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RESEARCH ARTICLE

In Vitro Effects of Culture Medium and Serum on Germ Cells in Testis and Epididymis of Male Wistar Rats [1]

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Summary

The present study was designed to examine the influence of culture media and serum on survival of *in vitro* rat sperms. The effects of different culture media with or without 10% fetal bovine serum (FBS) on spermatids and spermiums of Wistar rats were assessed *in vitro* between 2004 and 2005. Spermatozoa were cultured in Gamete-20, RPMI-1640, alphaMEM (AMEM), Dulbecco's modified Eagle's medium (DMEM) and Eagle's MEM (MEM). The number and morphology of cells was recorded at 4 and 24 h. Under all conditions the number and the viability of the cells decreased with time but parameters were positively affected by the presence of FBS. Viability rates of spermatids and spermatozoa reduced at 24 h of culturing. The survival and morphology were the best in RPMI with serum and worst in MEM without serum. These results indicate that different composition of culture media and FBS are important for maturation and survival of spermatozoa. Carefully selected culture media can play important roles in the generation of functional sperms as well as in the success of *in-vitro* fertilization (IVF) procedures.

Keywords: *Culture, Maturation, Medium, Serum, Sperm, Rat*

Erkek Wistar Ratlarda Kültür Medyumunun ve Serumun Testis ve Epididimis Germinal Hücreleri Üzerine *in-vitro* Etkileri

Özet

Bu çalışma, kültür mediumu ve serumun *in vitro* rat spermleri üzerine etkisini incelemek amacıyla planlanmıştır. %10'luk fetal bovine serum (FBS) içeren veya içermeyen çeşitli kültür mediumlarının spermatid ve spermler üzerine etkileri Wistar tipi ratlar üzerinde *in vitro* olarak değerlendirildi. Spermatozoonlar Gamete-20, RPMI-1640, alphaMEM (AMEM), Dulbecco's modified Eagle's medium (DMEM) ve Eagle's MEM (MEM)'de inkübe edildi/kültür yapıldı. Hücre sayısı ve morfolojisi 4. ve 24. saatlerde kaydedildi. Tüm koşullarda zamanla hücre sayısı ve canlılığında azalma tesbit edildi. Parametrelerin FBS varlığında olumlu yönde değiştiği görüldü. Tüm spermatid ve spermatozoonların yaşam oranları 24 saatlik kültürden sonra azalırken; en iyi hücre morfolojisi ve yaşam oranları serumlu RPMI, en kötü hücre morfolojisi ve yaşam oranları ise serumsuz MEM'de tesbit edildi. Sonuç olarak kültür mediumunun bileşenlerinin ve FBS'nin, spermatozoonların olgunlaşması ve yaşaması için gerekli olduğu; uygun seçilmiş kültür ortamının, fonksiyonel sperm oluşturulmasının yanı sıra *in vitro* fertilizasyon (IVF) uygulamalarının başarısında da önemli rol oynayabileceği düşünülmektedir.

Anahtar sözcükler: *Kültür, Medium, Olgunlaşma, Serum, Sıçan, Sperm*

INTRODUCTION

In vivo spermatogenesis in mammals is a long and complex process regulated by multiple mechanism that interact with one another [1,2]. Many researchers have tried to develop cell culture systems in which *in vitro* spermatogenesis could be achieved [3]. Sperm maturation is the process by which the mammalian spermatozoon becomes fully mature to fertilize ovum *in vivo* or *in vitro*. During this stage, spermatozoa leave the testis (spermiation) and pass along the proximal region of vasa efferentia and proximal epididymidis. Just

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after the meiosis to the time of fertilization, the male germ cell undergoes differentiation and maturation [4]. This process includes the study of spermatogenesis, treatment of infertility, and modification of the male germ line. However, exact molecular mechanisms that regulates the organization among various somatic and germ cell types in the testis required for the complete process of spermatogenesis remain to be determined. Moreover, spermatozoa undergo capacitation in the female reproductive tract they acquire the potential to capacitate as they transit the epididymis [5,6].

The importance of investigating the factors that affect sperm maturation lies in the increase in the number of sperms and life-long fertilization. It is known that in rats one testis produces about 4.000 mature spermatozoa [7]. At the end of this process, differentiation of spermatogonia to spermatozoa takes place sometime between a few days to a few weeks and depends on interactions of cell-cell, cell-basement membrane and cell-environmental factors. Environmental factors include growth factors, hormones and adhesion molecules [8,9]. Stages of maturation can be induced under various conditions. For instance, incubating epididymal spermatozoa with primary cultures of epididymal epithelium not only promotes sperm motility but also enhances the capacity of spermatozoa to fertilize oocytes and extends the viability of spermatozoa *in vitro* [10].

Serum-free culture systems have long become important tools in investigating the biological characteristics of mammalian cells *in vitro* [11]. Mammalian cells have been shown to proliferate in serum-free hormonally defined medium without altering the cell type-specific characteristics. This has led serum-free culture to become a major resource to study cells *in vitro* and to identify novel growth factors or regulatory mechanisms for proliferation and differentiation. Using serum-free culture systems, it was determined that most cell types require specific growth factors and hormones to proliferate *in vitro* [11,12]. Unfavorable effects induced on spermatazoa allow definitive experiments to analyze the effect of individual factors on maturation. Even though maturation in different media has been studied, it was not possible to compare these and the effects of serum under these circumstances could not be looked into. To understand the mechanisms of maturation, it is imperative to know first in what circumstances the spermatozoa degrade and what needs to be done to improve. Therefore we aimed to investigate the serum effect in different media and find out the environment the spermatozoa are affected by most.

MATERIAL and METHODS

Present study was carried out in the Labroatories of Departments of Anatomy and Histology & Embryology of Celal Bayar University Faculty of Medicine between 2004 and 2005. Adult male Sprague-Dawley rats weighing 150±30 g were used in the experiment. The ethics committee of Celal Bayar University approved the design and protocol of the experiments (15.12.2004 - 2004/137). Reagents used for the isolation of germ cells, including stock amino acids (essential and non-essential), L-glutamine, sodium pyruvate, penicillin and streptomycin were from GIBCO-BRL (Grand Island, NY, USA). Media used for culturing spermatogenic cells [12,13] included minimum essential medium (MEM, Gibco-BRL, Life Technology, Maryland, USA) or, Dulbeco's Modified Eagle medium (DMEM, Gibco-BRL, Grand Island, USA), alpha minimum essential medium (AMEM, Gibco-Brl, Grand Island, NY, USA), RPMI-1640 (Gibco-BRL, Life Technology, Maryland, USA) and HEPES-buffered medium (Gamete™-20, Scandinavian IVF Science, Gothenborg, Sweden), supplemented with and without 10% FBS (Gibco-Brl, Grand Island, NY, USA).

Preparation of pachytene spermatocytes and early spermatids

Post-mitotic germ cell preparations were obtained from rats testes by mechanical dissociation [14]. The right testis was encapsulated into 24 ml of 0.01 M phosphate buffer (pH 7.4), and then disintegrated mechanically by stretching between two microscope slides, followed by repeated aspiration into a 1 ml syringe. The large tissue pieces were removed, and the remaining cell suspension was homogenized and distributed among the individual medium groups with or without serum supplementation. Cell viability was evaluated by Trypan Blue exclusion test and was found to be ≥95%. Pachytene spermatocytes, round spermatids, and cytoplasmic fragments of elongated spermatids plus residual bodies (CES/RB) were observed by Nomorski DIC microscope [14]. Spermatocyts processed in MEM were transferred into different media with or without 10% FBS and incubated at 37°C in an atmosphere of 5% CO_2.

Preparation of epididymal sperm

Following median laparotomy, the epididymis was excised with the whole vas deferens and placed in a

Petri dish. A small catheter was inserted into the vas deferens. The duct was then ligated around the catheter with a surgical thread and the proximal part of the cauda was excised with a scissor. The caudal spermatozoa were removed by retrograde flushing through the vas deferens. The flow rate of the PBS was adjusted by a pump. The fluid was centrifuged at 500 g for 10 min, and the spermatozoa were suspended in PBS. Spermatozoa were placed in a warmed Petri dish containing different media conditions at 37ºC for 0, 4 and 24 h. One drop from each dish was placed on a warmed microscope slide, and a coverslip was placed over the droplet. At least 10 microscopic fields were observed at 400X magnification using a phase-contrast microscope, and the percentage of motile spermatozoa as well as spermatozoa anomalies were recorded [15]. The coverslip was removed and the spermatozoa suspension was allowed to dry in air. The sample was stained with modified H-E and examined at 400X for morphological abnormalities. Three hundred spermatozoa from different fields were examined in each sample as described previously [15]. After the initial assessment of sperm quality, spermatozoa were added to the medium in each well of every four-microwell plate and immediately put into the incubator. Samples were taken from the each well of the twenty-four-microwell plates at 4 and 24-hour intervals, placed on prewarmed slides and sperm parameters were assessed. The mean of these four samples was taken as the actual value for a particular time for each sperm suspension [16,17].

Statistical analyses

Data are presented as mean±SD and statistical analyses were performed using GraphPad Instat v3.01 software for Windows (GraphPad Software, San Diego, California, USA). The effects of the different media and serum were compared using repeated measures 2-way analysis of variance (ANOVA). ANOVAs were followed by multiple range tests when appropriate [18]. Data are expressed as percentages in statistical analyses. Differences at $P<0.05$ were considered significant.

RESULTS

Smears representing *in vivo* conditions *(Fig. 1A)* were examined for cell survival and morphological abnormalities at the end of 4 h and 24 h waiting periods. The slides were coded randomly and cells were counted by a histologist. Four fields at 400X magnification were counted. Quantification of the differentiating germ cells at different intervals of *in vitro* culturing was performed with cells on smear. Detection of the cells was made as explained previously [19,20]. Round, elongating and elongated spermatids were distinguished according to the standard anatomical criteria based on the cell size, nuclear and cytoplasmic morphology. Round spermatids were characterized by their small size (8 µm in diameter), a round, dense and smooth dark nucleus positioned centrally or inclining towards the cell membrane and also the presence of acrosomic granule adjacent to the nucleus with the narrow rim of cytoplasm. Elongating spermatids had diameters of 4-6 µm and were asymmetric in appearance (oval shape). Their nucleus had peripheral displacement, with the cytoplasm placed at midlevel of the nucleus. Elongated spermatid had growing flagellum. Its cytoplasm, having a decreased volume, was displaced to the back. Its nucleus also had a mature appearance. The nuclei of spermatogonia were variable in size and contained deeply stained chromatin. The nucleus was surrounded by a thin rim of cytoplasm. The spermatocytes are formed by a continuous series of cells that differ in size, but have a similar nuclear appearance and consist of cells from leptotene to secondary spermatocyte stages. Sertoli cells were characterized by the presence of an irregular and elongated cytoplasm and the large nucleus contained a fine pale-staining chromatin and were often seen attached to other cells or in clumps. Pale-stained cytoplasmic fragments without any nucleus were seen and identified as residual bodies *(Fig. 1B)*.

The number of testicular germ cells in serum supplemented media did not differ significantly compared to Gamete-20. The rank order of the effects of media was: RPMI> AMEM> DMEM> MEM. However, a significant decrease in the number of cell in culture occurred between 4 and 24 h *(Fig. 2)*. This decrease was more pronounced especially in serum-free media, and the effects of all media were similar. Numerical changes were similar to morphological changes *(Fig. 1B)*.

Sperms obtained from the epididymis *(Fig. 3A)* did not show any difference in media with serum with respect to the parameters. However, in serum-free media, there were significant numerical *(Fig. 4)* and morphological *(Fig. 3B)* changes at 24 h in culture compared to 4 h. The effects of media on these changes were similar to the changes observed at 4 h.

At the beginning, all spermatozoa were intact, motile and without any observable structural defects,

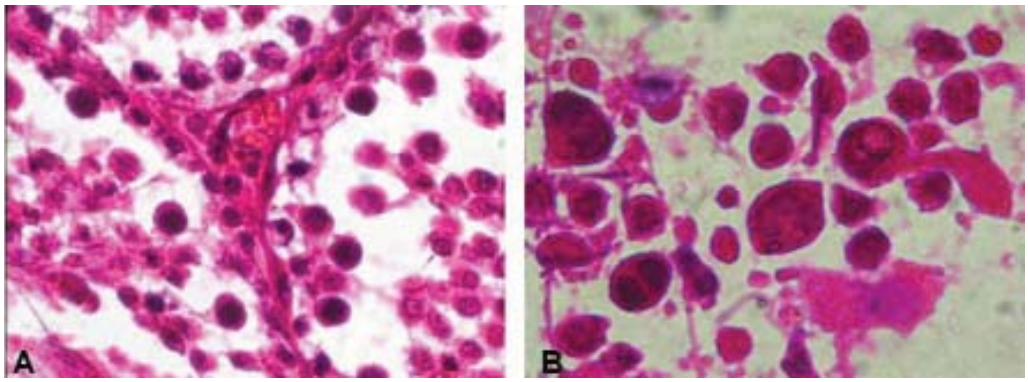

Fig 1. *A-* *In vivo* photomicrograph showing cross sections of testicular parenchyma before culture (400x magnification). ***B-*** *In vitro* photomicrograph of rat testis cells on smears stained with H-E (400x magnification)

Şekil 1. *A-* Kültürden önce testis parankiminden geçen kesit (400 büyütme) ***B-*** *In vitro* olarak Hematoksilen-Eozin ile boyanmış rat testis hücreleri (400 büyütme)

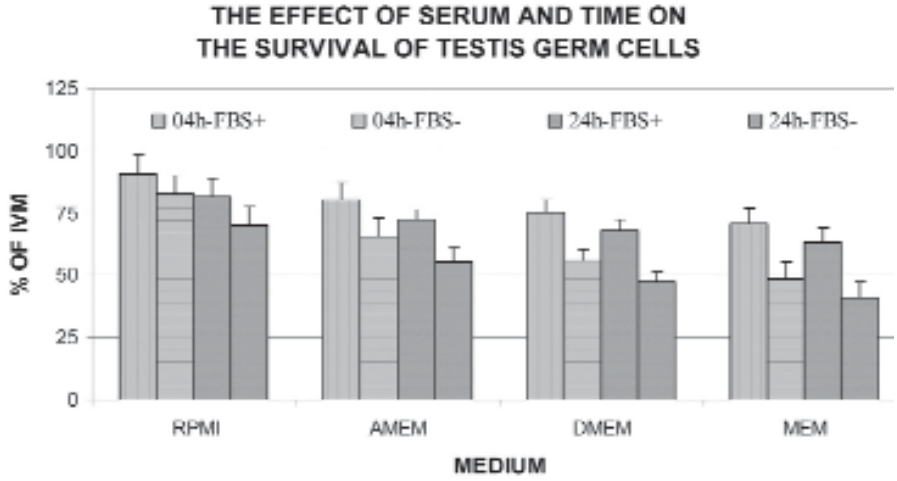

Fig 2. The effects of serum and time on the survival of testis germ cells. Comparison of cell counts in testes performed on smear images. Compared to Gamete-20 medium, there was a significant decrease in the number of cells at 24 ($P<0.05$) but not at 4 hours in all media. When serum was removed from the media, the decrease was more significant ($P<0.001$). The effect of media was significantly different from each other when serum was not added ($P<0.001$). The most detrimental effect was observed with MEM

Şekil 2. Kültür medyumuna serum ilavesinin ve zamanın testis germ hücrelerin yaşayabilirliği üzerine etkileri. Gamete-20 medyumu ile karşılaştırıldığında, tüm medyumlarda 4. saatte anlamlı bir fark gözlenmezken 24. saatte hücre sayısında anlamlı bir azalma izlendi ($P<0.05$). Kültür ortamından serum uzaklaştırıldığında azalma daha da belirginleşti ($P<0.001$). Serum ilave edilmediği zaman kültür medyumlarının etkileri birbirlerinden anlamlı olarak farklıydı ($P<0.001$). En belirgin etki MEM ile görüldü

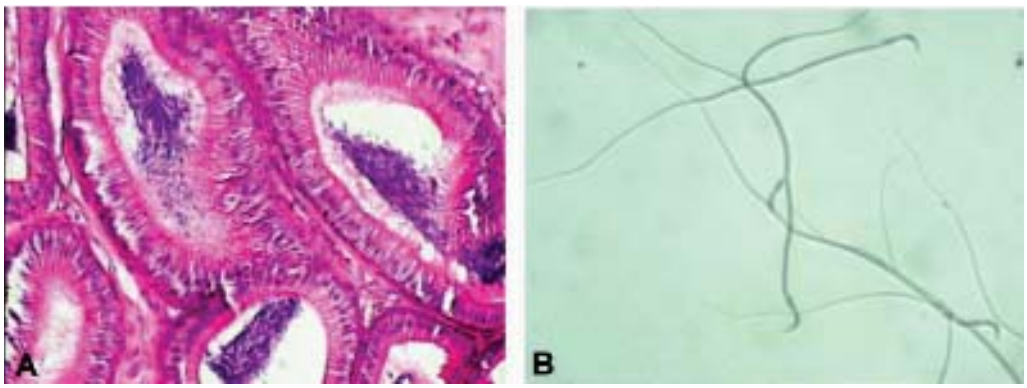

Fig 3. *A-* Photomicrograph showing cross sections of epididymis before culture (200X magnification). ***B-*** Light photomicrograph of live rat sperm using differential interference contrast (DIC) microscopy (400X magnification). Abnormality in morphology especially in serum-free media can be observed

Şekil 3. *A-* Kültür öncesi epididimis'den geçen kesit (200 büyütme). ***B-*** Differential interference kontrast (DIC) mikroskop ile elde edilmiş görüntülerde canlı spermler (400 büyütme). Özellikle serumsuz ortamda morfolojik bozukluk görülmektedir

and were regarded as normal. To explore optimal conditions for *in vitro* sperm survival, we examined the effects of several media used for rat sperm culture on motility of rat spermatozoa *(Fig. 5)*. Of the media tested, RPMI medium was found to be the best and MEM without serum was the worst for both survival and morphology. The percentage of motile epididymal sperm was significantly diminished in a time-related fashion by serum free medium compared to that of control Gamete-20 medium. The percentage of morphologically normal epididymal sperm decreased in animals exposed to serum free medium. Epididymal sperm abnormalities were characterized by an increased number of intact sperm with misshapen heads or tail defects and an increased number of isolated heads.

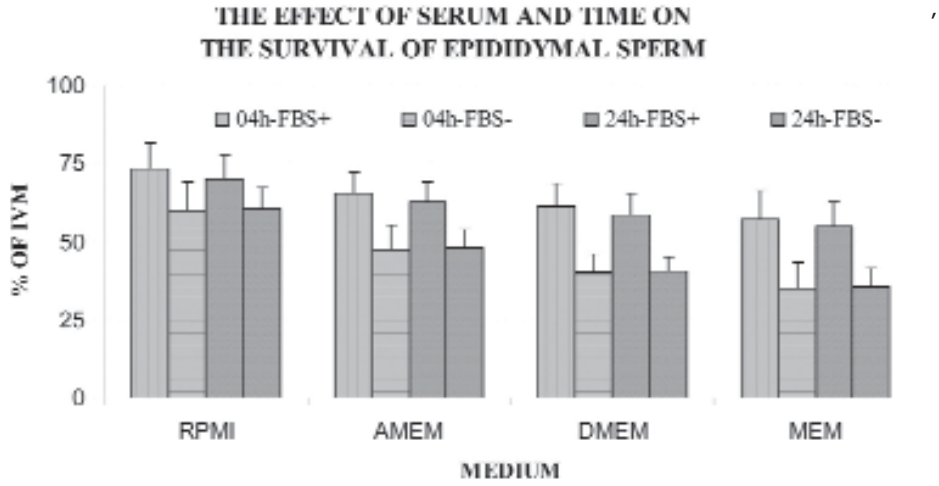

Fig 4. The effects of serum and time on the survival of epididymal sperms. Compared to Gamete-20 medium, there was a significant decrease in the number of sperms at 24 (P<0.05) but not at 4 h in all media. Removal of serum from the media rendered the difference more significant (P<0.001). The effect of media was significantly different from each other when serum was not added (P<0.001). Especially MEM exhibited the worst effect while RPMI was the best medium

Şekil 4. Kültür medyumuna serum ilavesinin ve zamanın epididimis'deki spermlerin yaşayabilirliği üzerine etkileri. Gamete-20 medyumu ile karşılaştırıldığında, tüm medyumlarda 4. saatte anlamlı bir fark gözlenmezken 24. saatte hücre sayısında anlamlı bir azalma izlendi (P<0.05). Kültürden serum çıkartıldığında farkın daha da belirgin hale geldiği dikkati çekmekte (P<0.001). Serum ilave edilmediği zaman kültür medyumlarının etkilerinin birbirlerinden anlamlı olarak farklı olduğu görülmekte (P<0.001). En kötü etki MEM ile görülürken RPMI en iyi medyumdu

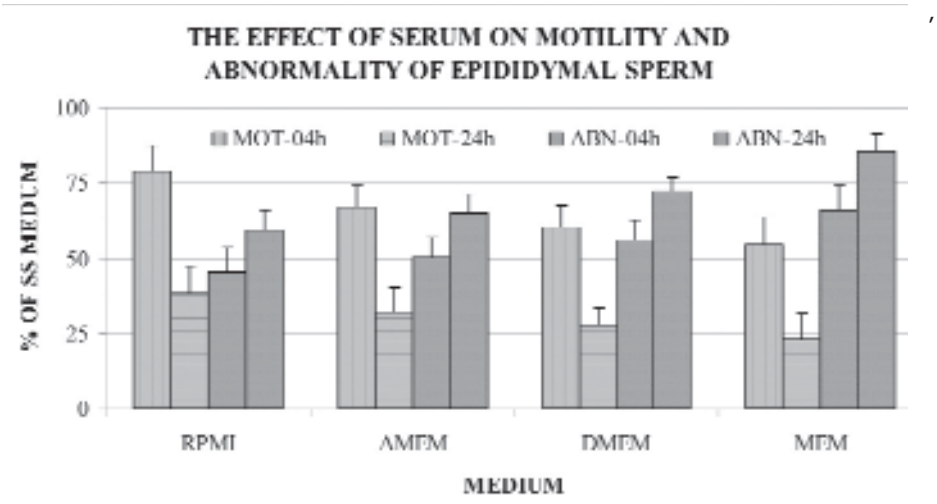

Fig 5. The effects of serum on the motility and abnormality of the epididymal sperm. When the motility (MOT) and morphology (ABN) of the sperms obtained from the epididymis were examined, there were no abnormalities at the beginning. However, significant alterations were observed at 24 h (P<0.001). With serum-free (SF) medium, motility decreased (P<0.05) and abnormality increased significantly (P<0.05). By looking at these parameters there is a difference among media and the best medium was RPMI whereas the worst was MEM

Şekil 5. Serum ve zamanın epididimis'den elde edilen spermlerin motilitesi (MOT) ve morfolojisi (ABN) üzerine etkileri. Epididimis'den elde edilen spermlerin motilitesi (MOT) ve morfolojisi (ABN) incelendiğinde, başlangıçta bir bozukluk olmadığı halde 24. saatte anlamlı değişiklikler ortaya çıktı (P<0.001). Serum içermeyen medyumlarda (SF) motilitenin azaldığı (P<0.05) ve şekil bozukluğu olan spermlerin arttığı (P<0.05) görüldü. Bu parametrelere bakıldığında medyumlar arasında fark olduğu, en iyi medyumun RPMI, en kötü medyumun ise MEM olduğu tesbit edildi

DISCUSSION

Production of differentiated spermatozoa is a result of successive mitotic and meiotic divisions that occur during spermatogenesis, a complex and tightly regulated process [21]. At the basis of this process are the spermatogonial stem cells, capable of maintaining their number as well as generating differentiating cells. Because of this pivotal role, viable systems for proliferation of spermatogonial stem cells under well-defined culture conditions would be an important tool in spermatogenesis research as well as a therapeutic method for regeneration and repair of impaired spermatogenesis. *In vivo* mammalian spermatogenesis is a long and complex process controlled by a multiple and mutually interacting mechanism [1,2]. There have been efforts to develop cell culture systems in which *in vitro* spermatogenesis could be achieved [3]. Early studies made use of relatively simple systems in which whole segments of seminiferous tubules were maintained in culture for several days [22,23]. Because of the low incidence of fertilization with spermatids [24,25], other investigators have tried to achieve *in vitro* maturation of spermatid [26,27]. There is also a need to investigate the effects of cryopreservation on the viability of spermatogenic cells used for *in vitro* conception, because the presence of nonviable spermatids will reduce the overall incidence of fertilization. *In vitro* culture of round spermatid may enhance spermatid maturation and therefore, probably, improves the incidence of fertilization [26]. However, cryopreservation of testicular tissues would be useful especially for prepubertal boys who are not able to produce spermatozoa, as well as older boys and young adults hence avoid the need for assisted reproduction in later life [28]. Aslam and Fishel [26] found that spermatogenic cells can be cryopreserved successfully and their viability would be retained when cultured for a short period. *In vitro* environment used in studies on maturation and viability after cryopreservation is important due to the medium and maturation factors contents as well. Research studies allow detailed investigation of the factors influencing the environment [2]. Sperm cells depict numerous alterations during maturation including morphological modifications, such as shedding of the cytoplasmic droplet or alterations to the acrosome and biochemical changes including the structural stabilization of chromatin and other organelles with disulphide bonds, and changes to the composition and biophysical properties of the membranes. Naturally, many changes have emerged in our study especially due to the effects of serum in different media with various chemical compositions and it was noted that especially serum has positive effect on both survival and morphology.

During *in vitro* culture, the spermatogenic cells remained viable for only a short time. The results showed that, in each group, the cells lost their viability gradually during the first 24 h of *in vitro* culturing. Almost 25-30% of the cells in the control group lost their viability between the 4 and 24 h. Most of the cells in Gamete-20 were alive confirmed by Trypan blue exclusion test. Sperms with testicular and epididymal origin in other groups lost their motilities and morphologies and viabilities to some extent in especially serum-free media. Among the compared media with that respect, the best medium was RPMI and MEM the worst. The media ranked as RPMI > AMEM > DMEM > MEM. This was true for serum-free (SF) and serum supplemented (SS) media in cultures.

Sperm maturation is affected by cell-cell adhesion, cell-matrix adhesion and the interactions between cells and many factors including growth factors. It is a well-known fact that the testosterone release of intersititiel Leydig cells affects spermatogenesis in terms of quantity and quality [29]. FSH and LH contents of the serum are responsible for sperm maturation [30,31]. It has been established that high temperature significantly affects maturation in cryptorchidism [10]. Sa et al. showed that co-culture of round spermatids on Vero cell monolayer could lead maturation of these cells up to the elongating/elongated stage and even to mature spermatozoa [27]. In the present study, we also found that medium and its content affect the sperm survival and morphology. These effects in our study may be stemming from the differences in pH, bicarbonate and other chemical composition of the media. Besides chemical composition, incubation temperature (37°C) may also be responsible of our results since it is closer to the temperature sperms sustain in undescended testis. The optimum temperature for normal spermatogenesis/spermiogenesis is 32-34°C and higher temperatures have been shown to inhibit amino acid incorporation and tubulin polymerization [32,33]. Different media have been investigated for maturation and culture of spermatozoa with various results. The present study showed that Gamete-20 and RPMI with serum media produced higher survival and better morphology than others. It is possible that Gamete-20 and RPMI media contain some components that are beneficial for spermatozoa. In this respect, supplementation of culture media with glutamine,

taurine and glycine has proven beneficial in developing bovine embryos [34,35]. The amino acids could be acting as energy substrates, pH regulator or as a pool for de novo protein synthesis [35,36]. However, the exact mechanism by which the lifespan of spermatozoa is extended remains unknown.

The primary aim of such studies is to define mechanisms that can increase maturation clinically. There are numerous methods to assess sperm maturation and each method attempts to show a correlation to fertility. Purvis and Christiansen stated that conventional methods to evaluate sperm quality and male fertility are not sufficient and any assessment should involve several tests of sperm cell function to increase the fertility [37]. Saacke [38] classified sperm quality traits into two categories, either viability related or morphological traits. Physiological or pathological conditions, handling of the sample during collection and processing by the technician affect viability traits most. Motility has long been considered a major criterion in the assessment of male fertility. Fleming and Saacke [39] and Clarke et al.[40] have reported that motility is a poor indicator of fertilizing potential for bovine sperm. On the other hand, Linford et al.[41] and Kjaestad et al.[42] have reported that, when used in conjunction with sperm velocity, it is a reliable indicator of bovine fertility. In a study comparing rat IVF with epididymal sperm obtained from the head or the cauda, Blandau and Rumery [43] reported that progressive motility correlated with IVF fertility. Caput epididymal sperm move in a circular motion while caudal sperm moved in a forward pecking motion and their fertilization rates for the rat were 8 and 93%, respectively. Franken [44] argued that progressive motility is the best indicator of male fertility.

In a cell-cell adhesion study, Sertoli cell-germ cell communications were disrupted by exposure to haloacid resulting in defects in the process of spermiation [45]. Furthermore, quantitative studies to demonstrate the effects of proteins that are added to the environment showed that the number of sperms in the cauda epididymis decreased significantly and there was a significant increase in the number of testicular spermatids retained beyond stage VIII of the spermatogenic cycle. Qualitatively, there was a significant increase in the number of cauda epididymal sperm with tail defects and significant decreases in the sperm motion parameters. To what extent, if any, alterations in sperm morphology can be held accountable for alterations in sperm motion or fertility is yet to be elucidated. Finally, there was a dose-related increase in the number of tubules with atypical residual bodies. These residual bodies were evident in testes of animals exposed to 24 mg/kg BCA. The dose of 8 mg/kg was selected as the lowest dose for the definitive study based on this finding and evidence of delayed spermiation at 24 mg/kg. Moreover, whether it depends on factors present in the environment or acts upon increased temperature, increased oxidative stress is amongst other factors that influence motility and morphology [2,46,47]. Taken together, these results indicate that intercellular communication and extra-cellular matrix are important for spermatogonia multiplication and that FBS promotes the survival of spermatogonia under *in vitro* conditions.

Modifications of the nutrient composition of all media and the interactions between media and serum alter germ cell survival and morphology due to their effect on adhesive properties of cell membrane. The objective of this study was to elucidate the relationship between media with different compositions and serum in terms of maturation. Cells are affected by the composition of especially the serum-free media, by losing its motility and morphology before undergoing maturation, and die. Unraveling the mechanisms underlying this interaction would be critical in correcting the impaired parameters with supplementation of certain factors. Therefore, it would be possible to achieve progress in sperm maturation, usability of testicular spermatozoa and cryopreservation among people having problems with fertility.

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