating trace elements in free radical generation. Flavonoids do act directly in ROS scavenging or protect the antioxidant system of the animal body. The antioxidant action of flavonoids is associated with the structural arrangement of functional groups with respect to nuclear structure. Radical scavenging and metal ion chelation activity of flavonoid are influenced by structural arrangement, replacement, and a total number of hydroxyl groups present in a particular group. The position of B-ring hydroxyl in a flavonoid compound is the focusing point during ROS or RNS scavenging process as it contributes hydrogen and an electron to the hydroxyl, peroxyl or peroxynitrite ions and results in the production of more stable flavonoids radical (Pietta, 2000; Kumar and Pandey, 2013).

Hepatoprotective Activity: There are several flavonoids compounds including anthocyanins, catechin, apigenin, quercetin, naringenin, rutin, and venoruton have been documented for their hapatoprotective property. Development of clinical hepatic signs is more obvious under different chronic diseases like diabetes; therefore, dietary supplementation of flavonoids is more effective compared to other chemical compounds. It has been reported that the expression of glutamate-cysteine ligase catalytic subunit (Gclc), increased level of glutathione, and decreasing ROS in the liver of diabetic mice were observed after flavonoids supplementation. In addition, increased expression of Gclc in the hepatocyte resulted in lower

level of hepatic ROS and proapoptotic signaling. The inhibitory release of proinflammatory cytokines by flavonoids protects the liver against the progression of fatty liver (Tapas et al., 2008; Madrigal-Santillán et al., 2014).

Antimicrobial Activity: Antimicrobial activity of flavonoids has been recognized against microbial infection and this property has also been tested during *in vitro* trials. The tested flavonoids have showed great extent of response against a wide range of microbes. Each flavonoid possesses the potent antimicrobial ability. Antibacterial flavonoids involved multiple actions in the cellular targets instead of a single site for action. Specific (covalent bond formation) and nonspecific (hydrogen bonding and hydrophobic) mechanism of action is involved in the formation of protein complex. This protein complex disrupts the microbes' cellular functions by inactivating the microbial adhesins, enzymatic activity and transportation of cellular proteins. Moreover, changes in microbial membranes have also occurred due to lipophilic property of flavonoids (Cushnie and Lamb, 2005; Tanwar and Modgil, 2012).

Similarly, different reports are available about the possible effect of flavonoids on viral diseases. It has been found that like other antiviral agents flavonoid also possess the capacity to inhibit the release of the enzymes linked with cell cycle of viruses. An antiviral feature of flavonoid provides beneficial opportunity to treat the viral diseases with minimal side effects (Nijveldt et al., 2001; Orhan et al., 2010; Agrawal, 2011).

Anti-Inflammatory Activity: The incidence of chronic diseases becomes low with greater plant-based foods intake in the daily diet. Inflammation is the first invasion of any disease and this sign has been cured by supplementing the flavonoids; however, the broader aspect of flavonoid and the components related anti-inflammatory action need more clarification. It has been described that the restricted functions and formation of pro-inflammatory mediators like eicosanoids, cytokines, adhesion molecules and C-reactive protein possess the possible anti-inflammatory mechanism of actions of flavonoids (Rathee et al., 2009; Serafini et al., 2010). *In vitro* studies have shown that some flavonoids have a strong anti-inflammatory effect and inhibit the influence of the expression of adhesion molecules. They reduce the incidence of oxidized low density lipoprotein-induced inflammatory effects through the expression of Toll-like receptor–nuclear factor-kB (NF-kB) signaling pathway in peripheral blood mononuclear cells (Harwood et al., 2007).

Anticancer Activity: Anticancer action of flavonoids has been well documented. Consumption of fruits and vegetables in daily diet lower the incidence of occurrence of prostate, lung, stomach, and breast cancer. Low risk of lung, endometrium, oesophagus, stomach, and colon cancer development has been observed in moderate wine drinkers.

Different mechanisms have been suggested for the anticancer effect of flavonoids on incidence and progression of cancer. The down regulation of mutant p53 protein and Ras proteins results in arrest of cell cycle, inhibition of tyrosine kinase, low expression of heat shock proteins and high binding ability to estrogen receptor (Sandhar et al., 2011; Kumar and Pandey, 2013).

1.7. Flavonoids Effects on Reproductive Functions in Animals

Efficacy of flavonoids on reproductive attributes in animals is another main feature which has been tested under *in vitro* and *in vivo* conditions. Under *in vivo* conditions, it has been shown that flavonoids could enhance the sex gametes quality. Similarly, these compounds have been tested during *in vitro* experimental trials. Use of these compounds especially in sperm freezing extenders is very advantageous. The superfluous of ROS production and an imbalance in ROS level and the antioxidant defense system in the sperm lead to oxidative stress during cryopreservation. This incidence is becomes double due to the presence of polyunsaturated fatty acid in sperm membranes. One of the possible approaches to minimize ROS formation during sperm freezing is, to boost the antioxidant system by adding the flavonoids instead of other physiologic or non-physiological antioxidants. In addition, amphipathic nature of flavonoids readily intermingles with the lipid bilayer and protects the sperm during oxidative stress. Its affinity to membrane relies on the degree of hydroxylation, molecular structure and side chain length (Moretti et al., 2012). Flavonoids especially the quercetin is supposed to be a potential regulator of reproduction and it influences the reproductive events by mediating via mTOR signaling system. mTOR signaling pathway is a vital signaling cascade that regulate the cell growth, proliferation and survival (Makker et al., 2014). In addition, the inhibition of mTOR signaling pathway subsequently affects the granulosa cell proliferation (Yaba et al., 2008).

It has been observed that the plant extracts contain flavonoids are capable to treat the experimentally induced polycystic ovarian syndrome. These agents regulate the LH surge by interacting the GABA system, also increase the follicular development and endometrial tissue organization (Zangeneh et al., 2010). Flavonoids significantly dealt with the problem of postmenopausal uterine endometrium thickness and vaginal dryness problems because flavonoids have a weak estrogenic activity. Flavonoids also impede with the biologically destructive chemical reactions in the reproductive organs and scavenge the ROS (Zaid et al., 2010). An increment in progesterone concentration could show that flavonoids

supplementation through its molecular configuration may provoke protective effects against induced oxidative stress (Shimada et al., 2005). In contrast, some of these plants extracts are known to have antifertility influence by their action on hypothalamo-pituitary-gonadal axis or directly effect on reproductive organs and subsequently inhibit the ovarian steroidogenesis (Santini et al., 2009; Nordeen et al., 2013).

1.8. Quercetin: Biochemical and Anti-Stress Properties

Amongst the classes of flavonoids, quercetin is the most common naturally existing flavonol in vegetables, fruits, tea, and red wine. Antioxidants capacity of the flavonoids rely on the molecular configuration. The positions of hydroxyl groups and different aspect linked with chemical structure are the key components for ROS scavenging activity of quercetin. The quercetin has the ability to alter the membrane-dependent functions like lipoperoxidation which is related to flavonoid molecular configuration and their ability to coalesce and penetrate the membrane lipid bilayers (Saija et al., 1995).

Quercetin is categorized by a phenyl benzo(c) pyrone-derived configuration contain two benzene rings which are interconnected with a heterocyclic pyran or pyrone ring (Morand et al., 1998). The flavonol aglycone is generally present at the 3- position of the unsaturated C-ring with a sugar moiety, creating O-b-glycosides in quercetin (Kumar and Pandey, 2013). Quercetin is plant extract and these extracted quercetin glycosides are hydrolysed to liberate the aglycone and finally purified. The quercetin (3,5,7,3',4'-pentahydroxyflavone) serves as radical when to react with unpaired radical by donating a proton and becomes radical itself. However, resulted unpaired radical is removed by resonance that forms the quercetin radical weak and less reactive with minimum energy (Mariani et al. 2008). Usually, three structural groups (B ring o-dihydroxyl groups, the 4-oxo group in conjugation with the 2,3-alkene, and the 3- and 5-hydroxyl groups) stabilize the quercetin molecule and help to act as an antioxidant (Hollman and Katan, 1997). The functional groups have the ability to donate the electrons to the rings which increase the resonance form number in addition to those created by the benzene structure (Mariani et al., 2008). It is recognized for its remarkable clinical applications such as anti-inflammatory, antiallergic, antiviral, antibacterial, and anticancer activities. Similar to other flavonoids, quercetin also protects the cells against detrimental consequences of oxidation stress and reduces the cellular injuries by eliminating the oxygen radicals, by

defending against lipid peroxidation and by metal ions chelation mechanism (Coskun et al., 2005).

1.8.1. Effect of Quercetin on Sperm Quality

Quercetin has the capacity to suppress the free ROS/NOS radicals at different levels. It reduces the formation of the superoxide ion, by suppressing of lipid peroxide radicals formation and limiting ROS through metal ion chelating. The ROS eliminating capacity of quercetin protect the sperm effectively at the testicular level and during the sperm chilling and freezing procedure. In this aspect, several studies have been carried to challenge the efficacy of quercetin. In the following lines, an overview has been complied by extracting the conclusions of conducted studies.

1.8.1.1. Effect of Quercetin on Sperm after Oral Supplementation

Dose and time duration of treatment by oral supplementation of the quercetin affect the sperm quality and it could be the potential source to treat the subfertility cases. The provision of quercetin has a positive impact on testicular function and consequently sperm quality (Taepongsorat et al., 2008). The protective mechanism of quercetin is associated with the antioxidant potential, however, it has been also noticed that it can act as pro-oxidant and as weak estrogen under normal physiological conditions. The binding of quercetin with estrogen receptors could be one factor that further influences the endocrine milieu, Sertoli cell function and, subsequently sperm quality (Ranawat et al., 2013).

1.8.1.2.Effect of Quercetin on Sperm Parameters during Incubation

The sperm loose the motility, viability, and integrity with the passage of time during the incubation. In this context, many studies have been conducted to maintain the sperm quality over the hours by exogenous supplementation. Similarly, quercetin has been tested in several species to maintain the sperm viability during incubation. It has been reported that incubating ram sperm with quercetin did not affect the sperm motility at initial hours; however, with the

passage of time it maintains the motility (Nass-Arden and Breitbart, 1990). An improvement in stallion sperm has been documented in the presence of quercetin in capacitation medium and it protected the sperm to undergo the premature capacitation (McNiven and Richardson, 2006). In addition, a protective influence of quercetin on human sperm during long-term storage or in the presence of stress inducer has been known (Mazzi et al., 2011; Moretti et al. 2012) and it maintains the sperm viability of infertile men during the ICSI/IVF procedure. It has been hypothesized that quercetin maintains the sperm quality by controlling the sperm glycolytic rate and maintain the extracellular pH, ATP content of the cells, mitochondrial activity and redox system to reduce lipid peroxidation. On another hand, there is a report about the negative impact of quercetin on sperm motility during incubation and it has argued that negative impact of quercetin is related to decreased activity of Ca^{+2}-ATPase (Khanduja et al., 2001). Significant improvements in sperm motility, viability and morphology have been observed by supplementing the quercetin in incubation medium in the presence of hydrogen peroxide. Quercetin also protects the sperm antioxidant defenses and downregulating the MDA level (Ben Abdallah et al., 2011).

1.8.1.3.Effect of Quercetin on Sperm Parameters during Cooling and Freezing

Semen chilling or cryopreservation has advanced the animals breeding many folds by the long-term preservation of sperm and world-wide propagation of valuable genetic material. There are some deleterious factors associated with sperm preservation procedures that reduce the sperm viability and fertilizing capacity. Amongst those detrimental factors, the oxidative stress induced by the cooling and freezing method is the major factor. In this scenario, a strategy to incorporate the antioxidant in the sperm storage media is being practiced for better sperm survival during freezing and thawing. Similarly, the fortification of quercetin to semen diluters has also been carried out in different species during sperm chilling and cryopreservation and improved outcomes have been achieved.

The long-term rabbit sperm storage in the presence of quercetin had maintained the sperm viability and DNA integrity, and significantly lowered the lipid peroxidation level. Lower doses of quercetin provide beneficial influences; however, supplementation with higher doses in the extender led to toxic effect (Johinke et al., 2014). An improvement in the post-thaw human sperm motility, viability, DNA integrity and oxidation has observed when quercetin was added to the freezing extender (Zribi et al. 2012). Cryopreservation of ram sperm

in the presence of smaller doses of quercetin did not provide any toxic effect to sperm; however, the higher doses resulted in reduce mitochondrial potential comparatively (Silva et al., 2012). It has been reported that quercetin addition to stallion sperm cryodiluents reduced the DNA damage during sex-sorting and improved motility, reduced DNA damage. Moreover, its inclusion also significantly enhanced the zona binding ability of cryopreserved sperm (Gibb et al., 2013). It has been observed that sperm of each species behave differently to the quercetin during the cryopreservation; however, overall an improved sperm cryosurvival has been obtained.

It has been also observed that bovine cryopreserved sperm readily underwent acrosome reaction and reduced the acrosin activity when LPC is used; however, the supplementation of quercetin in incubation medium delayed acrosome reaction process by lowering the acrosin activity (Aguirreburualde et al., 2012). In contrast, it has been seen that quercetin adopts a different mechanism to induce capacitation in frozen–thawed bovine sperm by modulating the redox state and the oxidative metabolism (Cordoba et al., 2007).

1.8.1.4. Protective Effect of Quercetin Against Different Toxic Agents on Reproductive Parameters

Urbanization and wide usage of chemicals or non-chemical products in our daily life and environmental pollution greatly exposed the human to several health hazards. The hazards linked to environmental toxicants are not restricted to general health problems, it also greatly influenced the reproductive health. A large number of *in vivo* and *in-vitro* studies have been conducted in lab animal models using different environmental toxicants to observe the changes at testicular and cellular level. These toxic agents also affect the endocrine milieu that further hinders the sertoli cells function. Alike to quercetin, several antitoxic compounds have been tested to combat the toxicant effects in testes. Following paragraphs have been reviewed about the protective effect of quercetin on testicular and sperm cells during exposure to toxicants.

The environmental pollutants such as diesel exhaust particle (DEPs) significantly affect the testicular tissue, sperm morphology, and sertoli cells activity. The impairment is associated with DEPs by increased incidence of lipid peroxidation and quercetin alleviates the adverse effects induced by DEP (Izawa et al., 2008; Mi et al., 2010). Similarly, the exposure of rat to the 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) also induce a toxic effect on testicular histogram and sperm picture. Quercetin has minimized the toxic effect of TCDD on testicular

tissues and sperm quality by decreasing the lipid peroxidation and boosting the antioxidant enzymes (SOD, CAT, and GSH-Px) (Ciftci et al., 2012). Carbon tetrachloride (CCl4) is also a model toxicant which causes the testicular changes and sperm abnormalities in the exposed animals. To combat the toxic effect of CCl4 on testicular tissue and sperm, quercetin has been tested and beneficial results have been obtained. Quercetin promoted the anti-apoptotic activity in testes tissues of CCl4 exposed animals by culminating excessive ROS (Sonmez et al., 2014). Cadmium induces the histopathological changes in testes, reduces the sperm motility and disturbs the endocrine milieu in treated mice. The oral provision of quercetin has provided the beneficial effects of cadmium instigated changes in testes and sperm damage (Farombi et al., 2012). Moreover, an improvement against Aroclor 1254, which is an environmental endocrine disrupter, in spermatogonial cells of chicken has been documented by supplementing the quercetin in the incubation medium (Zhang, 2005).

In addition, heavy metal environmental pollutants are also a great concern owing to having estrogenic activity. Several reports are available to comprehend role and impact on male reproductive health. It has been revealed that these environmental pollutants are endocrine disrupters by their weak estrogenic potency and long-term exposure lead to testicular dysfunction. The quercetin intervention could ameliorate the adverse effect of estrogenic agents on testes tissues and restores spermatogenesis, maintain endocrine milieu and improve the sperm quality during estrogen exposure (Bharti et al., 2014). It has been expected that improved male reproductive functions are linked with the antioxidant potential of quercetin during estrogen exposure. The quercetin reduced histological damages induced by removal of excess ROS in the testis. Moreover, it has also antiestrogenic property that stimulates the androgen synthesis and reduces the apoptotic activity in germ cells (Li et al., 2010).

Different therapeutic agents like cisplatin (Aldemir et al., 2014), docetaxel (Altintas et al., 2015), fenitrothion (Saber et al., 2015) and ethanol (Uygur et al., 2014) greatly influence the male reproductive functions. Long-term usage of these compounds resulted in abnormal testicular histology and spermatogenesis, endocrine disruption and imbalance in redox state. However, the supplementation of quercetin in intoxicant animals has performed a role for the resumption or maintenance of testicular echotexture, sperm survival and removal of excessive ROS from the reproductive tissues. Likewise, the beneficial impacts of quercetin against streptozotocin (STZ)-induced diabetes in animal models have been reported. Generally, the hyperglycemia has shown to cause testicular dysfunction and deterioration in sperm quality by significant oxidative stress. It has been noted that dietary supplementation of quercetin recovers

the testicular dysfunction and sperm quality by alleviating the detrimental effect of ROS in diabetic patients (Khaki et al., 2010; 2011; Alkhamees, 2014).

The physical injuries to testes like testicular torsion lead to an ischemic condition in testes which is corrected by surgical intervention. However, resumption of spermatogenesis in affected animals requires an ideal compound as an adjunctive therapy. In this context, different studies have been carried to correct spermatogenesis in affected animals by dietary supplementation of quercetin. It has been assumed that the beneficial effects of ischemia and reperfusion injury in testes might be related to its antioxidant, antiplatelet and anti-inflammatory property of quercetin (Aktoz et al., 2010; Aldemir et al., 2012).

1.8.2. Effect of Quercetin on Oocyte and Embryonic Quality

Quercetin possesses strong antioxidant property which is beneficial for reproductive health and subfertility and it has been recognized safe compound to use as a diet supplement. Alike to improve male reproductive function by quercetin, it has also been tried to assess the efficacy and safety of quercetin for female reproductive health. In contrary to the numerous studies on male fertility, fewer reports are available about the effects of quercetin on female reproduction (Wojcik et al., 2010). Generally, the dynamic function of ovary results into overproduction of ROS especially during final stages of follicular development and near the incidence of ovulation, and this over production of ROS is halted by the active enzymatic antioxidant system. Generally, a slight increase of ROS is necessary for the meiotic resumption from diplotene arrest in mammalian oocytes. However, superfluous of ROS in ovary or imbalance in redox state leads to oxidative stress under disease or stress condition (Agarwal et al. 2005). The increased oxidative stress affects oocyte quality enclosed in the follicle and subsequently fertilization and pregnancy rates (Agarwal et al., 2005). Studies regarding the use of quercetin in female reproductive function have been summarized in the following paragraphs.

Cadmium is the environmental pollutant which significantly affects both male and female fertility. Cadmium-induced cytotoxicity in granulosa cells and affects the mitochondria activity by inhibiting the BCL2, XIAP and triggering the caspase-3 expression. A protective effect of quercetin was noticed that culminate the lipid peroxidation, maintain the antioxidant enzymes and reduce the apoptosis through modifying the XIAP, BAX, BCL2 and caspase-3 signal expression during cadmium-induced toxicity (Jia et al., 2011). One study has also

demonstrated the anti-abortive capacity of quercetin and described that it might modify the maternal-fetal interface immunity balance against the abortion inducers (Zhao et al., 2011). It has been documented that environmental endocrine disruptors like Aroclor 1254 cause the endometrial injury which can be healed by the provision of a moderate dose of quercetin (Xu et al., 2014). Similarly, the protective antioxidant role of quercetin against teratogenic effects of theophylline in rat embryos has been reported (Karampour et al., 2014). In contrary, no beneficial impact of quercetin has been reported during T-2 toxin-induced oxidative stress in incubated granulosa cells but a modulating effect of quercetin on antioxidant defense system (SOD and GPx) has been observed (Capcarova et al., 2015).

It has been described that quercetin has a potential to improve the embryo implantation under oxidative stress. It protects the developing embryo against oxidative stress by the elimination of ROS, stabilizing the mitochondrial function, boosting the T-AOC, GPx and CAT (Yu et al., 2014). Moreover, quercetin also reduces the adverse effect of oxidative stress during embryo implantation and placental development by reducing the uterine blood glucose which is the potential factor to oxidative stress (Braga et al., 2012). The improvement in embryonic development is associated with quercetin provision. At low-level quercetin showed higher embryonic development by the elimination of ROS from the culture media whereas higher doses were detrimental to developing embryos (Kang et al., 2013; Orlovschi et al., 2014).

The negative impact of quercetin on the granulosa cells activity under normal conditions has been described. Suppression of proliferation/PCNA and promotion of Bax/apoptosis in granulosa cells are the main events which are associated with quercetin supplementation. It was observed that FSH promote the granulosa cells to proliferate and suppress the apoptotic process whereas quercetin reverts these process and inhibits the mTOR intracellular signaling system that is required for FSH action on ovarian cells (Štochmalová et al., 2013). A dual role of quercetin has been observed on the uterus and stated that lower dose modifies the uterine morphology whereas higher dose results in uterine stromal and glandular proliferation and can increase the uterine neoplastic incidence (Shahzad et al., 2014).

1.9. Aims and Objectives

Based upon the previous studies, the current study has been hypothesized that quercetin would reduce the detrimental effects of summer heat stress on reproductive performance of rabbits. Therefore, the objectives of the current study were:

1.9.1. To determine the effect of quercetin on sperm quality in summer heat exposed rabbits

1.9.2. To examine the effect of quercetin on histopathology and apoptotic markers in the testes of heat exposed rabbits

1.9.3. To evaluate the effect of follicular development, oocyte quality and oocyte maturation rate obtained from heat stressed female rabbits

1.9.4. To observe the effect of quercetin on apoptosis of granulosa cells in follicles of summer heat exposed females rabbit

2. MATERIALS AND METHODS

The present study was conducted in the Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Adnan Menderes University, Işıklı, Aydin (37°43'42N 27°56'14'E), Turkey. The rabbits were kept in the small animal unit (rabbitry) of Faculty of Veterinary Medicine throughout the study period. The procedures were practiced on the rabbits according to the guidelines of Animal Ethics Committee of Adnan Menderes University (Turkey). An approval was endorsed by the Animal Ethics Committee of Adnan Menderes University (ADÜ-HADYEK No. 64583101/2014/153) about the use of animals for scientific purposes.

2.1. Temperature-Humidity Index Calculation

The daily ambient temperature and relative humidity were recorded at two time afternoon (at 13:00 and 17:00 pm) during mid-July to last week of September using hygrothermometer (AT 303C, Qingdao Tlead International Co., Ltd. Shandong, China). The instrument was placed in the rabbitry to observe the variations in air temperature and relative humidity. The temperature-humidity index (THI) was calculated by following the equation adapted by Marai et al. (2001):

$$THI = db°C - [(0.31 - 0.31 \times RH) \times (db \times °C - 14.4)]$$

Where:

db°C = Dry Bulb Temperature in Celsius

RH = Relative Humidity Percentage.

The heat stress level was categorized based on the THI values which are as follows: less than 27.8 = absence of heat stress, between 27.9 to 28.9 = moderate heat stress, between 28.9 to 30.0 = severe heat stress and higher than 30.0 = very severe heat stress.

2.2. Experiment 1

2.2.1. Animals

Twelve male White New Zealand rabbits with the age 10-12 month, and mean body weight (2.9±0.1) were included in the present study. The bucks were caged individually in wire enclosures (70, 50, 35 cm^3) in a naturally ventilated house. A normal daylight was provided (16-17 hrs.) and no additional supplementary electric light was provided. Clean fresh water was available for rabbits at all times. The rabbits were trained for semen collection during the rearing period using teaser doe and rabbits showed good sperm parameters during the training were selected further for the experimental trail.

2.2.2. Feed Preparation and Feeding Trial

The maintenance diet was formulated according to the recommended values by NRC, 1977. The given ingredients (Table 1) were ground in the powder form and pellets were prepared. For the treatment group, the quercetin was mixed in mesh feed and pelleted. The length and diameter of the pellets were kept about 10 and 4mm, respectively. The proximate analyzes of feed (Table 1) was carried out using a standard procedure which as follows: the dry matter was determined by oven drying at 105°C overnight and crude protein by Kjeldahl nitrogen analysis. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) content were measured. Crude fat was estimated Soxhlet apparatus by diethyl ether.

Table 1. Ingredients and Proximate Analysis of Nutrients

Feed Ingredients	**(%)**	**Chemical Composition**	**(%)**
Corn	31	Dry matter	87.8
Wheat bran	25	Crude protein	17.4
Dried distillers grains soluble	15	Crude Fat	4.8
Sunflower meal (36% protein)	9.5	ME MJ/kg	10.73
Soybean	7	NDF	25.65
Safflower	5	ADF	11.4
Sunflower meal (28% protein)	3.5	Ash	4
Limestone	2.5		
Salt	1		
Vitamin-minerals mixture	0.1		

The rabbits were randomly divided into two equal groups ($n = 6$ per group). Each rabbit was provided 150 grams/day of maintenance ration. The control group was fed the basal diet and treatment group was offered basal diet supplemented quercetin (Quercetin hydrate, 337951, Sigma-Aldrich, St. Louis, USA) @ 30mg/kg/day (Bhaskar et al., 2013). The selected rabbits were housed in the ventilated room during the experimental trail. Before the start, the rabbits were allowed ten days for acclimatization to the heat conditions. After acclimatization, the both groups were offered the experimental feed for a period of 8 weeks.

2.2.3. Semen Collection and Reaction Time

During the feeding trial, the male rabbits were used for semen collection at different intervals. Semen was collected from all the selected rabbits during adaptation period. The frequency of semen collection was once a week from each buck. The semen was collected at day 0, 15, 30, 48 and 56 of feeding trial. The semen was collected by single operator at morning time between 8:00 to 9.00am. Semen was collected using locally made artificial vagina. Prior to semen collection, the artificial vagina (AV) was assembled and the inner jacket kept warm with adequate pressure by introducing the water with a temperature of 45°C and blowing air. The AV was lubricated using vaseline at the exposed end and avoided extra lubrication. A graduated collection tube was attached to the one end of AV. The care has been taken not to allow the AV to get too hot or cold because it reduces the efficiency of semen collection and avoided the sample to get contamination with urine. A matured cyclic doe was exposed to the bucks as a teaser during the collection. After collection, the ejaculates were observed for the color and presence of gel. Any deviation from the opaque white color of ejaculates samples was noticed. If gel plug was present, it was removed immediately following the collection.

The reaction time was recorded in seconds using the stopwatch and spell between the introductions of teaser doe in the pen to mount, was considered as the reaction time for a buck (Gado et al., 2015).

2.2.4. Semen Quality

2.2.4.1. Ejaculate Volume, Semen Osmolarity and Sperm Concentration

The semen volume for each ejaculate was recorded in microliters (µL) dually by noticing the value in a graduate collection tube and by using the micropipette (100-1000 µL). The ejaculates were mixed properly with a micropipette in the laboratory after collection. A sample of 50 µL was taken in small Eppendorf tube (500 µL) specially designed for osmometer. Before the calculation, the osmometer was calibrated and samples were run for osmolarity value measurement. The osmolarity of each sample was obtained using a freezing-point depression osmometer (Osmomat-3000, Gonotec, Germany). The sperm concentration was evaluated using standard Thoma chamber. The semen was diluted at the ratio of 1:200 (v:v) in water. After dilution, a homogenized drop (10 µL) loaded in the both chambers and allowed for 5 min to settle the sperm. The sperm cells were counted in the central grid area of the both chamber and calculated the given formula. The sperm concentration was denoted in million per millilitre.

Sperm count/ml = (Numbers of sperm counted in 400 small squares) × Dilution rate (200) × Depth of slide (10) × 1000.

Table 2. CASA Setup Characteristics for Rabbit Sperm Motility Characteristics

Parameters	**Abbreviations**	**Specifications**
MOT (%)	Total motile sperm	> 25 µm/s
PROG (%)	Progressively motile sperm	> 100 µm/s
VAP (µm/sec)	Average path velocity	The average velocity of the smoothed cell path
VCL (µm/sec)	Curvilinear velocity	The actual velocity measured over the actual point to point track followed by the sperm
VSL (µm/sec)	Straight line velocity	The average velocity measured in a straight line from the beginning to the end of the track
STR (%)	Straightness index	The average value of the ratio VSL: VAP
LIN (%)	Linearity index	The average value of the ratio VSL: VCL
WOB (%)	Wobble	A measure of the oscillation of the actual trajectory of its spatial average path (VAP: VCL).
ALH (µm)	Amplitude of lateral head displacement	The mean width of the head oscillation as the sperm cells swim
BCF (Hz)	Beat cross frequency	The frequency of sperm head crossing the average path in either direction

2.2.4.2. Motility Characteristics

The semen samples were diluted (1:4 ratio) with TCG solution [Tris 276.5 mM (hyrdoxymethyl)-aminomethane 08387, Merck, Darmstadt, Germany, Glucose 76.8 mM

08337, Merck, Darmstadt, Germany and Citric acid 90.9 mM 403727 Carlo Erba Reagents SAS Val De Reuil, France] prior to motility assessment. The motility assessment in each sample was performed by using CASA system (SCA®-Sperm Class Analyzer, Microptic S.L. Viladomat, Barcelona, Spain) connected with a phase contrast microscope (Olympus, CX41, Japan) along the camera and adjustable heated stage. The diluted samples (3 µL drop) was placed over a prewarmed glass slide and covered with a coverslip (22 × 22 mm^2). Before the use, the specifications of CASA were standardized for evaluation of rabbit sperm motility. The particle size was set 10 to 80 micron to focus the sperm head. Multiple fields (up to 100 sperms/field at ×10) in less than 2 seconds/field were analyzed and each field was selected at different places over the slide. As minimum as 500 sperm characteristics were recorded for each sample. Rabbit sperm motility characteristics assessed according to set up of CASA described in Table 2.

2.2.4.3.Sperm Viability

Sperm viability was observed in each diluted (1:4, v/v with sperm concentration 100×10^6/mL) sample by using propidium iodide (PI; P4170 Sigma-Aldrich, St. Louis, USA) staining. A sample of 50 µL was incubated with 2.5 µL PI (5 µL; 200µg/mL stock solution) for 5 min at 37°C. A fixative about 2.5 µL (4% Paraformaldehyde dissolved in PBS) was used to halt the sperm motion prior to observation. A wet mount was prepared using microscopic glass-slide and a minimum of 200 sperm were tallied under ×40 magnification with a fluorescence microscope (Olympus BX53, Japan) equipped with differential interphase contrast (DIC), camera (Olympus DP26, 5 M.P), multiple fluorescence filters (Olympus U-FBN/UFBW) and image software (Olympus Imaging cell sens software CS-EN-V 1.6). The excitation and emission of PI-stained cells were between 530 to 615 nm using U-FGW filters. The nonviable sperm with damaged plasma membrane exhibited the yellowish-red fluorescence and viable with normal plasma membrane did not emit any florescence (Fig. 1).

2.2.4.4. Sperm Acrosome Integrity

To determine acrosome integrity, the diluted semen samples (50 µL) were incubated with *Arachis hypogea* (peanut) agglutinin Fluorescein isothiocyanate conjugate [(5 µL;

200µg/mL stock solution); FITC-PNA, L7381 Sigma-Aldrich, St. Louis, USA] for 5 min at 37°C. After incubation, the samples were fixed using 4 % paraformaldehyde solution. Afterwards, a drop of sample was placed on the glass slides and coverslipped. In each sample, about 200 sperm cells were assessed at a magnification of ×40 using epifluorescence microscope (specification as mentioned above sub-heading 2.2.5). Two panels; one under bright field and the second using epifluorescence, were examined. This stain intensely labels the acrosomal region of acrosome-reacted sperm, which emits uniform applegreen fluorescence while acrosome-intact spermatozoa do not show any fluorescence at the anterior part of the acrosome head (Fig. 2). The bright panel of microscope indicates the total number of sperm whereas the percentage of acrosome-reacted sperm was calculated in fluorescence illumination panel. FITC-PNA stained cells excitation and emission were between 500 to 540 nm using U-FBW filters.

2.2.4.5.Functional Mitochondrial Potential

The activity of mitochondria in the mid-piece was determined using the mitochondrial-specific dye rhodamine 123 (Rh123; R8004 Sigma-Aldrich, St. Louis, USA). Briefly, 10 µL of Rh123 (0.01mg/mL diluted in PBS) was added to 500 µL diluted semen sample and samples were left for incubation at 37°C in water bath for 30 min. A fixative solution (4 % formaldehyde) was added to the incubated sample to check the sperm motility. A drop of 10 µL was put on the glass slide and covered with a coverslip. The slides were observed for a minimum 200 sperm immediately at ×40 magnifications using a fluorescence microscope. The sperm with bright fluorescence at the mid-piece were considered to have high mitochondrial activity, whereas sperm with no or little fluorescence were supposed to have low mitochondrial activity at the mid-piece region (Fig. 3). Excitation and emission of Rh123 stained cells were in the range of 490 to 520 nm using U-FBN filter.

2.2.4.6. Sperm Morphology

Briefly, the percentage of morphologically abnormal sperm was determined by fixing a 25 µL portion of each fresh sperm sample in 250 µL of Hancock solution. A drop of 5 µL was placed over the slide and observed at ×100 magnification (oil immersion objective) under

phase contrast microscope. A total of 200 sperm in each sample were counted and differentiated for head, mid-piece and tail abnormalities (Fig. 4). These abnormalities were numbered and classified as acrosomal changes (missing or knobbed acrosome), head abnormalities (pyriform, micro, macro or elongated head), mid-piece (double or retroaxial), proximal or distal cytoplasmic droplet, and tail defects (curved, coiled, dag defect or double tail) (Bondar et al., 2000).

2.2.5. Testicular Histopathology and Apoptosis

2.2.5.1. Testis Collection

At the end of the trial, all the rabbits from HS and Que groups were slaughtered. Testes were removed quickly, cleaned the extra surrounding tissues or fat and examined apparently for any lesions. Normal testes were processed further for histopathological findings.

2.2.5.2. Testicular Histopathology and Testicular Immunohistochemistry by TUNEL Assay (terminal deoxynucleotidyl Transferase-mediated dUTP nick end-labeling)

The testes samples were fixed in Bouin's solution for 48 hrs. and then, the fixed specimens were dehydrated in alcohol and embedded in paraffin. The embedded tissues were sectioned of 5 µm, deparaffinised and stained with hematoxylin and eosin (HE). The testicular tissue was examined and evaluated in random order under blindfold conditions with standard light microscopy by a pathologist. In the histopathological examination of testicles, we observed for any deviation from the normal architecture of seminiferous tubules, germinal and sertoli cells. The degenerated/necrotic germ cells, tubular atrophy, the presence of exfoliated germ cells in the lumen and interstitial vacuoles were differentiated by using light microscopy (Olympus BX 51, Japan).

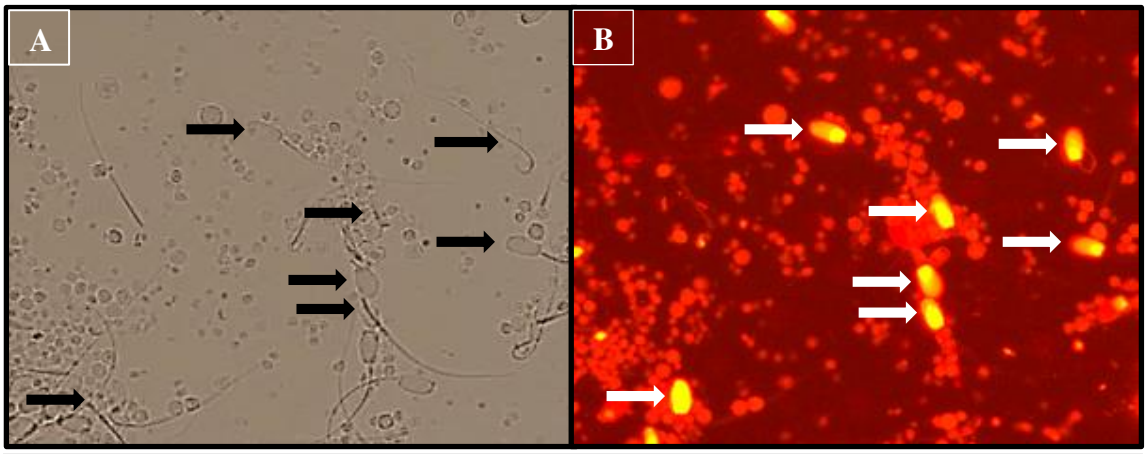

Fig. 1. Representative two panels (A&B) showing the rabbit sperm viability from freshly ejaculated semen. A: Total sperm (black arrows) observed under bright microscopy (×40) and B: sperm labelled with the propodium iodide (yellow fluorescence at the head region indicated by white arrow) observed under fluorescence microscope (×40).

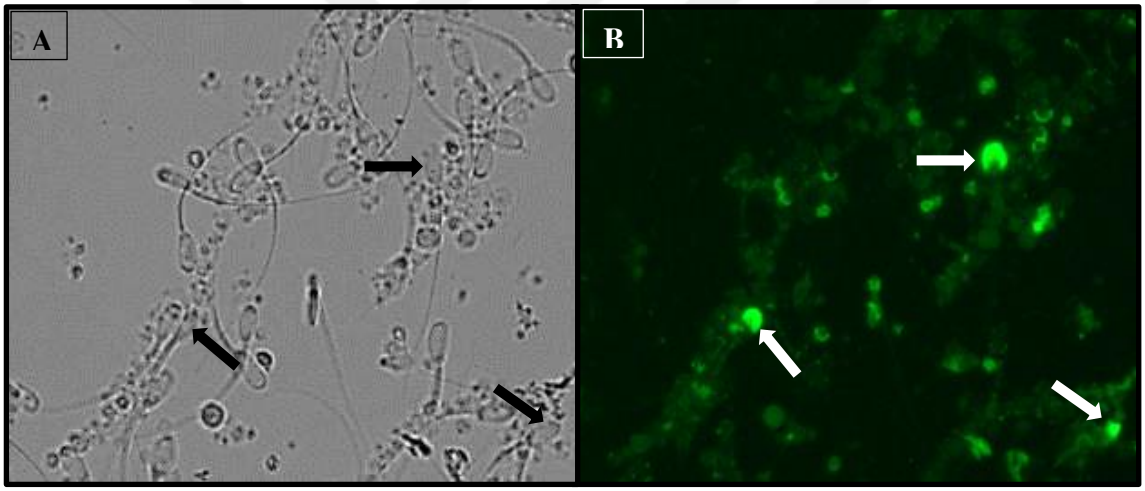

Fig. 2. The photomicrographs of sperms acrosome under bight and florescence microscopy (×40). The black arrows (A) points the normal sperm under bright field and white arrows (B) inidacte the acrosome-reacted sperm exhibiting the apple green fluorescence at acrosomal region.

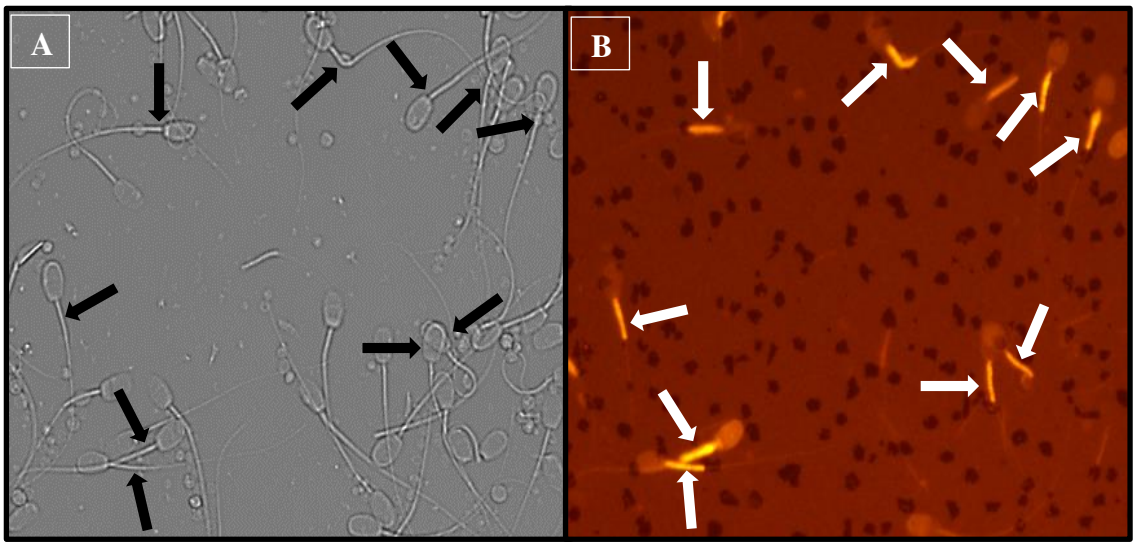

Fig. 3. The micrographs showing the unstained (A) or stained with Rhodamine 123 (B) rabbit sperm at 400 × magnification. The left panel represent the total number sperm cells (black arrow) under bright field (A); and right panel showing the sperm (black arrows) with high potential mitochondria at mid-piece region using fluorescence microscopy (B).

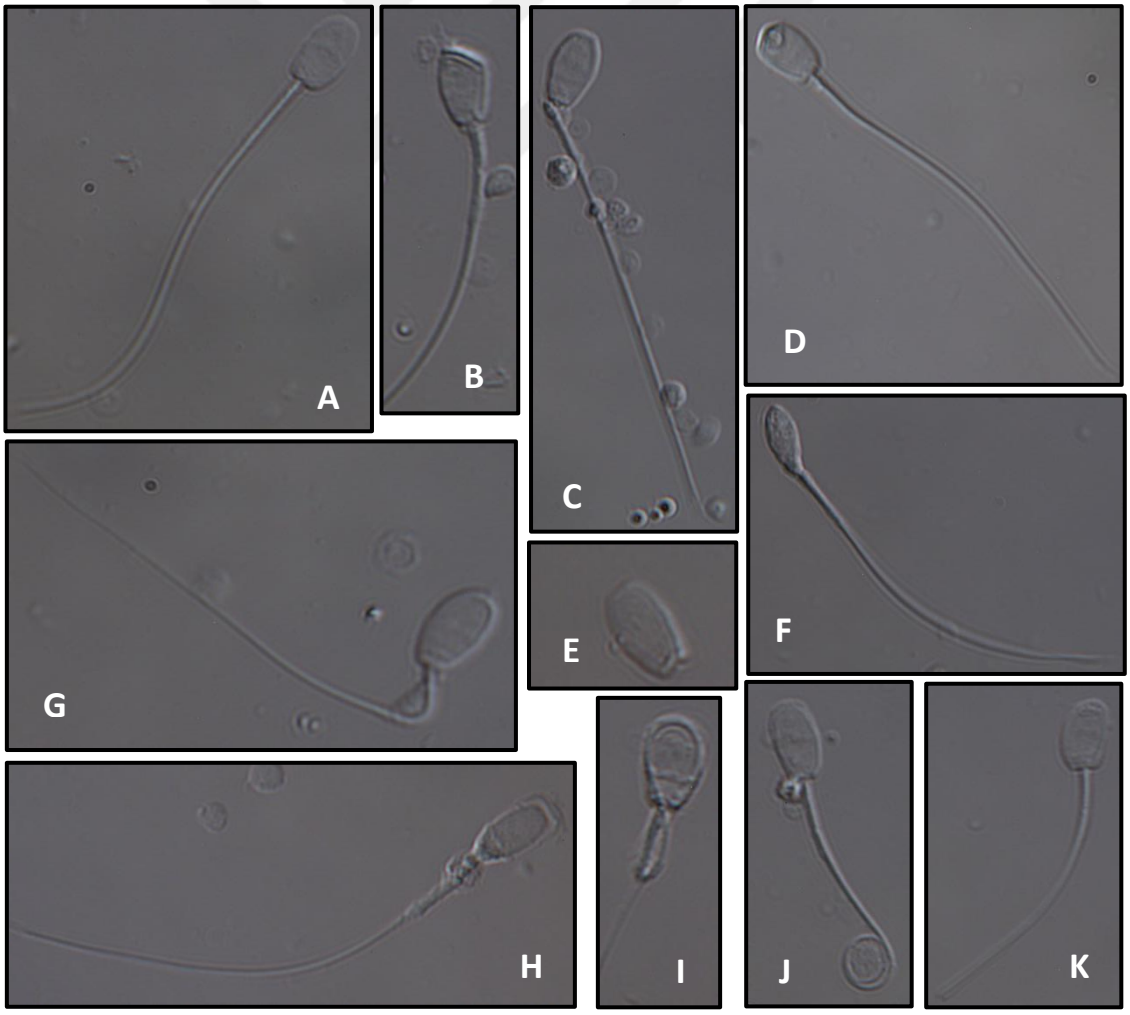

Fig. 4. The micrographs represents the normal (A) and abnormal rabbit sperm morphologies [head (B-F), mid-piece (G-H) and tail (I-K)] observed under bright field (×100).

In apoptotic cells, the DNA damage was observed by using commercially available TUNEL kit (Millipore, APOPTAG® Plus Peroxidase *In Situ* Apoptosis Detection Kit). Briefly, the sections were paraffinized and deparaffinized in alcohol serial washings. Afterwards, the samples were washed with PBS and incubated in proteinase K (20 mg/mL) for 15 min at room temperature and then again washed twice in distilled water for 2 min. Later, the slides sections were kept for 5 min in 3 % H_2O_2 solution and washed twice with PBS for 5 min. For TUNEL reaction, the sections were incubated in humid chamber at 37°C for 1 hour using 50µL drop of Terminal deoxynucleotidyl transferase (TdT) and fluorescein-dUTP (29-to 59-deoxyuridine triphosphate). The reaction was observed under fluorescence microscope. The prepared sections were shacked with 1/34 percent distilled water kit solution (Stop/Wash) for 15 seconds and then further incubated for 10 min at room temperature in the same solution and washed with PBS (3×1 min). Afterwards, the slides were covered with the prepared anti-digoxigenin peroxidase conjugate and incubated for 30 min at room temperature and then again washed with PBS (4×2 min). Finally, the samples were kept in 0.045 % (v/v) containing 0.08 % H_2O_2 (w/v) 3, 3-diaminobenzidine tetrahydrochloride (DAB) solution for 3-6 min to induce the chromogenic reaction. At the end, the reaction was observed under light microscopy and samples were washed with distilled water (3×1 min). The sections were stained with Mayer's hematoxylin and dehydrated in serial alcohol dilutions. The prepared slides were covered with coverslip. The negative control sections were also prepared in the same manner, however, those were not treated for terminal transferase TUNEL reaction. The slides were observed in a random fashion by selecting 5 different fields (10 tubules/field) to count the TUNEL-positive cells using bright microscope under ×20 magnifications. TUNEL positive staining indicates the extent of DNA breakage and the affected cell types that illuminate as brown in color.

2.3. Experiment 2

2.3.1. Feeding and Management of Female Rabbits

A total of twenty-six adults New Zealand White female rabbits about 8-9 months old with body weight (3.2±0.3kg) were selected. Similar environmental conditions were also provided to female rabbits as mentioned above in the sub-heading (2.2.1.). Females rabbits were divided into two equal groups (n = 13 each group) and fed the basal diet to control group whereas treatment group was fed the quercetin supplemented in the basal diet for 20 days. Eight

females from each group underwent laparotomy procedure and five from each group were slaughtered. One week prior to laparotomy or slaughtering, female rabbits were also injected $PGF_2\alpha$ (Lutelen, 0.2 mL/animal, cloprostenol, Topkim, Istanbul, Turkey) intramuscularly to synchronize the cycle.

2.3.2. Animal's Preparation and Laparotomy Procedure

The experiments were carried out according to the protocol approved by the animal care committee and in compliance with institutional guidelines. Basic sterilized surgical instruments (scalpel, scissors, forceps, clamps, and surgical drapes) and supplies (Xylazine and Ketamine) were used. Animals were kept off-feed and water for a period of 12-16 hrs. before the surgical procedure. After fasting, the animals were administered intravenous pre-anaesthetic medication (Xylazine 2 %, 1.8 mg/kg IV, Alfazyne Alfasan international BV Woerden, Holland) and followed by anesthesia (Ketamine, 100 mg Ketasol 10 %, 15 mg/kg IV, richter pharma ag, Wels, Austria) through venepuncture of peripheral ear vein. The anesthetized rabbits were positioned in dorsal recumbancy and then the abdominal area (xyphoid to pubis) was clipped and povidone iodine antiseptic solution was applied for sterility.

The animal was covered with a sterile drape to avoid contamination at the surgical site. Laparotomy operations were conducted by two surgeons under general anesthesia, aseptic, and sterile surgical conditions. A midline incision was made through the skin and subcutaneous tissues between pubis and umbilicus line. Linea alba was incised to approach the abdominal cavity. The uterine horns were located, held by thumb forceps and washed with the sterile PBS (Phosphate buffered saline, P4417 Sigma-Aldrich, St. Louis, USA). The follicles (>1-3 mm) were recognized and counted. The follicles were aspirated using a 1 mL syringe equipped with a 26-gauge needle containing sterile PBS. The aspirated oocytes were processed in the lab for further work. After completing the oocyte aspiration, the powdered antibiotic was sprinkled to circumvent any contamination. The surgical site was closed in two layers: muscles and fascia together, and skin. Simple interrupted suture pattern was performed for the closure of both layers by catgut and acrylic thread. After the closure, the antiseptic spray was applied and antibiotic (Baytril-K 5 %, Enroflaxacin, 0.1 mL/kg IM, Bayer GmbH, Kiel, Germany) was injected intramuscularly. Animals were kept under the normal conditions and cared intensively for three days post-operation.

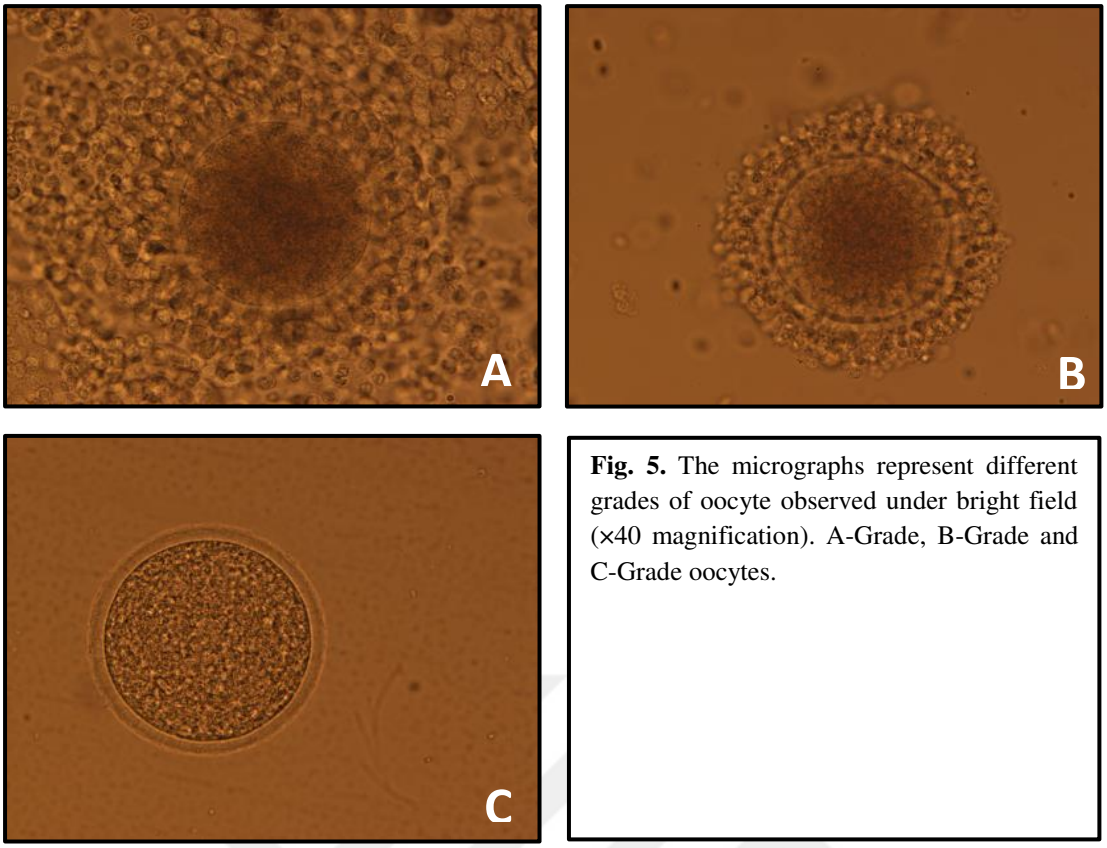

Fig. 5. The micrographs represent different grades of oocyte observed under bright field (×40 magnification). A-Grade, B-Grade and C-Grade oocytes.

2.3.3. Grading and Measurement of Cumulus Oophorous Complex (COCs)

After oocyte collection, the oocyte were washed thrice in TCM-199 medium (Medium-199 M4530 Sigma-Aldrich, St. Louis, USA) and suspended in TCM-199 during grading and measurements. The collected compact COCs were classified into three basic categories with respect to the presence of a number of cumulus oocyte complex layers (Fig. 5). A-grade represented the oocytes which completely surrounded by a maximal number of cumulus cell layers; B-grade oocytes were covered by 3 to 4 with cumulus cell layers and C-grade oocyte showed to have no any cumulus layers (denuded cell). The COCs dimensional were measured between zona to zona (µm) and whole COCs in the horizontal and vertical plane (µm) by using an eye-piece with the in-built caliber of 100 lines (each line represent 10 micrometers) fitted in dissecting stereomicroscope (Olympus SZ51, Japan) at ×40 magnification.

2.3.4. *In-Vitro* Oocyte Maturation, Cumulus Expansion and Nuclear Stage

The compact oocytes of A and B grades were selected and suspended in the maturation media for 24 hrs. The basic medium was TCM199 and it was supplemented with 50 mM Hepes

(HEPES sodium salt H3784 Sigma-Aldrich, St. Louis, USA), 0.3 mM sodium pyruvate (Sodium pyruvate P5280 Sigma-Aldrich, St. Louis, USA), 10 % fetal bovine serum (FBS, F0804 Sigma-Aldrich, St. Louis, USA), 10 µg/mL follicle stimulating hormone (FSH, Folltropin 700 IU Bionichi Animal health Europe ltd Dublin Ireland), 5 IU/mL human chorionic gonadotrophin hormone (hCG Pregnyl 1500 IU NV Organon Oss Holland), 100 IU/mL penicillin G potassium (Penicillin G 1000,000 IU, I.E. Ulagay Istanbul Turkey), 50 µg/mL streptomycin sulphate (Streptomisin Sulfat 1 g, I.E. Ulagay Istanbul Turkey). The pH value of the maturation medium was adjusted about to 7.4. The medium was sieved by using 0.22 µm filter for sterility prior to use. The medium-TCM199 was equilibrated 4-6 hrs. before incubation. In the plastic dish (Cell Culture Dish 430166, Corning Incorporated, NY, USA), about 5-6 droplets of 50 µL were prepared and covered by sterile mineral oil (Mineral oil, M8410, Sigma-Aldrich, St. Louis, USA). About 8-10 oocytes were incubated for maturation in each droplet.

Cumulus expansion in COCs, subjected to the maturation media was observed at 24 hrs. of incubation under a stereomicroscope (Olympus SZ51, Japan). It has been categorized as not expanded, partially expanded, or fully expanded (Fig. 6).

The fluorescence DAPI staining was performed to assess the nuclear status and the presence of the first polar body in *in vitro* matured oocytes. The cumulus cells were removed by gently pipetting after maturation period. The denuded oocytes were fixed in a solution of 4 % paraformaldehyde (Paraformaldehyde, 04005, Merck, Darmstadt, Germany) for 30 min at room temperature. Subsequently, the oocyte were incubated in 0.5 % Triton X-100 (Triton X-100, X100, Sigma-Aldrich, St. Louis, USA) in DPBS solution for 30 min for permeability. After permeabilization, oocytes were incubated in buffer containing 2 % BSA (Bovine Serum Albumin, A9418, Sigma-Aldrich, St. Louis, USA) for 1hr. Finally, the oocytes were stained with DAPI (DAPI 4'6-diamidino-2-phenyindole, dilactate, D9542, Sigma-Aldrich, St. Louis, USA) for nuclear contents. The stained oocytes were mounted on the glass slide with a coverslip and examined using epifluorescence microscope. The nuclear status was classified into GV, GVBD, MI, and MII stages (Fig. 7). The numbers of oocytes lost during the staining procedures or those that could not be classified as germinal vesicle (GV), germinal vesicle break-down (GVBD), metaphase-I (MI) and metaphase-II (MII) during evaluation were recorded. DAPI produces a blue fluorescence with excitation at 360 nm and emission at 460 nm. The rate of nuclear maturation was defined as the number of oocytes that were at the MI or MII stage of meiosis relative to the total number of oocytes cultured *in vitro*. Oocytes that

arrested at the GVBD stage or that only progressed to the MI stage were considered immature (Spindler and Wildt, 1999).

2.3.5. Ovaries Collection, Weigh and Antral Follicle Count

In the second half of the experiment, five female rabbits in each group were slaughtered at the end of the feeding trial. After opening the abdominal cavity, the ovaries were removed and weighed using weighing balance (Denver Instruments, SI 114, sensitivity 0.1mg, Biochemia, NY, USA). The follicles were tallied on the ovarian surface visually.

2.3.6. Ovarian Histology and Immunocytochemistry

After apparent examination, the ovaries were fixed into a 4 % buffered neutral paraformaldehyde solution. All fixed samples were dehydrated with ethyl alcohol (50–100 %) and embedded in paraffin. The embedded tissues were sectioned at 5 μm and stained with hematoxylin/eosin (HE). Two histological sections from each ovary were observed under a light microscope for sorting the follicle population (Olympus BX 51, Japan). The observed follicles were classified as primordial, primary, secondary and antral follicles based upon the presence of granulosa cells layers surrounding oocyte.

For histopathological purpose, the sections were stained with HE staining and numbers of primordial, primary, secondary and antral follicles were observed in a section from each ovary. For the presence of apoptotic granulosa cells in follicles, the sections were processed for TUNEL procedure. Similar procedure was followed as described above section (sub-heading 2.3.3). The apoptotic cells were counted separately for each follicle type (primordial, primary, secondary or antral). The occurrence of TUNEL positive cells in follicles were averaged for each follicle group.

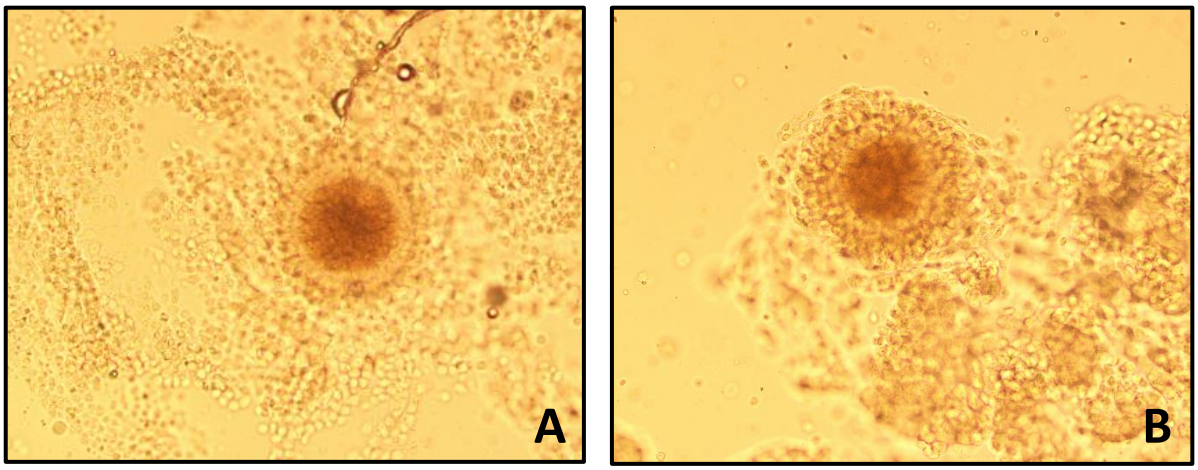

Fig. 6. The representative micrographs indicate the fully expanded (A) and partially expanded (B) oocytes after 24 hrs of maturation period (Bright field; ×20 magnification).

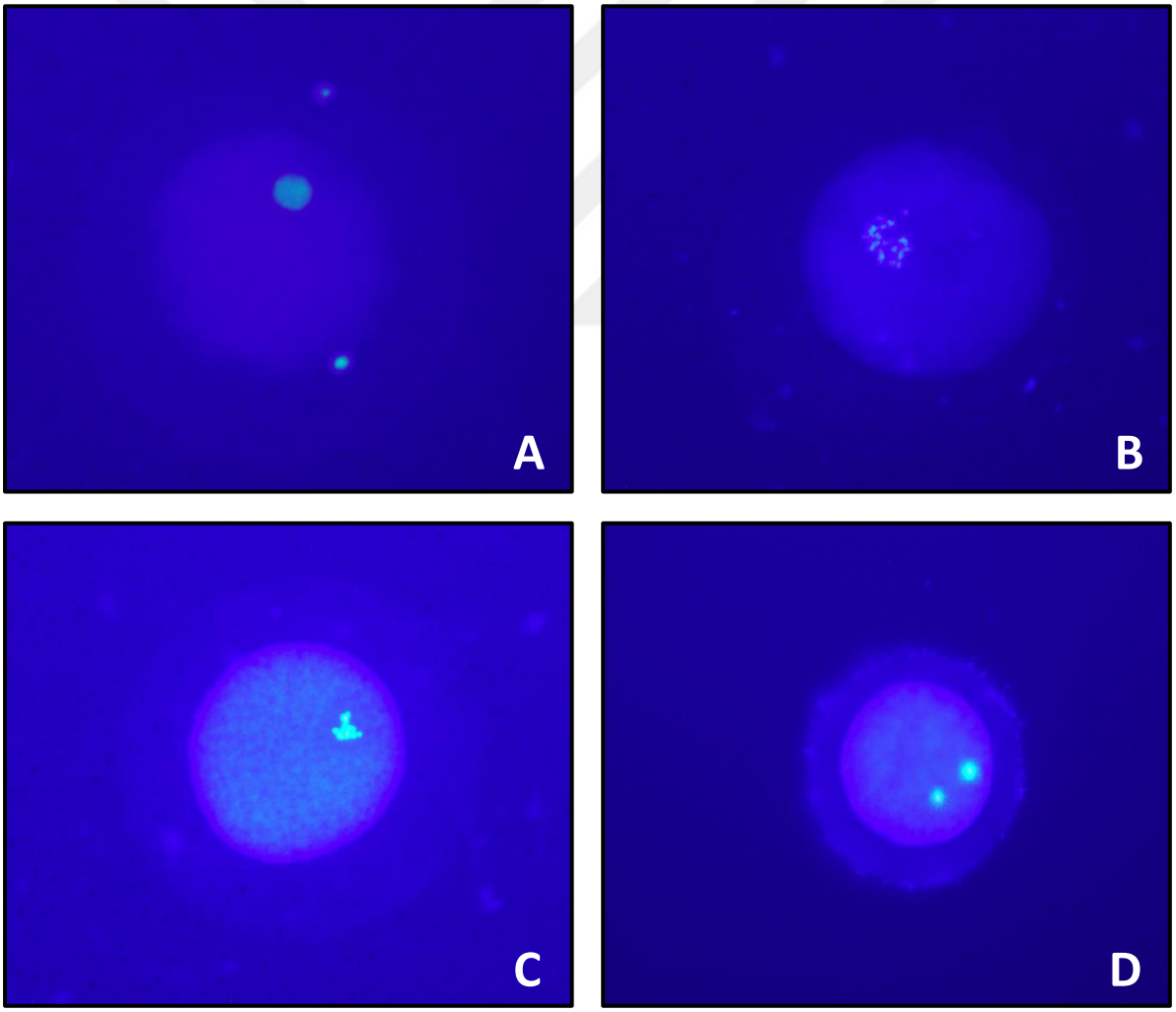

Fig. 7. The micrographs showed the matured oocyte stained with DAPI for nuclear maturation fluorescence. The germinal vesicle (A), GVBD (B), MI (C), and MII stage (D) under fluorescence (×40 magnification).

2.4. Serum MDA Estimation

At the time of slaughtering, blood was collected in 15 mL capacity serum tube. The tubes were slanted for 5 min and centrifuged at 4000 rpm for 10 min for complete serum separation (EBA 20, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany). The separated serum was kept in Eppendorf tube and stored at -20°C until assayed. The serum MDA level was estimated following the previously described method (Ohkawa et al., 1979). The measurement of MDA level was indicated by thiobarbituric acid reactive substances (TBARS) in serum which based on the reaction between MDA and thiobarbituric acid. A volume of 1.75 mL of the solution [0.67 % thiobarbituric acid (Trichloroacetic acid 272424 Sigma-Aldrich, St. Louis, USA) and 20 % 2-Thiobarbituric acid (2-Thiobarbituric acid T550-0 Sigma-Aldrich, St. Louis, USA)] was added to 0.25 mL of serum sample and final volume of the mixture was 2 mL. The samples were boiled at 95°C for about 30 minutes. The boiled samples were cooled down with ice cubes. In cooled samples, a volume of 2 mL of n-butanol was added. The mixture was vortexed properly and centrifuged at 3000 rpm for 10 min at 4°C. After centrifugation, the supernatant were separated and the absorbance was measured in the supernatant by the spectrophotometer (Shimadzu UV-1601, Kyoto, Japan) at 532 nm against blank using distilled water. MDA concentration was expressed as µmol/mL by multiplying the given formula:
Σ"Δ535" =1.56×10"6" M"-1" cm"-1"

2.5. Statistical Analyses

All the data were analyzed using statistical software (SPSS; version 17.0.1 Chicago, IL, USA). In Experiment 1, the physical and microscopic sperm variables at different time periods between control and quercetin supplemented groups were compared by using the student *t*-test. The proportion of apoptotic cells in testis was analyzed by performing the *chi*-square test.

In Experiment 2, the student *t*-test was performed to analyze the follicle number, number of oocyte per ovary, and oocyte dimensions in control and quercetin groups. The Fischer Exact test was used to analysis the oocyte grading, nuclear maturation rate, and apoptosis rate in different follicle type between the treatment groups. The oocyte expansion rate was analysed through the *chi*-square test. Oneway-ANOVA was used to test tha data of follicles poupation type between the treatment groups. The serum MDA concentration was also analysed through student *t*-test.

3. RESULTS

3.1. General Comments

Out of seven male rabbits in the control group, one died during the study period and data of dead rabbit was excluded. During the experiments, no significant ($p>0.05$) change in air temperature and humidity was observed. The average temperature, relative humidity, and THI were 33.5±1.7°C, 39.5±5.7 % and 29.9±1.2, respectively. The THI value showed the severe heat stress condition during the experiments (Fig 8).

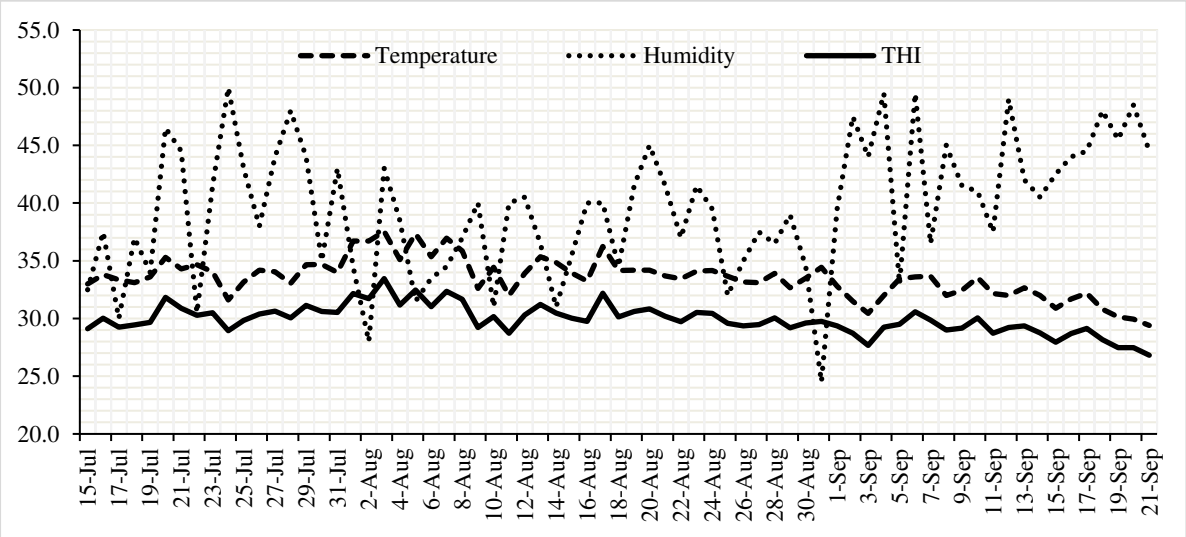

Fig. 8. The daily variations in ambient temperature [°C (hyphen)], relative humidity [% (dotted line)] and THI (solid line) during the study period.

3.2. Experiment 1

3.2.1. Effect of Quercetin on Reaction Time, Semen Volume, Semen Osmolality and Sperm Concentration

The effect of quercetin on reaction time, semen volume, osmolality and sperm concentration have been presented in Table 3. The data described that reaction time did not change ($p>0.05$) between the HS-Que and HS rabbits throughout the study period. Similarly, the average reaction time (sec) in both HS-Que (5.4±1.2) and HS (4.1±0.4) groups remained

same (Table 3.7; p>0.05). The results indicate that mean volume was similar (p>0.05) across the groups at D 0 and 15 of the collections. However, a significantly (p<0.05) higher semen volume was collected at D 30, 48 and 56 in HS-Que compared to HS rabbits group. Likewise, average semen volume in HS-Que group was also greater (p<0.05) than of HS group (Table 3.7). The semen osmolarity at each observation was similar (p>0.05) across the treatment groups. The average semen osmolarities in HS-Que and HS groups were 368.6±21.4 and 402.8±22.1 mOsm, respectively (p>0.05; Table 3.7). The results showed that sperm concentration was not different (p>0.05) between the HS-Que and HS rabbits at D 0, 15 and 30 of semen collection, nevertheless, it was higher (p<0.05) at D 48 and 56 of observation in HS-Que group. The average sperm concentration was significantly (p<0.05) higher (561.6±61.7 vs. 364.1±33.8) in quercetin treated group compared to HS (Table 8).

Table 3. Effect of Quercetin on reaction time, Semen Volume, Semen Osmolality and Sperm Concentration in HS Rabbits

Parameters	**Day**	**HS-QUE**	**HS**	***p*-value**
Reaction time (Sec)	0	2.0±0.4	3.0±1.1	0.438
	15	6.4±4.4	3.2±0.8	0.497
	30	8.6±4.4	3.4±0.9	0.285
	48	4.8±1.3	3.8±0.9	0.558
	56	5.2±1.3	7.4±2.2	0.427
Osmolarity (mOsm)	0	398.6±11.1	484.0±44.6	0.101
	15	331.0±64.1	483.6±71.5	0.15
	30	367.0±60.3	331.5±5.9	0.285
	48	396.2±63.5	377.0±18.4	0.779
	56	350.0±37.1	324.0±7.8	0.513
Semen volume (µl)	0	550.0±148.3	507.0±63.8	0.797
	15	692.0±80.0	615.0±57.8	0.458
	30	780.0±49.2	506.0±77.6	0.018
	48	840.0±29.1	462.0±59.1	0.000
	56	862.0±33.2	510.0±93.6	0.008
Sperm concentration ($\times 10^6$/ml)	0	454.0±24.0	472.0±86.7	0.847
	15	441.8±28.4	375.8±34.1	0.176
	30	406.4±34.1	305.2±46.2	0.116
	48	546.4±50.8	255.6±37.9	0.002
	56	681.0±18.7	410.0±26.3	0.000

Significance = p<0.05; NS = p>0.05

3.2.2. Effect of Quercetin on Motility Characteristics in HS Exposed Rabbits

The progressive and total motility results have been described in Table 4. The progressive motility was similar (p>0.05) between HS-Que and HS groups at D 0 to 30 of observation; however, it was tended to be higher (p=0.08 and 0.07) at D 48 and 56 in quercetin supplemented rabbit group. The average progressive motility, irrespective of day of observation, was similar (p>0.05) across the treatment groups. The total motility was same (p>0.05) between D 0 and 15 of semen collections in treatment groups. Numerically higher motility (p=0.08) was noticed in HS-Que treated group at D 30 and a significant change (p<0.05) was observed at D 48 and 56 in HS-Que treated group compared to HS group. Similarly, the average total motility was significantly (p<0.05) greater in HS-Que treated group (78.1±3.7) compared to HS group (66.6±3.9) (Table 8).

Table 4. Effect of Quercetin on Progressive and Total Sperm Motility in Rabbits Exposed to HS

Parameters	**Day**	**HS-QUE**	**HS**	***p*-value**
Progressive motility (%)	0	43.2±11.1	38.7±10.1	0.772
	15	51.6±16.6	37.7±15.6	0.498
	30	55.3±8.0	43.5±6.2	0.292
	48	45.0±8.6	25.9±4.4	0.081
	56	52.1±7.7	32.8±4.4	0.074
Total motility (%)	0	78.3±10.4	80.1±5.8	0.885
	15	82.3±8.4	65.9±13.0	0.317
	30	80.9±7.5	55.4±9.5	0.081
	48	78.5±8.2	56.8±4.9	0.029
	56	86.4±7.4	59.6±4.4	0.003

Significance = p<0.05; NS = p>0.05

The effect of quercetin on sperm kinetics in HS rabbits has been presented in Table (5). The results showed a non-significant change (p>0.05) in VCL rate between 0 and 30 days of collections, a significant change (p<0.05) at D 48 and numerically higher (p=0.06) at D 56 in HS-Que and HS groups. A non-significant difference (p>0.05) was observed in VSL rate at D 0, 15 and 30 of observation between the groups, whereas, it was significantly higher (p<0.05) in HS-Que treated group at D 48 and 56. The VAP rate was also different significantly (p<0.05) at D 48 and 56 in HS-Que compared to HS subjected rabbits. A similar pattern (p>0.05) was also observed in LIN in treated groups as in VAP. HS-Que group exhibited a significantly

higher ($p<0.05$) WOB percentage, ALH and BCF level in rabbit sperm at D 48 of the observation.

3.2.3. Effect of Quercetin on Rabbit Sperm Viability, Acrosome Integrity and Functional Mitochondrial Potential During Exposure to HS

The effect of quercetin on sperm viability, acrosome integrity and functional mitochondrial potential in HS rabbit semen has been presented in Table 6. A non-significant difference ($p>0.05$) at D 0, 15, and 30 and significant ($p<0.05$) improvement was observed at D 48 and 56 in sperm viability and acrosome integrity in HS-Que group. The average sperm viability was same ($p>0.05$) between the groups, whereas, the average sperm acrosome integrity was significantly higher ($p<0.05$) in HS-Que group than HS. The functional mitochondrial potential of sperm was same ($p>0.05$) at D0 whereas significantly higher ($p<0.05$) number of sperm with functional mictochondria were observed at D30 and 48 of observation in HS-Que compared to HS rabbit sperm. The average sperm functional mitochondrial potential was significantly higher ($p<0.05$) in HS-Que treated group compared to HS (Table 8).

3.2.4. Effect of Quercetin on Sperm Morphology

The effect of Quercetin on sperm morphology abnormalities in HS rabbits are presented in Table 7. There was a non-significant difference ($p>0.05$) in head abnormalities at D 0 and 15 between HS-Que and HS groups. A significantly lower percentage of head abnormalities were observed following on D 48 and 56 in HS-Que group than HS. In addition, the average head abnormalities were significantly ($p<0.05$) lower in HS-Que group compared to HS. A non-significant difference ($p>0.05$) at D 0 and significant change ($p<0.05$) at D 15 and 30 between HS-Que and HS was observed in mid-piece abnormalities incidence. The lower tendency of midpiece abnormalities incidence was observed in HS-Que group at D 48 and 56. The average midpiece abnormalities were significantly ($p<0.05$) less in HS-Que treated group. A non-significant difference ($p>0.05$) at D 0, 15 and 30 and a significant difference ($p<0.05$) at D 48 and 56 was observed in tail abnormalities between HS-Que and HS groups.

Table 5. Effect of Quercetin on Sperm Kinetics (VCL, VSL, VAP, LIN, STR, WOB, ALH and BCF) in HS Rabbits

Parameters	**Day**	**HS-QUE**	**HS**	***p*-value**
VCL(µm/Sec)	0	58.7±10.5	54.2±10.8	0.773
	15	78.1±21.0	60.0±14.8	0.531
	30	52.7±7.0	41.9±13.2	0.499
	48	71.3±6.6	42.6±4.3	0.013
	56	71.3±8.8	49.6±3.1	0.066
VSL(µm/Sec)	0	13.1±2.6	11.0±3.1	0.628
	15	20.8±5.7	16.5±5.7	0.536
	30	12.8±1.7	10.3±3.5	0.555
	48	17.2±2.2	7.3±3.5	0.004
	56	19.0±2.1	11.6±1.2	0.024
VAP(µm/Sec)	0	25.7±5.1	23.7±6.2	0.811
	15	39.3±10.9	29.2±8.6	0.484
	30	25.8±3.7	19.4±6.2	0.419
	48	33.3±4.4	17.2±1.6	0.012
	56	35.6±4.1	20.2±1.9	0.016
LIN (%)	0	22.0±1.5	18.8±1.6	0.203
	15	26.4±0.7	25.0±4.1	0.531
	30	23.7±1.2	24.7±3.0	0.765
	48	24.0±1.1	17.7±1.4	0.024
	56	26.7±0.8	19.0±1.0	0.020
STR (%)	0	49.4±1.6	45.7±1.0	0.101
	15	52.9±1.3	52.5±5.1	0.680
	30	51.0±1.2	52.2±3.1	0.736
	48	51.6±1.1	44.5±1.5	0.018
	56	55.7±0.3	48.7±1.9	0.101
WOB (%)	0	44.8±1.6	42.3±1.9	0.362
	15	49.8±0.7	46.5±3.9	0.347
	30	46.3±1.5	47.8±2.7	0.651
	48	46.4±1.8	39.8±1.6	0.046
	56	50.2±0.7	48.07±2.6	0.343
ALH(µm)	0	2.1±0.2	2.0±0.2	0.581
	15	2.5±0.4	2.1±0.2	0.571
	30	1.9±0.1	1.8±0.2	0.69
	48	2.3±0.1	1.6±0.1	0.004
	56	2.1±0.1	1.8±0.1	0.171
BCF (Hz)	0	6.8±1.4	4.3±0.9	0.182
	15	8.6±2.3	8.4±3.4	0.787
	30	6.0±1.0	6.4±1.8	0.856
	48	9.3±0.8	4.1±0.5	0.002
	56	7.4±1.4	4.3±1.0	0.100

Significance = $p<0.05$; NS = $p>0.05$

Table 6. Effect of Quercetin on Sperm Viability, Acrosome Integrity and Functional Mitochondrial Potential in Rabbit Semen during Exposure to HS

Parameters	**Day**	**HS-QUE**	**HS**	***p*-value**
Acrosome reaction (%)	0	29.8±8.5	40.8±10.5	0.437
	15	24.4±1.1	27.4±9.1	0.200
	30	25.0±4.8	34.7±5.5	0.226
	48	10.0±3.3	32.6±8.0	0.031
	56	11.0±1.1	23.9±3.6	0.009
Sperm viability (%)	0	69.5±7.0	76.5±2.5	0.376
	15	65.7±3.5	67.3±9.0	0.874
	30	55.0±8.3	32.9±6.1	0.066
	48	59.4±6.3	40.3±2.2	0.022
	56	59.3±3.6	44.1±2.3	0.008
Functional mitochondrial potential (%)	0	64.7±4.2	55.9±8.3	0.374
	15	65.0±8.5	43.2±5.8	0.069
	30	70.1±5.3	33.5±3.4	0.002
	48	64.4±3.4	44.2±3.6	0.005
	56	69.9±3.3	58.0±4.9	0.080

Significance = $p<0.05$; NS = $p>0.05$

Table 7. Effect of Quercetin on Sperm Morphological Abnormalities in HS Rabbits

Parameters	**Day**	**HS-QUE**	**HS**	***p*-value**
Head abnormalities (%)	0	13.1±1.4	11.2±0.5	0.250
	15	13.6±1.2	12.9±0.6	0.623
	30	11.6±1.0	14.8±1.1	0.052
	48	7.3±0.5	11.7±0.8	0.001
	56	6.7±1.0	11.5±1.3	0.019
Mid-piece abnormalities (%)	0	8.9±1.6	6.7±0.3	0.223
	15	2.2±0.2	4.6±0.3	0.000
	30	8.3±1.0	13.3±1.9	0.049
	48	9.1±1.2	12.4±1.0	0.058
	56	8.3±1.1	11.1±0.8	0.074
Tail abnormalities (%)	0	4.9±0.2	4.8±0.8	0.829
	15	3.7±0.4	3.9±0.6	0.790
	30	4.0±0.4	3.9±0.5	0.883
	48	4.9±0.4	2.2±0.3	0.001
	56	4.4±0.4	3.4±0.2	0.066
Total abnormalities (%)	0	26.9±2.5	22.7±0.5	0.140
	15	19.5±1.6	21.4±0.3	0.292
	30	23.9±1.0	32.0±2.8	0.026
	48	21.3±1.7	26.3±1.4	0.062
	56	19.4±1.3	26.0±1.0	0.003

Significance = $p<0.05$; NS = $p>0.05$

The results presented in Table 7 showed a similar ($p>0.05$) incidence of total abnormalities at D 0, 15 and 30, whereas, a significantly ($p<0.05$) lower number of abnormalities were seen in HS-Que group than HS at D 48 and 56 of observation. Similarly, average total sperm morphological abnormalities were significantly ($p<0.05$) less in HS-Que treated group compared to HS group (Table 8).

Table 8. Effect of Quercetin on Sperm Quality Parameters in HS Rabbits

Parameters	**HS-QUE**	**HS**	***p*-value**
Reaction time (Sec)	5.4±1.2	4.1±0.6	0.389
Semen volume (µl)	881.4±153.5	510.4±40.0	0.024
Sperm concentration ($\times 10^6$/mL)	561.6±61.7	364.1±33.8	0.007
Reacted acrosome (%)	18.1±2.5	31.9±3.4	0.002
Sperm viability (%)	61.8±3.4	52.2±4.6	0.106
Mitochondrial potential (%)	66.9±3.0	47.0±4.9	0.001
Osmolarity (mOsm)	368.6±21.4	402.8±22.1	0.272
PM (%)	43.1±5.08	31.6±4.8	0.111
TM (%)	78.1±3.7	66.6±3.9	0.042
VCL (µm/Sec)	62.3±5.5	54.2±5.5	0.306
VSL (µm/Sec)	15.9±1.6	13.4±1.7	0.294
VAP (µm/Sec)	30.3±2.9	25.4±2.9	0.255
LIN (%)	25.0±0.7	23.5±1.1	0.268
STR (%)	52.1±0.7	50.8±1.2	0.394
WOB (%)	48.0±0.8	46.1±1.1	0.194
ALH (µm)	2.1±0.1	2.0±0.1	0.585
BCF (Hz)	7.2±0.7	6.5±0.8	0.565
Head abnormalities (%)	10.4±3.6	12.4±2.2	0.029
Mid-piece abnormalities (%)	7.3±3.4	9.6±4.1	0.04
Tail abnormalities (%)	4.3±0.9	3.6±1.2	0.021
Total abnormalities (%)	22.2±4.5	26.6±4.8	0.012

Significance = $p<0.05$; NS = $p>0.05$

3.2.5. Effect of Quercetin on Apoptosis of Testicular Tissue

Histological examination of testicular tissues revealed that the interstitial stroma (Leydig cells, vasculature and supporting stroma) appeared normal in both groups. The results of apoptosis of testicular germ cells have been presented in Table 9. A significantly higher ($p<0.05$) number of apoptotic germ cells in seminiferous tubules in HS group compared to HS-Que group. Both the groups have shown the apoptotic activity in germ cells, however, in the case of HS-Que group, the apoptosis was seen in spermatogonia stage, whereas, all the phases of germ cells underwent apoptosis in HS group (Fig. 9).

Table 9. Effect of Quercetin on Incidence of Apoptotic Testicular Germ Cells in HS Rabbits

Parameter	HS-QUE	HS	*p*-value
Apoptotic cells/ST	20.4±7.6	55.8±20.7	0.007

Significance = $p<0.05$; NS = $p>0.05$

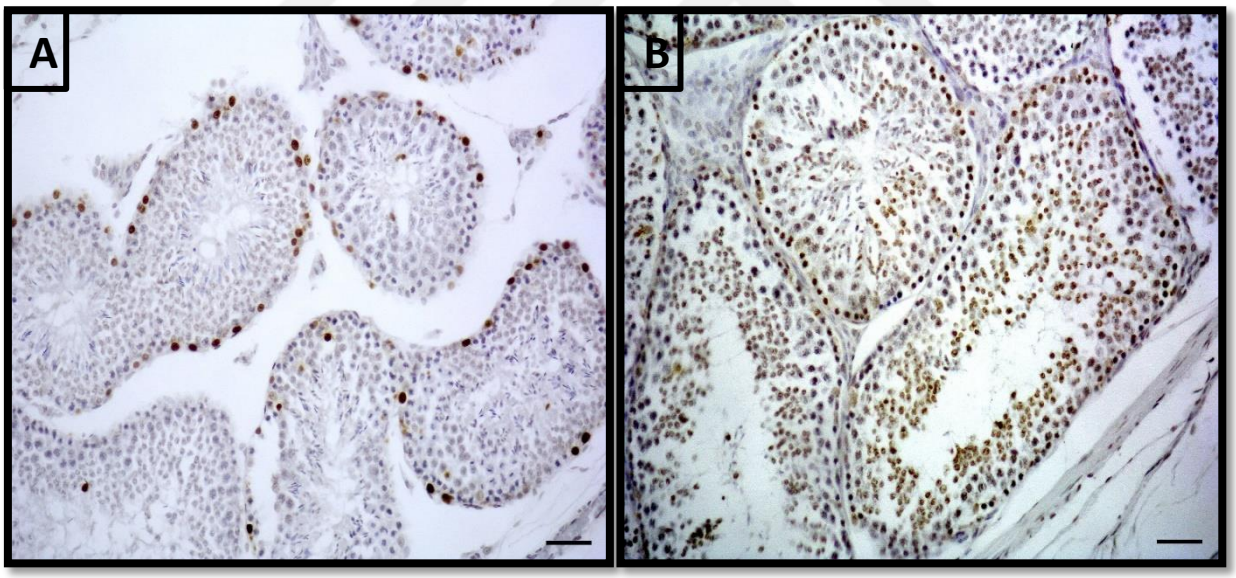

Fig. 9. Microscopic view of [A: Que group, ×20] and [B: HS group, ×40] testes after TUNEL staining of HS exposed rabbits. A few apoptotic germ cells are present in Que supplemented group (A) and whole population of germ cells showing higher apoptosis rate in HS group (B).

3.3. Experiment 2

3.3.1. Effect of Quercetin on the Number of Follicle and Oocyte Retrieval

The results described (Table 10) that quercetin supplementation during HS significantly improve the follicular (>1-3mm) development and further oocyte retrieval rate compared to non-supplemented HS group.

Table 10. Effect of Quercetin on the Number of Follicle and Oocyte Retrieval

Parameters	**HS-QUE**	**HS**	***p*-value**
Total No of ovaries	14	14	
No of follicles per ovary >1-3mm (n)	10.5±1.5	8.1±1.7	0.000
Total collected oocytes	129	93	
No. of oocyte recovered/ovary (n)	9.2±1.5	6.5±1.4	0.000

Significance = $p<0.05$; NS = $p>0.05$

3.3.2. Effect of Quercetin on Oocyte Grading and COCs Dimensions

The results showed that the number of A-grade oocytes collected from HS-Que group were greater ($p<0.05$) than that obtained from the HS group. Whereas, the number of B-grade retrieved oocytes was similar ($p>0.05$) between HS-Que and HS groups. Similarly, no difference ($p>0.05$) was observed in number and percentage of collected C-grade oocyte from HS-Que or HS group. The proportion of A, B and C-grade oocytes were significantly different ($p<0.05$) between the HS-Que and HS groups (Table 11).

Table 11. Effect of Quercetin on Oocyte Grading after retrieval

Groups	**A-grade oocytes (%)**	**B-grade oocytes (%)**	**C-grade oocytes (%)**	**Chi-square**	***p*-value**
HS-QUE	63.6	27.1	9.3	6.87	0.0322
HS	45.2	41.9	12.9		

Significance = $p<0.05$; NS = $p>0.05$

The oocytes dimensions showed that A-grade oocytes retrieved from HS-Que group have significantly ($p<0.05$) smaller zona to zona diameter with larger COCs size compared to HS group. The B-grade oocytes collected from HS-Que and HS groups had similar ($p>0.05$)

zona to zona and COCs size. The diameter between zona to zona in C-grade collected oocyte was same ($p<0.05$) across the treatment groups (Table 12).

Table 12. Effect of Quercetin on Oocyte Grading and COCs Dimensions

Parameters	**HS-QUE**	**HS**	***p*-value**
Grade-A Oocytes			
Total No.	82	42	
A-grade oocyte per ovary (SEM)	5.9±1.2	3.0±2.0	0. 000
Zona-Zona (µm)	143.5±2.3	152.3±2.3	0.023
COCs-COCs (µm)	645.6±33.8	458.3±32.1	0.001
Grade-B Oocytes			
Total No.	35	39	
B-grade oocyte per ovary (SEM)	2.5±0.7	2.7±1.2	0.472
Zona-Zona (µm)	140.6±4.6	150.0±4.4	0.157
COCs-COCs (µm)	185.9±6.4	187.1±4.1	0.89
Grade-C (Nude) Oocytes			
Total No.	12	12	
C-grade oocyte per ovary (SEM)	1.2±0.4	1.5±0.5	0.201
Zona-Zona (µm)	150.0±3.1	146.0±9.2	0.694

Significance = $p<0.05$; NS = $p>0.05$

3.3.3. Effect of Quercetin on Oocyte Expansion and Nuclear Maturation

Oocyte collected from the HS-Que treated and HS groups showed a similar percentage ($p>0.05$) of either fully or partially cumulus expansion after 24 hrs. maturation period (Table 13).

Table 13. Effect of Quercetin on Oocyte Cumulus Expansion Rate

Groups	***n***	**Full expansion (%)**	**Partial expansion (%)**	***Chi*-square**	***p*-value**
HS-QUE	69	55/69 (79.7)	14/69 (20.3)	0.062	0.80
CON	53	43/53 (81.1)	10/53 (18.9)		

Significance = $p<0.05$; NS = $p>0.05$

The results of nuclear maturation showed that there was a non-significant difference ($p>0.05$) in the proportion of GVBD, MI and MII stages between HS-Que and HS oocytes after

24 hrs. of maturation period. The percentage of degenerated oocytes were also similar (p>0.05) between the groups (Table 14).

Table 14. Effect of Quercetin on Nuclear Maturation Rate in the HS Rabbit Oocytes

Groups	GVBD (%)	MI (%)	MII (%)	Degenerated (%)	*Chi*-square	*p*-value
HS-QUE	4/69 (5.8)	10/69 (14.5)	48/69 (69.6)	7/69 (10.1)	5.90	0.11
HS	8/53 (15.1)	8/53 (15.1)	30/53 (56.6)	7/53 (13.2)		

Significance = p<0.05; NS = p>0.05

3.3.4. Effect of Quercetin on Follicle Development in HS Female Rabbits

The results showed that ovary weight was significantly greater in Que-HS group than the HS (Table 15). The quercetin supplementation significantly improved (p<0.05) the number of primordial and antral follicles during HS in rabbits. However, the number of primary and secondary follicle number was same (p>0.05) between the treatment groups (Table 15).

Table 15. Effect of quercetin on follicle population in HS female rabbits

Parameters	HS-QUE	HS	*p*-value
Ovary weight (gm)	0.21±0.04	0.16±0.03	0.009
Follicle type			
Primordial	133.4±13.5	21.4±4.9	0.000
Primary	16.0±2.2	21.0±8.9	0.260
Secondary	13.6±6.0	9.8±4.4	0.291
Antral	7.0±0.7	4.4±0.5	0.000

Significance = p<0.05; NS = p>0.05

3.3.5. Effect of Quercetin on Ovarian Cells Apoptosis

The TUNEL findings shows that apoptotic changes were not limited to the follicles, number of the apoptotic cells have also been observed in ovarian interstitial stroma.

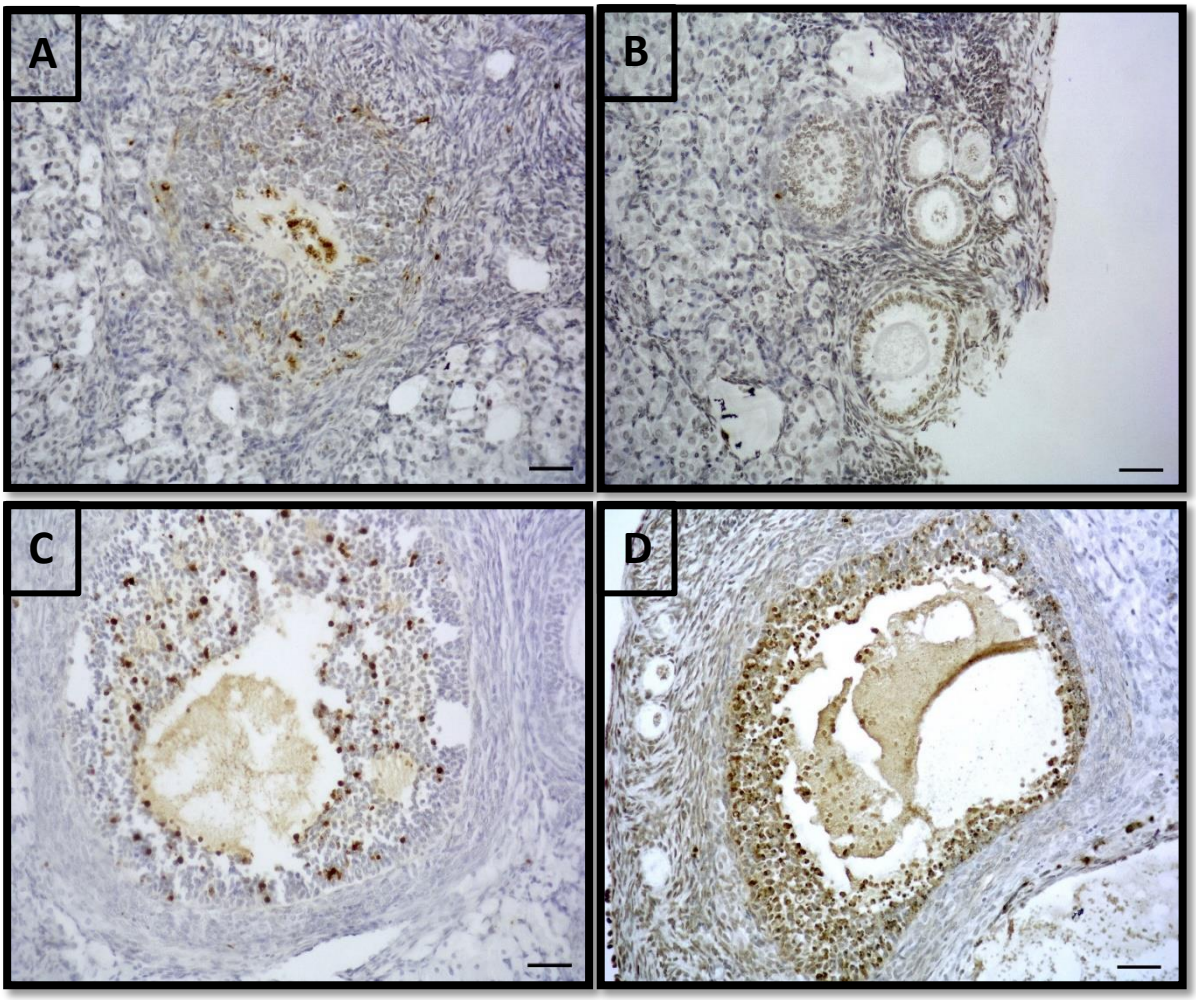

Fig. 10. Microscopic view of [A and C: Que group, × 40] and [B and D: HS group, ×40] of ovaries after TUNEL staining of HS exposed rabbits. A few apoptotic granulosa cells in primary, secondary and antral follicles are present in HS-Que group (A and C). Higher number of apoptotic granulosa cells is viewed in primary, secondary and antral follicles in HS rabbit group (B and D).

The results regarding follicular apoptosis have been presented in the Table (15). There was no apoptotic cell observed in primordial follicles in any treatment group. A higher proportion ($p<0.05$) of apoptotic cells were observed in primary and antral follicles in the control group compared to the Que supplemented group (Fig. 10), whereas, similar ($p>0.05$) proportion of apoptotic cells were seen in secondary follicles both HS and HS-QUE groups (Table 16).

Table 16. Effect of Quercetin on Apoptosis in Follicles

Parameters	**HS-QUE**	**HS**	***p*-value**
Primordial follicles	0	0	-
Primary follicles	30.0±10.0	64.0±20.7	0.011
Secondary follicles	40.0±10.6	44.0±11.4	0.582
Antral follicles	12.0±3.9	46.2±19.9	0.005

Significance = $p<0.05$; NS = $p>0.05$

3.4. Effect of Quercetin on Serum MDA Level

The MDA levels (Table 17) at the end of the trial showed that MDA was not ($p>0.0.5$) different between HS-Que and HS group; however, it was numerically higher ($p=0.08$) in the control group.

Table 17. Effect of Quercetin on Serum MDA Level in HS Female Rabbits

Parameter	**HS-QUE**	**HS**	***p*-value**
Serum MDA (nmol/mL)	17.4±2.5	19.8±1.7	0.08

Significance = $p<0.05$; NS = $p>0.05$

4. DISCUSSION

4.1. Heat Stress and Reproductive Functions

Although, the reproductive performance of an animal has a direct relation with physiological and nutritional status of the animal, heat stress affects animal's performance by reducing the productive and reproductive functions. The environmental conditions have also an equal contribution to the productive and reproductive performance of the exposed animals. During the summer months, an increase in the body temperature decreases the performance of animal by changing the animal's homeostatic system and intake of food. These factors collectively affect the male and female animal's reproductive performance widely. Generally, the malfunction of HPG-axis, changes in reproductive cycle, reduced oogenesis, low quality or degenerated oocyte, reduced ovulations, fertilization and embryo development are the major consequences of HS that successively lead to low pregnancy rate in the female. Similarly, in males the inefficiency of HPG-axis, less response of testicular cells to gonadotropins, reduced onset of spermatogenesis, degeneration of germ cells, and deterioration of sperm quality are also widely observed during exposure to high temperatures (Durairajanayagam et al., 2015). Other than the energy imbalance, high incidence of oxidative stress and genetic makeup may also play a detrimental role in the reproductive functions of the animals. In this scenario, several reports are available in different species to highlight the effects of HS on the male and female reproductive performance. Many strategies have been employed to alleviate the detrimental effects of HS. The supplementation of antioxidant is one of the strategies that have provided advantageous effects on sperm or oocyte quality under HS. As a continuation of the earlier studies, the supplementation of quercetin to heat exposed rabbits had provided the beneficial effect on sperm quality, testicular texture, and reduced the apoptosis incidence in the developing sperm cells. The higher number of follicles and good quality oocyte with low apoptotic activation had been obtained following quercetin supplementation to the HS female rabbits.

4.2. Effect of Quercetin on Semen Quality

The estimation of the seminal fluid volume is an essential component of sperm analysis in human and mammalians because it suspends the sperm, provide protection against harsh conditions and serve as medium to transfer of sperm into the female reproductive tract. Moreover, it contains all the essential nutritious content which nourishes the sperm during its travel from testis to the site of ejaculation in the female genital tract (Roberts and Jarvi, 2009). In the present study, an improvement in semen quality in the ejaculates has been observed after the quercetin supplementation in HS rabbits and no deviation in semen volume has been observed in non-supplemented HS rabbits. This pattern in seminal fluid is similar to the earlier studies where HS animals were fed with dietary supplements (Kamel, 2012; El-Tohamy et al., 2012; Zeweil et al., 2013; Hashem et al., 2013; Elnagar, 2010). The unchanged or low semen volume in HS rabbits might be associated with low activity of accessory glands under high environmental temperature (Marai et al., 2008). In addition, low activity of accessory glands has a relationship with testosterone level as these glands work under the influence of testosterone concentration. There are many studies in different species which clearly elaborated the detrimental influence of HS on testosterone level. The changes in testosterone level due to HS affect the accessory glands functions and, in turn, lower the semen volume. In addition, increased ROS in accessory glands during stress is also another factor which resulted in low seminal volume (Alshahrani et al., 2013). Increased semen volume upon quercetin supplementation might influence the activity of accessory organs by its excessive ROS scavenging property.

Libido is a potential variable for the assessment of reproductive efficiency in large and small animals, and the reaction time, which is measured as the time between introductions of male to the pen or cage to mounting or successful ejaculation is used to assess the libido level. It is believed that male with higher libido has the capacity to produce more viable and adequate fertile sperm in an ejaculate during the specified time period. The reaction time is variable in each species and generally, its duration is affected by environment, age, and managemental conditions. In the current study, no difference was observed in the reaction time of HS male rabbits by dietary supplementation of quercetin. Similar results were observed in HS rabbits by supplementing the Vitamin E (Hashem et al., 2013). However, the higher reaction time has been observed in different studies when experimental animals were exposed to the higher temperature (Okab, 2007; Safaa et al., 2008; Pei et al., 2012). A similar situation has been described in a study when the boar were exposed to heat for a long-term (Elile et al., 2014). The authors suggested that if animals have continuous exposure to heat, resumption to normal reaction time takes more than the expected time period because of lower circulating

testosterone level (Murphy et al., 2001; Elile et al., 2014). According to earlier reports, the continuous low libido is related to the impairment of Leydig cells function by excessive ROS which subsequently affects testosterone level (Hansen, 2009; Tremellen, 2008). Although, the testosterone and libido relationship has postulated; in addition, involvement of nervous system, pheromone and HPG axis also plays a great role in reproductive behavior (Fabre-Nys et al., 2015).

The osmolarity of the ejaculate is an important variable because osmolarity influences the sperm motility, viability and fertility. The acrosome reaction and oocyte penetration are linked with osmolarity change, as human sperm has osmo-sensitive calcium entry pathway that helps for the initiation of acrosome reaction (Rossato et al., 2002). In the present study, there was no change in the osmolarity value between the groups during the trial. Teodoro et al. (2012) reported no change in wolf semen osmolarity across the seasons. Bhakat et al. (2014) reported that bull seminal osmolarity increased during the hot season which might be linked with changes in core body temperature, feeding or higher water loss. In elephant, the osmolarity of semen has a positive correlation with macro minerals and negative correlation with sperm motility (Kiso et al., 2013). It has been described that the cations Na^{+2} and K^{+2} play a role in the osmotic balance of seminal plasma osmolality. Any deviation in Na^{+2} and K^{+2} values results in poor semen quality, as the both hypo- and hyperosmotic conditions had a detrimental effect on sperm survival (Tvrda et al., 2013). Similarly, Karaca et al. (2002) described that HS changes the ion concentration in poultry breeder semen that ultimately affect the sperm quality by changing the osmotic balance in the seminal plasma. In the current study, the osmolarity values were higher than the reported for normal rabbit semen osmolarity. This increase could be associated with the presence high Ca^{+2} or K^{+2} that occurred due to the osmotic imbalance during the high loss of water content through respiration, change in the body temperature or feeding. The estimation of correlation between seminal plasma osmolarity and ion concentration during the different seasons could be helpful to elaborate the effect of the osmotic change on sperm quality and fertility in different species.

Sperm concentration in rabbits is greatly affected by summer heat stress. The reduction in sperm concentration is gradual (4-6 week) in response to the summer heat stress (Okab, 2007; Pei et al., 2012). The reduction in sperm concentration is more prominent in rabbits during the summer compared to the other seasons (Mathur et al., 1989; Roca et al., 2005; Marai et al., 2002). In the present study, a significant improvement in sperm concentration was observed following the dietary supplementation of quercetin to the heat stressed rabbits. To enhance the sperm concentration of heat stressed rabbit, different dietary supplements have

been tested (Kamel, 2012; El-Tohamy et al., 2012; Zeweil et al., 2013; Hashem et al., 2013; Elnagar, 2010). The improved sperm concentration during HS is related with ROS scavenging property of quercetin. In future, the effect of dietary quercetin on rabbit sperm concentration during different seasons could reveal more valuable information about the antioxidant property of quercetin.

The sperm motility is a common parameter in almost each mammalian species and it has great value in the species with internal fertilization as sperm travel forwardly to reach the site of fertilization (Chrenek et al., 2007; Holt and Van Look, 2004). The CASA values of progressive motility in quercetin group tended to be higher than control whereas total motile sperm were significantly higher in quercetin group. In earlier studies, when different dietary supplements were fed to HS rabbits, a positive (Elnagar, 2010; Hassan et al., 2012; Kamel, 2012; Zeweil et al., 2013) or no (El-Tohamy et al., 2012; Hashem et al., 2013) impact of supplements have been observed. It has been stated that improvement in sperm motility following dietary antioxidants supplementation could be due to the activity of endothelial nitric oxide synthase that in turn enhances the motility (Donnelly et al., 1997) or scavenging effect of quercetin on ROS (Zhang, 2005). In addition, the enhanced sperm motility could be ascribed to the excessive release of fructose in semen during transfer from testis to the female genital tract or in ejaculation (Zeweil et al., 2013; Yousef et al., 2003). It has been well described the motile sperm subpopulation had reduced in fresh ejaculates following heat stress (Marthur et al., 1989; Roca et al., 2005; Okab, 2007; Safaa et al., 2008; Maya-Soriano et al., 2015). As described hereby, HS negatively influenced the progressive and total sperm motility which might be linked with the changes in mitochondrial activity by excessive ROS production under heat stress (Agarwal et al., 2008). The excessive ROS production reduced the motility by decreasing in axonemal protein phosphorylation and sperm immobilization. The combined effect of both factors result in reduction of membrane fluidity which plays role in sperm-oocyte fusion (de Lamirande and Gagnon, 1995). In addition, diffusion of ROS across the cell membrane hindered the activity of glucose-6- phosphate-dehydrogenase which maintains the glucose level for sperm motility (G6PD). There are some opinions that chronic exposure to heat stress does not influence the sperm motility and preserve the sperm fertilizing ability (Rahman et al., 2014; Hamilton et al., 2016). According to those, animals submitted to heat stress for a long period had no change in motile sperm percentage and it could be linked with acclimatization behavior of exposed animals to keeping a cooler area or decreasing temperatures at night time (Maya-Soriano et al., 2015).

Sperm motility is considered as an important characteristic of assessing the fertility of an individual. However, the use of the subjective motility assessment has a poor association with fertility rate. Use of computer-based programs for motility assessment provides more detailed and precise description of motion characteristics of studied sperm. It has been stated the VCL, LIN and ALH could be helpful to determine the fertility of sperm in a semen ejaculate (Liu et al., 1991). Maya-Soriano et al. (2015) reported that the exposure of rabbit to hot condition had the detrimental influence of the sperm movement kinetics which could lead to the low fertilizing rate. The sperm motion kinetics in ejaculates obtained from quercetin supplemented group had higher values compared to non-supplemented. Therefore, it seems that inclusion of quercetin would be helpful to improve the sperm motion kinetics which is linked with fertility mechanism.

The estimation of sperm viability in fresh, stored or cryopreserved semen provides the clue for the future fertility of sperm. There are many methods which elaborate the sperm viability simply, efficiently and economically (Gliozzi et al., 2003). The procedure employed in the present study is advantageous over other defined staining methods, as it gives detail of the sperm livability and membrane integrity at the same time. In the present study, the viable sperm increased after four weeks interval of quercetin supplementation. In contrast, no improvement in sperm viability has been achieved in non-supplemented HS rabbits. The similar elevated sperm viability has already been observed during short or long-term HS exposure in rabbits (Maya-Soriano et al., 2015; Safaa et al., 2008; Okab, 2007; Roca et al., 2005; Marthur et al., 1989). It has been reported that use of dietary antioxidants agents like pomegranate peel (Zeweil et al., 2013), ascorbic acid and betaine (Hassan et al., 2012), royal jelly (Elnagar, 2010), selenium and folic acid (Kamel, 2012; El-Tohamy et al., 2012), vitamin E and propolis (Hashem et al., 2013) mitigate the sperm against damaging effects of excessive ROS produced due the HS. As the sperm contains the unsaturated fatty acids that also make it more prone to the ROS damage. The intrinsic antioxidant capacity is not enough to protect the plasma membrane; therefore, the extrinsic antioxidant present in seminal plasma or in the storing media provided protection against ROS (Zini et al., 1993). Additionally, the excessive ROS reduce the sperm membrane fluidity and DNA integrity (Aitken, 1999); therefore, supplementation of antioxidant agents under stress is a better strategy to protect the sperm against the detrimental effects of ROS produced due to HS.

It is evidently described that HS remarkably suppress the important physiological events like capacitation, acrosome reaction, zona binding capacity etc., which are required for the successful fertility. Amongst those, acrosome has prime importance and acrosome reaction

is triggered by ROS present in the uterine environment; however, the surplus flow of ROS deteriorates the acrosomal integrity which prevents the sperm to bind with zona. The acrosome mitigation against the HS by quercetin is the evidence of its antioxidant capacity. Maya-Soriano et al. (2015) have documented that chronic heat stress has no influence over acrosome integrity due to the adaptation of exposed animals in raising area. In contrast, most of the authors reported that HS induce the acrosome reaction in a large proportion (Safaa et al., 2008; Okab, 2007; Roca et al., 2005). The incidence of reduced acrosomal integrity is associated with high lipid peroxidation rate and alteration in the content of polyunsaturated fatty acids or lipoproteins of sperm plasma membranes in epididymis as a result of elevated temperature (Zini et al., 2001).

HS has a damaging influence on mitochondria by the excessive production of ROS. Generally, the mitochondria yield minute amounts of ROS under physiological situations by oxidative phosphorylation and sperm is readily removing the small amounts of ROS released by the mitochondria. However; large amount of ROS has negative effect on the genetic material, proteins and lipid based cell organelles as well as the mitochondria. The detrimental effect of ROS produced due to HS on the mitochondria structure has been documented (Qian et al., 2004). As, the mitochondria do not contain the GSH (Fernandez et al., 1997); therefore, the susceptibility of mitochondrial structure to ROS increased twice during the stress. In the present study, an improvement in mitochondrial activity has been observed in HS rabbit sperm following the quercetin supplementation compared to the non-supplemented group. This increment in mitochondrial activity by quercetin remarkably indicates the antioxidative activity of quercetin.

Alike to the other rabbit sperm parameters, sperm morphological abnormalities are also increased by high ambient temperature. In the present study, a higher percentage of sperm with morphological abnormalities were recorded in HS rabbits. This observation has a harmony with previous studies (Mathur et al., 1989; Finzi et al., 1995; Okab, 2007; Kamel, 2002; Roca et al., 2005); however, few reports (Maya-Soriano et al., 2015; Finzi et al., 1995) inferred that the longer HS condition has less impact compared to the short exposure. The chronic heat exposure might be related to the defending mechanism, which can be termed as an adaptation to higher temperatures (Finzi et al., 1995). Generally, the rabbit sperm are thermo-sensitive and assessment of morphological abnormalities in HS animals could be a good indicator of fertility. The total sperm abnormalities were significantly greater at the fourth week of the observation and it was followed by decreasing values in both quercetin supplemented and non-supplemented group. This variability has also been reported earlier by Finzi et al. (1995) in

heat-exposed rabbits for a long duration. It is proposed that ROS has great association with morphological alteration in sperm and the degree of impairment that depends on the nature and production of ROS in response to ambient temperature (Durairajanayagam et al., 2015). An earlier report indicated that sperm has no susceptibility to HS when frequent testicular insulation is applied, although a great portion of abnormal sperm in ejaculates has been observed after initial insulation (Hamilton et al., 2016).

A modulatory effect of quercetin on sperm abnormalities has been inferred. A similar action of royal jelly (Elnagar, 2010), selenium and folic acid (Kamel, 2002) have been observed on sperm morphologies in HS rabbit. It has been stated that the occurrence of sperm morphological abnormalities occur during the course of maturation process while travelling from caput to the cauda epididymis. As caput epididymis is rich in blood supply and incidence of ROS is also increased under stress condition. Therefore, a well-protected mechanism is required to combat the excessive ROS. In this scenario, the incorporation of quercetin was the ameliorative strategy against the ROS production under high ambient temperature.

4.3. Effect of Quercetin on Testicular Histopathology and Apoptosis

Apoptosis leads to a programmed cell death in a physiological manner in response to alteration in the cellular morphological and biochemical environment which in turn forces the cell to undergo the death process. Generally, the apoptosis plays a major role during spermatogenesis, in normal or pathological conditions (Vaux and Flavell, 2000) to stabilize an equilibrium among germ cells, sertoli cells and cellular homeostasis in the testis (Baccetti et al., 1996). The higher rate of germ cells suffers from the apoptotic activity in subfertility or infertility cases (Lin et al., 1997). There are numerous stress inducers or chemical compounds that induce the germ cell apoptosis in the testes (Bharti et al., 2014; Uygur et al., 2014; Sonmez et al., 2014). Several studies have been reported the effect of various stress factor on the testicular apoptosis (Yin et al., 1997; Uygur et al., 2014). In the present study, we have observed high proportion of apoptotic germ cells in testis of HS group. In HS-Que group, the testicular germ cells also underwent to apoptosis; nevertheless, all stages of spermatogenesis were involved in apoptosis in case of HS group. It appears that HS markedly increases the oxidative stress that in turn increases the apoptosis rate in non-supplemented animals. The oxidative stress-apoptosis phenomenon has been explained in different reports in HS animals (Yin et al., 1997; Kanter et al., 2013). In the present study, the spermatogonia have been observed in a

greater sensitivity to apoptosis due to ROS produced under HS, and similar observations have been reported earlier (Maneesh et al., 2005). The quercetin supplementation prevents the overproduction of ROS under HS which leads to improved testicular environment and subsequently curtailed the germ cells death through apoptosis. The current report is in agreement with the earlier reports regarding the beneficial impact of quercetin on testicular germ cells against different toxins (Bharti et al., 2014; Uygur et al., 2014; Sonmez et al., 2014). It has also been speculated that quercetin might be involves in downregulation of the expression and activity of apoptosis-related marker proteins which in turn maintain the cell survival under HS.

4.4.Effect of Quercetin on Follicular Development and Oocyte Retrieval

The higher ambient temperature affects the process of folliculogenesis, uterine milieu, and subsequent fertility. The heat imposed females deteriorate the oocyte quality by reducing competence and lead to oocyte aging which further minimizes the fertilization and development. In this regards, several reports are available where oocyte quality has been evaluated through *in vitro* maturation and fertilization. In the present study, the effect HS on the follicular development, oocyte quality, and oocyte developmental competence and ameliorative impact of quercetin has also been evaluated as well. Usually, the summer HS is attributed to low pregnancy rates due to the reduced follicles recruitment and development. In the present study, low follicles and oocyte numbers have been observed in HS group. A similar observation has been made in earlier reports (Garcia-Ximenez and Escriba, 1996; Yassein et al., 2008) in rabbits during summer heat stress. In contrast, a higher oocyte pool was collected from the rabbits reared, under controlled climatic conditions (Daoud et al., 2012). However, the number of oocytes collected was not in consonance with the previously done studies which stimulated follicular growth (Garcia-Ximenez and Escriba, 1996; Yassein et al., 2008; Daoud et al., 2012). It has been reported that the hormonal stimulation does not provide any improvement on the follicular development, nuclear maturation or hormonal secretion in rabbits during stress conditions (Arias-Álvarez et al., 2013).

The follicles contain a well-established antioxidant defense system which protects the functional and structural integrity of the encased oocyte and surrounding granulosa cells. Generally, the ROS is necessary for the ovulation process under normal physiological conditions; however, the higher accumulation of the ROS in follicle under stress has

detrimental effects on growing oocyte and cause the aging of the oocyte. The viability of the oocyte surrounding granulosa cells is also questionable during the excessive production of the ROS. Therefore, a balance between ROS and antioxidants system inside the follicle might be essential for the viability of oocyte and granulosa cells under normal and stressed conditions (Tamura et al., 2008). In the present study, the females were supplemented with dietary quercetin as an antioxidant. The higher number of oocyte retrieval in supplemented group inidicates that the presence of high ROS had been scavenged by quercetin provision in heat exposed female rabbits. The oocyte per ovary retrieval rate was the same with an earlier observation when Vitamin C was fed to HS female rabbits (Yassein et al., 2008). In addition, Beazley and Nurminskaya (2016) stated that oral quercetin intake enhances the follicular development and further fecundity in young female mice. The higher follicle development in quercetin might be associated with low ROS level, as the HS increased the ROS production or decrease the antioxidant system activity that leads to the higher ratio of atretic follicles (Lopez and Luderer, 2004). Earlier it has been observed that low concentration of FSH enhances the follicular ROS and subsequent apoptosis process and treatment of FSH had stimulatory effect on GSH synthesis which further maintain the follicle development (Tsai-Turton et al., 2007). Therefore, the dietary quercetin could be a potential factor in ROS elimination if there is low FSH concentration during the follicle development and maintaining the antioxidant defense system. The oocyte with a dimeter more than 80 % of the final diameter, has attained the capacity to resume the meiotic process. The average oocyte diameter in rabbit is about 147 µm (without zona pellucida) (Adams, 1958). In the present study, the lower diameter (143 µm) was observed in A-Grade oocyte in the quercetin treated group. However, the oocyte with this diameter is able to resumes the meiotic activity (147µm). Similarly, it has been reported that bovine oocyte resumes the full meiotic competence until MII stage if its size exceeds 100µm (Fair et al., 1995). Whereas, Otoil et al. (1997) showed that only bovine oocytes having 115 µm or bigger diameter attained full developmental competence to blastocysts. More studies are required to investigate the relationship between the oocyte size and maturation and further embryo development in rabbit.

4.5. Effect of Quercetin on Oocyte Maturation

HS has detrimental effects on ovarian follicle dynamics and oocyte competence development by premature aging in oocytes in response to high ROS production. The low

oocyte maturation rate and less steroidogenic activity of follicular cells under HS commonly reduce the fertility level (Maya-Soriano et al., 2012). In this regards, several attempts have been made to elaborate the mechanisms behind the HS effects on the oocyte maturation and fertilization capacity. The role of the antioxidant system on oocyte maturation is well-understood; however, role of antioxidants on oocyte maturation and embryo development during stress condition need to be explored to sketch a protective strategy. The present results showed that dietary quercetin supplementation to HS females have no influence on retrieved oocyte maturation rate. However, the oocyte maturation rate was higher when antioxidant (vitamin C) was supplemented to HS female group (Yassein et al., 2008). The quercetin has been tested as a supplement to improve the maturation and further embryonic development; however, there is great variability among results. Effects of low doses of quercetin were more prominent for oocyte maturation and ROS quenching (Orlovschi et al., 2014; Kang et al., 2013). The eCG or FSH was been not employed for the ovarian stimulation in the present study, therefore, it is the possibile that the oocytes could not maintain the internal antioxidant system. It has been already described that FSH stimulation is helpful for maintaining antioxidant (GSH) level in the developing follicle (Hoang et al., 2009). It can be speculated that the ROS is the major contributing factor in establishing the aging process in the oocyte, it can induce some irreversible changes which dietary supplementation is not enough to repair.

It has also been documented that oocytes produced under stressed conditions have less maturation capacity compared to those containing less level of ROS. The supplementation of antioxidant in oocyte maturation or injecting the antioxidant to female overcomes the stress factor (Lian et al., 2013). It has revealed that the multifactorial mechanism is involved during impairment of oocyte maturation under HS (Wolfenson et al., 2000; Roth et al., 2008). The quality of oocyte deteriorated by HS and reduced the oocyte maturation also influenced by meiotic chromosomes aberrations (Hamam et al., 2001), alterations in GV-stage oocytes (Gendelman and Roth, 2012b), and decreased expression of maternal genes involved in oocyte maturation (Gendelman et al., 2010) during the HS. The oocyte maturation depends upon the proper functioning of the nuclear and cytoplasmic organelles (Duszewska et al., 2010), and both compartments are highly sensitive to the HS. The exposure of oocyte to the HS disrupts the microtubules and microfilaments in cell cytoskeleton (Gallicano, 2001) that influence the oocyte maturation (Coss and Linnemans, 1996). The role of cumulus cells can never been neglected in oocyte maturation process and interaction of oocyte and cumulus cells is essential factor for oocyte maturation (Yokoo and Sato, 2004). Under HS condition, the cumulus cells synthesized the limited protein (Chian and Girard, 1995), unable to keep the GAP junction

tightly and low transformation of regulatory agents for thermoresistance mechanism of the oocyte is observed. HS oocyte is usually deficient in GSH; therefore, the delivery of the GSH by cumulus cells to the oocyte helps to combat the oxidative stress (Edwards and Hansen, 1997). A study has shown that quercetin attenuates the cadmium induced toxicity in cumulus cells in chicken follicles by reducing the MDA, maintaining the antioxidant system and decreasing the apoptotic activity by downregulation of related receptors (Jia et al., 2011). All of the other explained mechanisms, the incidence of the apoptosis in oocyte under HS is the main involved factor for low oocyte maturation rate. In this regard, Roth and Hansen (2004) have shown that meiosis inhibition is more prominent in HS oocyte due to the occurrence of apoptosis. Similarly, it has been stated that HS initiate the apoptotic process in the cytoskeleton of the oocyte and it further continues in actin-microtubules and microfilaments.

4.6. Effect of Quercetin on Apoptosis in Follicles

The ovarian activity is a critical aspect for the maintenance of fertility as it involves in steroidogenesis, follicular recruitment, ovulation and subsequent production of necessary hormones for the oocyte maturation, fertilization and successful establishment of the pregnancy in mammals (Devine et al., 2012). In the present study, the potential aspect of follicular apoptosis at primary, secondary or antral stages have been investigated during HS. In addition, ameliorative impact of the quercetin as an antioxidant has also been examined in HS animals. The apoptotic activity in follicles has markedly decreased in quercetin supplemented animals during HS exposure. The incidence of apoptosis was maximal in primary and antral follicles, however, primordial follicles remained unaffected. As it has been explained earlier that apoptosis has positive association with over production of ROS (Tilly and Tilly, 1995); therefore, antioxidant capacity of quercetin eliminates the excessive ROS and further reduces the apoptosis incidence in follicles under HS.

The high sensitivity of the antral follicles might be associated with oxidative stresses that induces apoptosis in the granulosa cells. Moreover, the reduction of GSH concentration under stress could be one another possibility of high apoptosis in antral follicles in HS animals. The report of the Chen et al. (2014) describe that triggering the HSPs in tissues exposed to moderate HS provoking the systemic inflammatory and redox action and the administration of quercetin helps to body to adapt the hot climates by maintaining the interleukin and superoxide dismutase levels. Among the follicles, especially antral follicles, with high GSH level and

antioxidant provision inhibit the process of apoptosis in follicles and granulosa cells, therefore, determination of antioxidant markers or MDA level in follicular fluid could be helpful to elaborate the mechanism of ROS on granulosa cells apoptosis under HS (Devine et al., 2012).

5. CONCLUSIONS

Based upon the present findings, it is concluded that heat induces significant effects on the sperm quality in exposed male rabbits. The exposure of male rabbit to HS decreases the seminal volume and dietary quercetin alleviated the HS with increase the seminal volume. The sperm concentration improved in HS male rabbit when quercetin is supplemented in diet. The quercetin supplementation has positive impact on progressive and total motility in HS exposed rabbits and sperm motion kinetics also improved in response to quercetin supplementation. The provision of quercetin under HS increased the percentage of sperm viability, acrosome integrity and maintain sperm mitochondrial potential in rabbits. Lower incidence of sperm abnormalities is obtained in collected ejaculates after quercetin supplementation in HS male rabbit. In addition, less number of germ cells undergoes the apoptosis when quercetin is provided during HS.

In conclusion, the exposure of female rabbits to increased temperatures, greatly influences the follicle development and oocyte retrieval. The quercetin supplementation in response to HS, improved the follicle development and oocyte retrieval in female rabbits. In addition, the provision of quercetin significantly improved A- grade quality oocytes under HS. The quercetin has modulating effect on the oocyte maturation rate which was retrieved from HS female. The development of primordial, primary and antral follicle population greatly increased in HS female rabbits during dietary quercetin provision. Moreover, quercetin reduced apoptosis in granulosa cells of developing follicles under HS by curtailing the serum MDA level in female rabbits. As a concluding remark, it could be that dietary quercetin supplementation, as a strong antioxidant, improves the sperm and oocyte quality in HS rabbits by eliminating the excessive ROS.

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AKADEMİK YAYINLAR

1. MAKALELER

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2. BİLDİRİLER

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