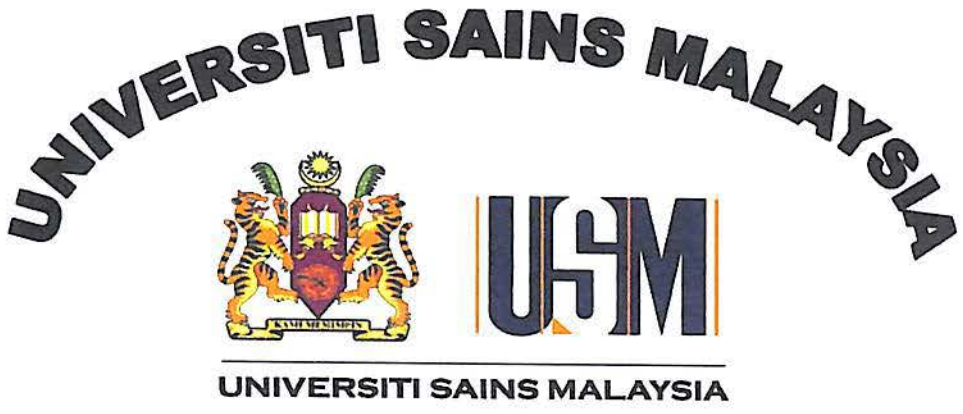

THE STUDY OF MORPHOLOGICAL FEATURES OF SPERMATOZOA UNDER DIFFERENT TEMPERATURE BY SCANNING ELECTRON MICROSCOPE

Dissertation submitted in partial fulfillment
for the Degree of Bachelor Science in Forensic Science

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2009

CERTIFICATE

This is to certify that the dissertation entitled

The Study of Morphological Features of Spermatozoa under Different Temperature by Scanning Electron Microscope

is the bonafida record of research work done by

CHANG KAH HAW

during the period 16th December 2008 to 30th April 2009

under my supervision.

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Life is not measured by the number of breaths we take, but by the number of moments that take our breath away. ~Abisola Oyawa~

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ABSTRACT

Semen is the body fluid frequently found in sexual crimes. It is principally comprised of spermatozoa suspended in seminal plasma. Spermatozoa with distinct and characteristic appearance can be detected and confirmed microscopically. In this study, the morphological features of spermatozoa under different temperature exposures were observed and examined by scanning electron microscope which was greatly used for the observation of discrete cell surfaces in three dimensional views at high magnification.

10 samples collected at Cytology section of Pathology Laboratory, Hospital Universiti Sains Malaysia, were divided into three different portions and kept into three different temperatures, including 55°C, 4°C and room temperature for a 5 day period. All the samples were observed by scanning electron microscope after exposure to respective temperature. Different scores were given to varied morphological features of spermatozoa observed. Through the scoring of morphological features, statistical analysis was conducted for the determination of any significant change. Result of the examination showed significant difference in the morphological features of spermatozoa for both the experimental samples as compared to the control samples.

After the high temperature exposure on the samples, significant changes in the shape and surface of the head, acrosome morphology and mid-piece of spermatozoa were found. Tapered end head was seen on heat exposure. In addition, depressions and fossa were found on the surface of the head with damaged acrosome morphology. Bent mid-piece of spermatozoa was also significantly observed with high temperature exposure.

On the other hand, significant changes were found with the samples on cooling. Tail and mid-piece of spermatozoa differed significantly with control samples on exposure to low temperature. Hair-pinned and severe coiled tails of spermatozoa were found in cooled samples. Mid-piece of spermatozoa was also disrupted with thinning and bending on low temperature exposure. Differences in the morphological features of spermatozoa on exposure to different temperature can be used as a tool to establish the condition of a sexual crime scene.

INTRODUCTION

Crime is an action of offence against an individual or state which is punishable by law. An area where a criminal act has taken place is called a crime scene. The processing of crime scenes are directed to same goal which involves examination, recognition, preservation, documentation, and collection of physical evidence from the scene of crime. All these tasks are done for the purpose of identification, comparison, individualization, and reconstruction as well as investigation (Horswell, 2000).

As claimed by Horswell (2000), the transfer of different types of biological material among the victim, the perpetrator and the crime scene can be found in any crime. Such transfers may be happened either in a unidirectional or bidirectional manner. In the forensic point of view, the analysis of biological materials can become the important and crucial evidence in order to associate or exclude an individual with a particular crime.

Sexual assault is complex. A sexual crime can be defined as an offense or an unlawful behaviour that involves sexual element which occur against another individual. Such sex crimes include the criminal offence in which the victims are forced or coerced to involve or participate in activity of sex (Flora, 2001). O'Connell, Leberg and Donaldson (1990) stated sexual offense as an inappropriate sexual behaviour in which an individual has not given consent or is unable to give the consent for the sexual activity.

In sex crimes, victims' bodies as well as their clothing are part of that particular scene other than place where criminal act occurred. A sex crime may occur at primary scene as the location where the perpetrator engaged majority of his attack and most the time spent. However, there could also be secondary scene with lesser rapist-victim interaction. Apart

from that, an intermediate scene may also be found for the transport or movement of victim from primary to secondary scene (Savino and Turvey, 2005).

In a rape crime scene, the most useful forms of evidence in terms of law enforcement are the fingerprints and biological material containing DNA (Savino and Turvey, 2005). Various kinds of personal evidence deposited on various objects and surfaces frequently resulted from a sex crime. Semen is often found in sex crime. The identification of relevant body fluid provides valuable evidence for the investigation and prosecution (Allard, 1997). However, such evidence is often transient and subjected to adverse environmental conditions (Spalding and Bigbee, 2001).

Two crimes are never the same. Therefore, determination of the surrounding conditions of a scene of crime could aid in the investigation process. In this study, the morphological features of spermatozoa exposed to different temperatures were observed by scanning electron microscope. Differences observed in morphological features due to different exposures were examined. The outcome of this study may provide a clue on the temperature of a scene where a sexual crime had occurred or evidence with semen stains was found in order to assist in investigation.

REVIEW OF LITERATURE

Anatomy of male reproductive tract as well as the structure of accessory glands is important for the understanding on the formation of semen (Figure 1). Male has symmetrical reproductive organs in pair for the production of spermatozoa (Basmajian and Slonecker, 1989). Male genital organs include the testes, epididymis, vas deferens and the penis. Seminal vesicles, prostate glands as well as the urethral glands are the accessory glands in the reproductive organs of male. All of such organs are paired except the prostate and the penis (Martin and Hine, 2000).

Testis is the organ for the production of semen as primary reproductive organ or gonad. Testes are paired and ovoid in shape as measured in the length of 4 to 5 cm, 2.5 cm in breath and about 2 cm each anteroposteriorly situated in the scrotum. The testes lies obliquely in scrotum as the pointing of upper extremity in a slightly anterolaterally manner (O'Rahilly, 1986; Williams and Warwick, 1980).

A thick, dense, white, inelastic and fibrous capsule known as tunica albuginea covers each testis. It is dense membrane and is covered externally by tunica vaginalis as the visceral layer. Tunica vaginalis is the inferior extremity of processus vaginalis of peritoneum. Beneath tunica albuginea, it is tunica vasculosa as vascular layer. Blood vessels are held together by areolar tissues and form connections with all the lobules of testis (Williams and Warwick, 1980).

Testis is divided into lobules by a series of fibrous septa. A layer of flattened mesothelial cells cover a testis. Lobules as internal architecture of testis are estimated to be 200 to 300 in number and differ in size according to position in testis. Seminiferous tubules are lying

within the confines of lobules where development and production of microscopic spermatozoa takes place. About 200 to 300 seminiferous tubules can be found in each testis with the total length of approximately 70 to 80 cm (Williams and Warwick, 1980).

The epididymis is important for the maturation, storage and survival of sperm. It is made up of tubules packed together in loose connective tissue into a long, narrow and flattened body which lies on the posterior surface of the testis. In comma shape, it is excessively coiled and forms the first of efferent route. There are ciliated epithelial cells lining the epididymis. Throughout the length of epididymis, height and incidence of cells vary with constant general arrangement. Mucoid secretion is discharged from such cells for the carriage of spermatozoa (Glover *et al.*, 1990).

Vas deferens or ductus deferens enters pelvic cavity through inguinal canal. As connection between duct of epididymis and urethra, it is about 45 cm long with ciliated pseudostratified columnar tubules. A chamber called ampulla is formed behind the bladder as the vas dilated for storage of sperm. An ejaculatory duct is formed where the duct enters pelvic urethra by joining the duct of a seminal vesicle. The transportation of sperm is conducted to the pelvic urethra through vas deferens during the seminal emission (Glover *et al.*, 1990).

Male urethra is extended from urinary bladder to distal end of the penis. It is about 20 cm in length which is the route for passage of urine and male reproductive fluids. Being divided by three parts, prostatic urethra is connected to the bladder through prostate gland with presence of two ejaculatory ducts, membranous urethra as the shortest part extends

from bladder to peritoneum and the longest spongy urethra through the length of penis (Seelay *et al.*, 2006).

Besides epididymis, there are other accessory organs in male reproduction tract. The seminal vesicles with thick muscular coat contract during the ejaculation. Each gland is about 5cm long. The presence of goblet cells at the lining columnar epithelium produces secretions as parts of seminal fluid. Yellowish sticky liquids secreted from seminal vesicles are added to the semen (Jequier and Crich, 1986).

Prostate gland is a partly muscular, glandular and fibrous organ that surrounds the pelvic urethra. Columnar and pseudostratified epithelium cells with secretion blebs at their surface lines the gland. During ejaculation, this gland's secretions in an opalescent appearance are expelled and enter the prostatic urethra by a series of small ducts. The slightly alkaline fluid secreted into the semen activates sperms and prevents them sticking together (Jequier and Crich, 1986).

For bulbo-urethral glands or Cowper's glands, these rounded structures are situated near to the base of prostate. The ducts of bulbo-urethral glands are 2 to 3 cm in length. Both the glands secrete mostly the mucus and open into membranous urethra, and neutralize the acidic environment of urethra and protect the sperm. Urethral glands or glands of Littre contribute also to the fluid portion of the semen. They are small and mucus secreting with the presence of glandular epithelium on their surface. The mucus secreted is emptied into the urethra (Jequier and Crich, 1986).

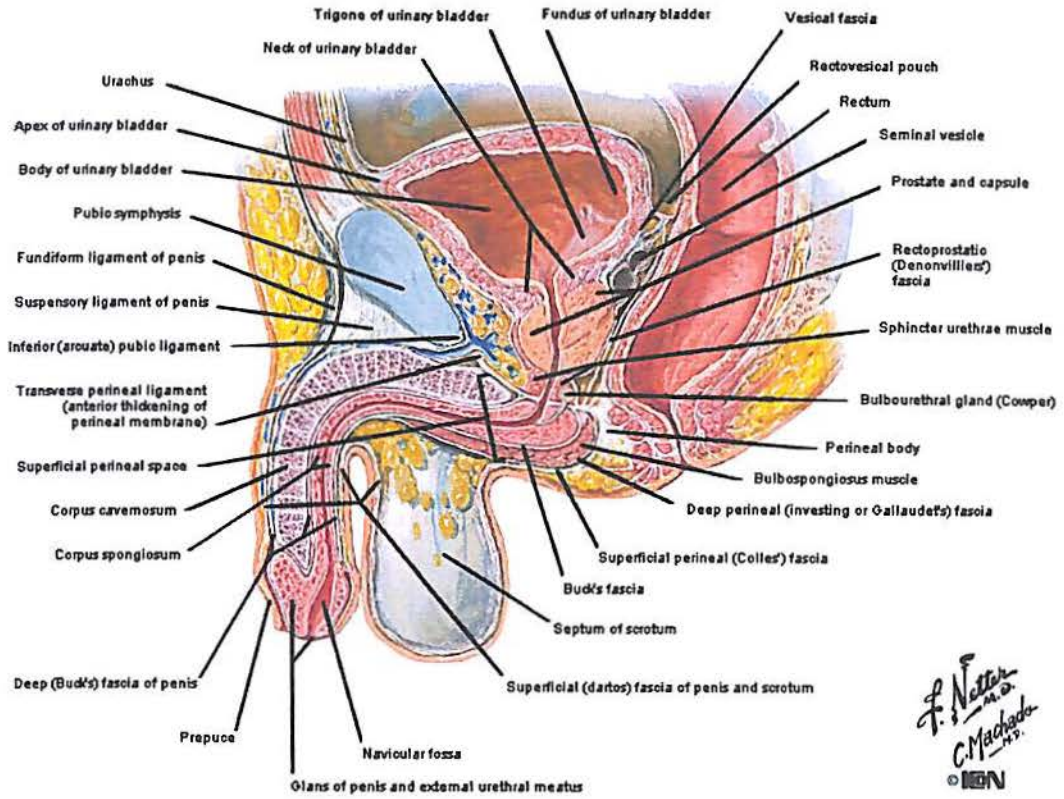

Figure 1: Anatomy of male reproductive system (Netter, 2003)

Semen or "whole semen" principally comprises the germ cells which are spermatozoa, suspended in a complex mixture of fluids called seminal plasma as ejaculated (Mann, 1964). It is an organic fluid with approximately 10% of spermatozoa, 90% of seminal plasma and also minute amount of other substances but the proportion of sperm and plasma is further determined by several different factors. These may include the size, storage capacity and output of different accessory glands of male reproductive tract.

Both the two components of semen, spermatozoa and seminal plasma differ in their origin, composition as well as the function. Spermatozoa originate in the testis from the germ cells or Sertoli cells of the seminiferous epithelium. On the other hand, seminal plasma acts as the nutritive and protective medium for the spermatozoa through the female reproductive tract (Mann, 1954).

In the publication of Mann (1954), it stated that spermatozoa are stored in epididymis until they are ejaculated after 12 to 16 days. Ejaculation as the discharge process of semen involves the forceful expulsion of semen from the urethra due to the contraction of urethra, skeletal muscles in the floor of pelvis and the muscles at the base of penis. The ejaculate volume typically ranges from 1 to 6 ml with an average volume of 3 ml (Mann & Lutwak-Mann, 1981). However, it depends on the time interval since last ejaculation, metabolic activity of the glands and presence or absence of partial ductal obstruction.

Production of spermatozoa through series of cell divisions in testis called spermatogenesis. Interstitial cells of testes increase in size and number after puberty and the development of lumens in seminiferous tubules give rise to beginning of sperm cells production. About 74 days are taken for a sperm cell to be produced. During spermatogenesis, successive stages of spermatogonia, primary spermatocytes, secondary spermatocytes and spermatids lead to the process of proliferation and transformation (Mann, 1954).

Spermatogenesis transforms spermatogonia into spermatozoa. Spermatogonia are produced within the seminiferous tubules of testis germ cells after divided by mitosis. They are diploid cells in the walls of seminiferous tubules that give rise to primary spermatocytes after another mitosis process. Two secondary spermatocytes are resulted by the primary meiotic division of primary spermatocyte, and each of these produce two spermatids after the second meiotic division (Mann, 1964; Mann and Lutwak-Mann, 1981).

Spermatid is the non motile cell that subsequently differentiates into a mature spermatozoon. The phase of meiosis is ended after the maturation of spermatozoa by the resultant spermatids through a process called spermiogenesis. After the formation of

spermatozoa in testis, they pass from the testes to epididymis and into urethra through the vas deferens and ejaculatory ducts (Mann, 1954).

Spermatozoa or sperm cells are male gametes which are haploid cells (Figure 2). A sperm cell contributes half of the genetic information to the diploid offspring when combines with an ovum to form a zygote. The sex of the offspring is determined by sperm cells. The stream lines of a spermatozoon are straight and parallel. Flattened ovoid head of a human spermatozoon has the maximum length of about 4 μm and a maximum diameter of 3 μm. In addition, the tail is about 45 to 50 μm in length (Williams and Warwick, 1980).

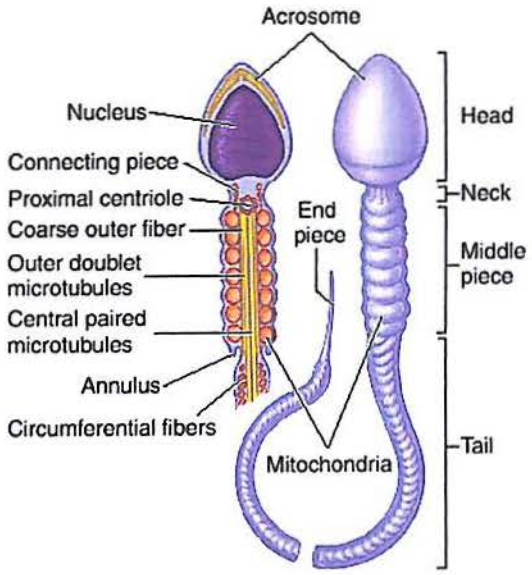

Figure 2: Schematic diagram of spermatozoon (Dorland's Medical Dictionary, 2005)

The head structure is principally comprised of a dense nucleus surrounded by a thin layer of cytoplasm. A minimum of cytoplasm and densely packed DNA at least six fold more highly condensed as compared to mitotic chromosomes in somatic cells can be found. The head structure contains at its anterior end with a thin secretory vesicle known as the

acrosome. Acrosome appears as a cap-like structure and can be differentially stained by standard histochemical stains (Ballantyne, 2000).

Mid-piece of a sperm cell is cylindrical appearance with about 1 μm in diameter and 7 μm long. It consists of axial bundle of fibrils called axoneme surrounded by mitochondrial sheath and further enveloped by cytoplasm and plasma membrane. Axoneme consists of a central pair of fibrils within a symmetrical set of nine doublet fibrils or 9 + 2 arrangement. There is an outer second ring of nine unequal size but coarser fibers arranged less symmetrically (Williams and Warwick, 1980).

Tail of spermatozoon is the motile part of that particular cell. Microtubules of axoneme and coarse dense fibers are continued uninterruptedly from the basal body through the mitochondrial sheath and the tail except 5 to 7 μm from the tip. Tail of spermatozoon may propel the sperm cell by whipping in an elliptical cone with a means of motility. They can move at about 1 to 3 mm/minute (Williams and Warwick, 1980).

According to Allard *et al.* (2007), the presence of semen with distinct and characteristic appearance can be confirmed with microscopic detection of spermatozoa. Examination for the presence of semen is of prime importance in allegations of sexual assaults. During investigation of sexual offences, intimate body swabs, clothing and bedding items are routinely submitted for examination. The detection of semen on such items and subsequent conduct of DNA profiling tests are often of vital importance, together with an evaluation as to the significance of findings.

Dried seminal stains on clothing and bedding may exhibit some or all of semen factors although months or even years after deposition. Study conducted by Soules *et al.* (1978) determined the rate of decay of sperm as it pertains to alleged rape. The examination of spermatozoa remains the primary test in alleged rape. According to Davies and Wilson (1974), spermatozoa were usually found up to 3 days on a surface after intercourse and were occasionally found up to 6 days. Washing tends to remove such body fluid, although there were reports of spermatozoa persistence after machine washing.

On previous studies conducted, spermatozoa are not a homogenous cell population with an appreciable part of pathological deviations cells. At least 30% of spermatozoa in the ejaculate should be morphological normal. No constant relationship was found between live and dead populations with respect to numerical differences between cell frequencies with abnormalities (Fredricsson *et al.*, 1977).

As spermatozoa are extremely small compared to other cells, examination of organelles such as cell membranes, acrosomes, mitochondria and tails skeletons require higher magnification along with special staining methods as claimed by Ozkavukeu *et al.* (2008). Therefore, advanced cell imaging technique is used to reveal possible detrimental effects on sperm from normal samples in order to achieve a basic knowledge of changes due to heating and cooling process.

According to Lachica and Garcia-Ferrer (1997), preservation of spermatozoa integrity is high with vital and important advantage for its use in court. In a room with fluctuating conditions as natural-like consideration, general preservation of the integrity of the spermatozoa was not obstructed and disrupted during the period. Therefore, it is possible

to identify spermatozoa even in long period concerning the general preservation of cell integrity.

Sperm morphology has been used for the evaluation of male fertility potential. With passage of time, accumulation of heads and tails were more frequently observed due to breaking and detachment of tails as well as fragmentation of sperms. However, microscopic examination of spermatozoa after extended periods of time after sexual aggression was still possible (Lachica and Garcia-Ferrer, 1997). From previous studies, percentage of normal forms of spermatozoa has been correlated with exposure to several factors, namely reproductive toxicants, disease and fertility (Davis and Gravence, 1994).

Previous study conducted by Baker and Clarke (1987) highlighted the need and requirement for more widespread agreement about definition of sperm morphology. The development of practical objective methods of assessment should also be provided. Ranges for metric dimensions provided for spermatozoa of normal head length (L), width (W) and the proportional relationship between them. $4.0\mu m \leq L \leq 5.5\mu m$, $2.5\mu m \leq W \leq 3.5\mu m$, and $1.5 \leq L/W \leq 1.75$ (WHO, 1992). Experienced observers were generally consistent in classifying sperm as normal or abnormal on observation and examination.

The evaluation of the morphology of human spermatozoa varies widely between and even within laboratories according to Menkveld *et al.* (1990). The criteria used for a normal spermatozoon are based on the appearance of spermatozoa found in the mucus of the upper endocervical canal (Menkveld *et al.*, 1990). The use of stricter criteria for evaluation of sperm morphology and establishment the within and between observer variations is a practical, reliable and repeatable method (Kruger *et al.*, 1988).

As stated by WHO (1999), a spermatozoon considered as normal according to strict criteria should have intact sperm head, neck, mid-piece and tail. Acrosomal region should be well-defined comprising of 40% to 70% of the area of sperm head. In addition, the mid-piece is slender, less than 1μm in width and about one and a half times the length of head, and attached axially to the head as well. For tail, it is straight, uniform, and thinner than the mid-piece, uncoiled and approximately 45μm in length. Normal spermatozoa have smooth oval configuration head and the most important, an absence of neck, mid-piece or tail defects (Fawcett, 1965; Zavos *et al.*, 1998; Eustache and Auger, 2003; Cassuto *et al.*, 2008).

With scanning electron microscope, normal human spermatozoa showed the criteria mentioned by WHO (1999) with an oval head with distinct acrosomal and post-acrosmal regions and well-defined outer plasma membrane. Mid-piece of sperm was long and encircled with a distinct concentric mitochondria sheath. The tail region is separated from the mid-piece by annulus (Lohiya *et al.*, 2004).

Abnormal forms of spermatozoa have been cytologically defined as cells that exhibit gross defects during observation. Examples of abnormal forms may include the presence of two heads, no head, incorrect insertion of mid-piece, or a broken flagellum, and by sperm head sizes as well as deviation form ideal oval shape of spermatozoa. Abnormal spermatozoa can be seen under electron microscope with membrane damage, resulting into bent mid piece and acrosome ballooning (Shibahara *et al.*, 2002).

Morphology of spermatozoa had been evaluated by various methods of electron microscopes, including transmission and scanning electron microscope. Scanning electron microscope is helpful in detection and understanding of discrete damage to cell surfaces as well as defects in sperm which are the limitation of other microscopic techniques (Sharma *et al.*, 2005). Scanning electron microscope was used in attempt to accurately score morphological differences in sperm (Liakatas, Williams and Hargreave, 1982).

Scanning electron microscope has the advantages in rapid screening process and also observation directly from the screen, zooming to high magnification and automation (Gopalkrishnan and Anand Kumar, 1990). Scanning electron microscope has the ability to detect certain subtle abnormalities of human spermatozoa. However, scanning electron microscope may have also disadvantages on the study of spermatozoa morphology. These include the small sample volume for analysis, the limitation of the observation field, and also the complicated and time consuming preparation procedure (Mortimer, 1994).

Scanning electron microscope was found to be more sensitive to expose surface alterations. This included all statistically significant morphological features of spermatozoa. On the observation by electron microscope, morphology of spermatozoa was studied based on the intactness of acrosome, mid-piece of sperm tail either coated with a smooth intact membrane or corrugation as well as the end-piece of sperm tail with damage including banding, breaks or splitting of tails (Grab *et al.*, 1993).

According to Baibekov, Asadov and Strizhkov (2007), exposure to high temperature affects the process of spermatogenesis and causes the morphological changes in the spermatozoa. The surface of spermatozoa cells was uneven with protrusions and

depressions on exposure and leads to significant changes in three dimensional ultra-structures of spermatozoa. As claimed by Baibekov, Asadov and Strizhkov (2007), polymorphism of heads and tails of spermatozoa by length, thickness and shape were observed with high temperature exposure. There were spermatozoa with scalloped heads and twisted tails appeared after exposure. Thick and thin tails bifurcated in the distal portions were also detected.

The effect of storage of human spermatozoa at room temperature as well as lower temperature was studied by Fredricsson and Kinnari (1979). Loss of vitality was found to be linear and not much variation with time within the temperature range from room temperature to refrigerator temperature. Apart from that, morphology of spermatozoa on storage as well as the living sperm population studied separately was only slightly impaired by storage (Fredricsson and Kinnari, 1979). This was supported by Chantler *et al.* (2000) with no significant detrimental effect on spermatozoa samples with short term cooling.

The following cooling and warming process declined the motility and function of human spermatozoa (Chantler *et al.*, 2000). Acrosomal abnormalities were evident as cracks by scanning electron microscope observation. Acrosomal changes and subacrosomal swelling were found to be significantly increased and also the morphologic abnormalities of tail increased significantly on such process. The parameter among tail abnormalities which showed significant change was the coiling of tail. An increase in the coiled tails was revealed which usually occurred after osmotic changes (Ozkavukeu *et al.*, 2008).