# Evaluation of the Quality of Deep-Frozen Cattle Semen and Selection of an Effective Method of Goat Sperm Preparation for *In Vitro* Fertilization

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## ABSTRACT

**The production and productivity of local goats can be improved to meet the demand for kids, meat and milk through *in vitro* breeding techniques which is capable of coping with the paucity of good quality breeding materials, where the application of Artificial Insemination and Embryo Transfer techniques are limited. The semen quality and sperm preparation to select the better portion devoid of unnecessary materials, play a major role in the success of *in vitro* techniques.**

**Two experiments were carried out to check whether the breed and the semen storage time affect the semen quality and to select a better sperm preparation method for *in vitro* fertilization. Fifty-eight cryopreserved semen straws of four cattle breeds and of five categories of storage period were subjected to semen quality (i.e. motility, dead sperm percentage and morphological abnormalities) analysis and no significant effect of either was found on motility and dead sperm percentage. There were significant effects of breed and storage period on sperm abnormalities. For sperm preparation, the classical sperm swim-up method and a simplified sperm swim-up method were compared with their motility and sperm concentration after prepared. A significant increment of the motility was observed in the sperms prepared by classical swim-up method and the final sperm concentrations were satisfactory in both methods. Though both methods are suitable for the preparation of sperms for *in vitro* fertilization, the simplified swim up method is preferred as it is more convenient and less expensive.**

**KEY WORDS:** IVF, Semen Quality, Sperm Preparation

## INTRODUCTION

There is a great potential for goat husbandry under many sustainable farming systems (Chandrasiri *et al.*, 1999) even though the goat production does not contribute significantly to the present livestock industry of Sri Lanka where the goat population is approximately 405,000 (Anon, 2004) out of these the majority belongs to the non-descript local types. The productivity of the local goats is at a lower level. In order to meet the future demand for kids, meat and milk of goats, the production and the productivity of the goat sector have to be increased. One of the major problems for the lowered production and productivity is lack of good quality breeding animals (Chandrasiri *et al.*, 1996). In addition, poor environmental conditions and selling of breedable and sometimes pregnant she goats for meat have worsened the situation.

There are many approaches to overcome this paucity of good quality breeding animals. Artificial Insemination (AI) is one such approach where semen of good breeding animals is used in insemination. Embryo Transfer (ET) technique is also a better approach where embryos are produced *in vivo*, recovered and transferred to other she goats. But, AI and ET too have their own limitations. The establishment of *in vitro* techniques; *In Vitro* Maturation (IVM), *In Vitro* Fertilization (IVF) and *In Vitro* Culture (IVC) to produce goat embryos withsemen and oocytes of good quality breeding animals is really a better solution, and these techniques can be highly exploited for improving the genotype and the population of the local goats. IVM, IVF and IVC techniques are still being developed in Sri Lanka. However, this technique has been successful in cattle (First and Parrish 1988) and in sheep (Cognie *et al.*, 1991) in many other countries.

Recovered oocytes from slaughterhouse ovaries can be matured *in vitro* and fertilized with pre prepared goat sperms either from fresh or cryopreserved semen of a good genetic origin to produce viable embryos.

Semen quality plays a major role for the success of this technique (Gunarajasingham *et al.*, 1995), and neither fresh nor cryopreserved semen is devoid of unnecessary materials: for instance, fresh semen samples contain glandular secretions, dead and damaged cells and deep frozen semen contains dead and damaged cells and chemical compounds added during processing and at deep freezing. Further, both types of semen, either fresh or cryopreserved, usually contain a considerable portion of dead and immotile sperms that could hinder the success of IVF procedures. Therefore, to remove unnecessary compounds and to screen the sperm of normal conformation and better motility for the success of these techniques, proper sperm preparation methods are required. The available sperm preparation methods have been mainly developed for humans & cattle. For goats, such methods are still being developed and selection of an effective method for this purpose is of much importance. Among the

common sperm preparation methods are sperm swim-up method, percoll gradient method, ficoll medium method and the method of centrifugation on a discontinuous density gradient. The most commonly used method for sperm preparation is the conventional swim-up method. However, it too involves several washings and centrifugations of semen that can make some damages to the sperm (Agarwal *et al.*, 1994; Shekarriz *et al.*, 1995; Henry *et al.*, 1996). Therefore, use of a simplified method with less technical steps would be more acceptable for sperm preparation (Zavos *et al.*, 2000).

Fresh semen is used in many of the sperm preparation methods. In the case of animals, use of fresh semen is difficult to practice as it requires much effort and expensive to rear and maintain studs for semen collection when required. Cryopreserved semen can be used instead, which has many advantages over fresh semen (Maxwel, 1984; Salamon and Maxwell, 1995). However, studies reveal that cryoprotectants induce marked changes on sperm surface membranes and even demembranation, and the plasma membrane of sperms is most vulnerable to freezing and thawing (Henry *et al.*, 1996). The quality of the cryopreserved semen has to be analyzed to check whether there are any limitations in the processing and preserving. During the long time storage, the semen quality (sperm concentration, sperm motility, live dead sperm ratio, abnormalities, etc.) can be reduced. Not only the storage time that can affect the semen quality, but breed dependant factors also can affect the quality of cryopreserved semen. Therefore, it requires identifying whether storage time and breed affect the semen quality, prior to the sperm preparations. Identification of the limitations may pave the way for the avoidances of them, and effective use of preserved semen will facilitate the success of future goat breed improvement programmes. The present study aims at evaluating the quality of deep frozen semen of different breeds, preserved for longer periods, selecting an effective method of sperm preparation for IVF in goat and facilitating the implementation of IVF technology for breed improvement in goat.

## MATERIALS AND METHODS

### EXPERIMENT 1

***Evaluation of the Quality of Cryopreserved Semen***

Cryopreserved semen straws of four Breeds, Friesian Australian Milking Zeebu (FrAMZ), Jersey (Jr), Friesian (Fr) and Friesian Sahiwal (FrSw) available at the semen bank of the Central Artificial Insemination Station (CAIS), Kundasale were selected and they were classified into 5 age categories according to the semen straw preserved year, as age >0, 19 – 15, 14 – 10, 9 – 5 and <5 years. Each semen straw was subjected to semen quality analysis.

A deep frozen semen straw was thawed at 37°C for 10-15 sec. and the semen of each straw was separately taken to khan test tube stored at 37°C in a water bath. This semen was subjected to quality tests.

#### 1.1 Sperm Motility

A drop of the semen was placed on a clean and warm (37°C) glass slide and covered with a cover slip. The same operator at the same conditions practiced visual detection through a light microscope (Nikon. Japan) at x100 magnification and the motility (total motility, sperm being able to propel themselves with a beating tail) was determined.

#### 1.2 Dead Sperm Percentage

Another drop of semen dose was placed on a clean and warm glass slide and two drops of eosin nigrosin solution prewarmed at 37°C were placed on the semen drop. This was mixed for 10 sec with an end of another slide and kept still for 50 sec. Then, a thin smear of the mixture was made on the slide and it was allowed to dry in air. The slide was examined under x400 power. Several areas of the smear were focused and sperms were counted at each area until the total count reached 100. Pinkish sperms were recorded as dead sperms.

#### 1.3 Correlation between Sperm Motility and Dead Sperm Percentage

Sperm motility and Dead Sperm Percentage was analyzed by Pearson's correlation coefficient using MINITAB software package.

#### 1.4 Sperm Abnormalities

A film of the semen was spread on a clean glass slide and it was air dried and fixed in flame. The slide was placed in an absolute alcohol solution for 4 min and air-dried. It was washed with distilled water and then with 96% ethanol. The smear was stained with Carbolfuchsin-eosin solution (William's stain) for 10 min then washed with water. Finally, the slide was placed on the stage of a light microscope (Nikon. Japan) and 100 sperms were counted separately for sperm abnormalities; head, mid tail and tail abnormalities (Annexure 1) at x400 magnification.

### EXPERIMENT 2

***Selection of a Sperm Preparation Method for IVF in Goats***

Two sperm preparation methods, the conventional swim up method and a simplified swim up method, each with ten replicates, were compared. For the swim up procedures, DM-H-SS medium (Crozet *et al.*, 1987) was prepared (Annexure 2) and the same medium was used for both procedures. This experiment was conducted at the Veterinary Research Institute, Gannoruwa, Peradeniya.

***Sperm Swim-up Method***

From the DM-H-SS culture medium, 1.25 ml was taken into a sterilized 5ml glass tube and 0.25 ml of the semen thawed at 37°C for 15 seconds in a water bath, was slowly underlaid using a sterile

Pasteur pipette and an aliquot of the semen was taken for initial motility analysis. It was slanted and incubated at 38.5°C for 60 minutes in a 5% $CO_2$ incubator (Forma Scientific, model 3111. USA.). One milliliter of the supernatant was separated and 2ml of DM-H-SS medium were added. Then it was centrifuged at 500g for 5mins. The recovered pellet was resuspended in 1ml of DM-H-SS. This solution was subjected to the tests for sperm quality (i.e. sperm concentration and motility).

*Simplified Swim-up Method*

From the DM-H-SS culture medium, 1.25 ml was sucked to a sterilized disposable plastic 5ml syringe and 0.25 ml of semen thawed at 37°C for 15 seconds in a water bath, was carefully sucked into the syringe without mixing the two and an aliquot of the semen was taken for initial motility analysis. The open end of the syringe was sealed with the needle itself. The syringe was slanted and incubated at 38.5°C for 60 minutes in a 5% $CO_2$ incubator (Forma Scientific, model 3111. USA.). The syringe was pressed until 1ml of supernatant was left in the syringe.

The remaining 1ml was used to the tests for sperm quality (i.e. sperm concentration and motility).

**2.1 Cleanliness of Prepared Sperms**

Availability of unnecessary particles in the microscopic field was observed by the same operator before the motility was taken.

**2.2 Sperm Motility**

Sperm motility was determined as described in 1.1 step.

**2.3 Sperm Concentration**

10μl of prepared semen is diluted in 990μl of formol saline and well shaken. Two parts of (10 μl each) the solution are loaded to each side of the haemocytometer (Mod_fuch's Rosenthal. 0.2mm, 1/16mm$^2$). Out of 9 large squares (each containing 16 small squares) 5 are selected and sperms are counted through a light microscope under x400 magnification.

*Calculation*

Volume of the small square = 0.2* 1/16 mm3
" large = 0.2* 1/16* 16 mm3
Volume of the 5 large squares = 0.2*1/16*16*5
= 1 mm$^3$
Dilution factor = 0.01
If the counted sperm no is ' n',
the concentration = n/1 mm3 x 1000 mm3 x 100

***Statistical Analysis***

All data distributions were tested for skewness using MINITAB Release 11 for Windows and SAS software package. Experiment 1 was conducted as a two factor factorial experiment in Incomplete Block Design. Data used in experiments were taken from a sample of normal population and were analyzed by Pearson's correlation coefficient and Student's t- test. The significant level was set at P<0.05.

## RESULTS AND DISCUSSION

### EXPERIMENT 1

**1.1 Sperm Motility**

There was no significant difference among the means of sperm motility of different breeds and different storage periods (Table 1). Though not significant, semen straws of FrSw breed shows the highest sperm motility followed by the breed Friesian.

**Table 1 - Sperm motilities of different breeds and age categories:**

| Breed | Mean | Age (yrs) | Mean |
|---|---|---|---|
| FrAMZ | 24.50$^a$ | >20 | 26.54$^a$ |
| Jy | 39.06$^a$ | 15-19 | 25.30$^a$ |
| Fr | 41.88$^a$ | 10-14 | 48.75$^a$ |
| FrSw | 44.00$^a$ | 5-9 | 40.89$^a$ |
| | | <5 | 45.25$^a$ |

Means in a column with the same letter are not significantly different.
Alpha = 0.05

**1.2 Dead Sperm Percentage**

There was no significant difference among the means of dead sperm percentages of different breeds and different storage periods. The breed FrAMZ showed lower dead sperm percentage and it showed higher dead sperm percentage in semen storage period over 15 years (Table 2).

**Table 2 - Dead sperm percentages of different breeds and age categories:**

| Breed | Mean Death% | Age (Yrs) | Mean Death% |
|---|---|---|---|
| FrAMZ | 65.63$^a$ | >20 | 64.77$^a$ |
| Jy | 54.56$^a$ | 15-19 | 69.20$^a$ |
| Fr | 56.12$^a$ | 10-14 | 48.13$^a$ |
| FrSw | 53.60$^a$ | 5-9 | 56.16$^a$ |
| | | <5 | 46.38$^a$ |

Means in a column with the same letter are not significantly different.
Alpha = 0.05

Both breed wise and storage time wise the dead sperm percentages are high. This does not affect much on the IVF technique as it uses preprpared sperms. However in AI technique, no prior semen preparation is done. Therefore it can result in lowered conception rates in Artificially Inseminated herds. Although not significant, the values of sperm motility in breed FrAMZ and age categories over 15 years are considerably low.

**1.3 Correlation of sperm motility and Dead Sperm Percentage**

There was a highly significant negative (Pearson) correlation (r = -0.882, P<0.01) between sperm motility and dead sperm percentage.

The negative correlation here implies that motility indicates the live death sperm ratio, and then, no need is there to go for the laborious staining procedures. However in this experiment only 100 cells were counted to get the dead sperm percentage due to time limitation and at least 200 cells have to be taken to increase the precision (Sugulle *et al.*, 2006).

**1.4 Sperm Abnormalities**

In breed wise, there was no significant difference in mean sperm head abnormalities while the mean sperm mid piece abnormalities of Jersey were significantly different from others. The mean sperm tail abnormalities of FrAMZ were significantly different from other breeds (Table 3)

**Table 3 - Sperm abnormalities according to breed:**

| Breed | Abnormalities | | |
|---|---|---|---|
| | Head | Mid | Tail |
| FrAMZ | 18.1[a] | 12.1[ab] | 17.9[a] |
| Jy | 14.9[a] | 8.4[b] | 11.1[b] |
| Fr | 18.3[a] | 13.4[a] | 10.4[b] |
| FrSw | 17.5[a] | 14.8[a] | 8.9[b] |

Means in a column with the same letter are not significantly different.
Alpha = 0.05

A significant difference in sperm head abnormalities was observed between 10 to 14 years and less than five years semen storage period categories. Sperm mid piece abnormalities were significantly high in both five to ten and less than five years categories while sperm tail abnormalities were significantly higher in 15 to 19 years category (Table 4).

**Table 4 -Sperm abnormalities according to storage time:**

| Age Category | Abnormalities | | |
|---|---|---|---|
| | Head | Mid | Tail |
| >20 | 16.8[ab] | 9.5[a] | 12.7[a] |
| 15-19 | 17.2[ab] | 9.4[a] | 18.2[b] |
| 10-14 | 13.6[b] | 8.4[a] | 8.9[a] |
| 5-10 | 17.4[ab] | 14.5[b] | 11.2[a] |
| <5 | 20.8[a] | 16.5[b] | 11.1[a] |

Means in a column with the same letter are not significantly different.
Alpha = 0.05

Here only the morphological abnormalities have been taken but abnormalities can be observed in chromosome or DNA level too. It requires advanced technologies to identify the DNA level abnormalities. However, a recent study reveals that there is a positive correlation between morphological and DNA level abnormalities (Younglai *et al.*, 2001; Sakkas *et al.*, 2000).

There was a high variance in collected semen samples. Even recently produced some semen straws showed lower motility, high dead sperm percentage and abnormalities, and this might be a result of lowered nitrogen level in the storage tanks.

**EXPERIMENT 2**

**2.1** ***Cleanliness of prepared sperms***

The prepared sperms from both methods were slightly particulated. This could be a hindrance for IVF as such particles might harbour bacteria like contaminations. Sperm washing in classical swim up method should have eradicated these particles but still particles are available. This research should proceed to next step; i.e. *in vitro* fertilization of oocytes with sperms prepared by both methods to check whether these particles are problematic.

**2.2** ***Sperm Motility***

There was no any significant difference between the initial motilities of the straws used for swim up method and simplified swim up method (P>0.05). In classical swim up method a significant increment in sperm motility was observed while the increment of that in simplified swim up method was not significant (Table 5).

**Table 5 - Motility differences between two sperm preparation methods:**

| | Method | |
|---|---|---|
| | Swmup | Sswmup |
| Initial motility | 71[a] | 72[a] |
| Final motility | 90[b] | 78[a] |

Swmup=swim-up method
Sswmup=simplified swim-up method
Means with the same letter are not significantly different.
Alpha = 0.05

**2.3** ***Sperm Concentration***

The means of sperm concentration after sperm preparation were not significantly different between two methods (Table 6).

**Table 6 - Sperm concentration after sperm preparation:**

| | Method | |
|---|---|---|
| | Swmup | Sswmup |
| Sperm concentration | 4.5[a] | 7.5[a] |

Swmup = swim-up method
Sswmup = simplified swim-up method
Means in the row with the same letter are not significantly different.
Alpha = 0.05

These results denote that even though both methods improved motility, the normal swim up

method is better with regard to the sperm motility while there is no difference in sperm concentration. Several scientists have shown that the minimum sperm concentration for IVF is lower than even $0.5x10^6$ /ml and the motility as lower as 30% was also acceptable for IVF (Ward *et al.*, 2003). Therefore, both methods have resulted in acceptable levels of motility and concentration in the prepared semen samples. This means that the simplified swim up method to recover the motile sperm from semen samples is as reliable as classical swim up method. One advantage of this method is the limited number of technical steps that it does not have a centrifugation step, which does not require particular expertise and saves chemicals; thus it is more practical and less expensive. Some researchers have shown that centrifugations could cause damages to the spermatozoal membranes (Agarwal *et al.*, 1994; Shekarriz *et al.*, 1995) while some other researchers have proven that no harm occurs to sperms due to centrifugation (Younglai *et al.*, 2001). The simplified method is a closed system compared to the normal swim up and this will enhance the safety from potential contaminations, which may lead to diseases. Skill and experience of the technician greatly influence the relative accuracy of motility evaluation and also such sort of data has to be analyzed by several technicians and inter-technician variances have to be minimized. In the present study, the gross motility has been taken but the important character is progressive motility, which demands much practice.

This research need to be broadened to evaluate the number of successful cell divisions in IVF from each method to get a high practical accuracy.

## CONCLUSIONS

There is no effect of storage time and breed on motility and dead sperm percentage but they affect on abnormalities.

The simplified swim up method is as effective as the normal swim up method, but the former is more convenient and more economical.

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## REFERENCES

Agarwal, A., I. Ikemoto and K.R. Loughlin (1994). Effect of sperm washing on levels of reactive oxygen species in semen. Journal of Andrology, 33:157–162. Available at: http://humrep.oxfordjournals.org/cgi/external_ref/access_num=7857166&link_type=MED. [Retrieved 20 May 2006].

Anon (2004). Livestock Statistics 2004. Livestock Planning and Economics Division, Department of Animal Production and Health, Getembe, Peradeniya.

Chandrasiri, A.D.N., S.H.A.Nihar, W.R.A.K.J.S. Rajapaksha, R.M.S.Malkanthi and A.S.B.Rathnayake (1999). Reproductive status of female goats slaughtered for meat in Sri Lanka. Animal production for a consuming world. 2000, Ed.G.M.Stone Vol: B. A suppliment of the Asian-Australian Journal of Animal Sciences 2000. Vol :13.

Chandrasiri, A.D.N., V.K.Gunawardana, R.M.S.Malkanthi and A.S.B.Rathnayake (1996). In vitro maturation, fertilization and culture (IVMFC) of goat oozytes. Sri Lanka veterinary journal 1996, 43: 1-2.

Cognie,Y., Y.Guerin, C.Guyader, N.Poulin and N.Crozet (1991). In vitro fertilization of sheep oozytes matured in vivo. Theriogenology 1991. 35: 12-15.

Crozet ,N., D.Huneau, V.Desmedt, M.C.Theron, D.Szollosi, S.Torres and C.Sevellec (1987). In vitro fertilization with normal development in sheep. Gamete Research. 16:159-170.

First, N.L. and J.J.Parrish (1988). Sperm maturation and in vitro fertilization. Journal of 11th international congress of animal reproduction and artificial insemination (1988). 5:161-168.

Gunarajasingham, D., H. Abeygunawardana, V.Y.Kuruwita, E.R.K.Perera and B.M.A.O.Perera (1995). Characterization of semen of Nilli Ravi buffalo bulls using standard and modified techniques. Tropical agricultural research 1995. 7 :152- 167.

Henry, S.A., J.Ho, D.Y.Munasinghe, H.L.Teo, V.Tok and C.Chan (1996). Sperm microstructure during sperm preparation for ART. Visual atlas of human sperm structure and function for assisted reproductive technology. 3: 51-52.

Maxwell, W.M.C. (1984). Current problems and future potential of artificial insemination programmes. In: Reproduction in Sheep. Eds. D.R. Lindsay and D.T. Pearce. pp 291 –298.

Salamon, S. and W.M. Maxwell (1995). Frozen storage of ram semen. Processing, freezing, thawing and fertility after cervical insemination. Animal reproductive science 1995. 37: 185 –249. Available at: http//www.andrologyjournal.org/cgi/content/full/25/2/#REF41.[ Retrieved on May 2006].

Sakkas, D., G.C. Manicardi, M. Tomlinson, M. Mandrioli, D. Bizzaro, P.G. Bianchi and U. Bianchi (2000). The use of two density gradient centrifugation techniques and the swim-up method to separate spermatozoa with chromatin and nuclear DNA anomalies. Human Reproduction,WHO Laboratory Manual for Examination of Human Semen(2000)Cambridge University Press, Cambridge. 15: 5, 1112-1116.

Shekarriz, M., D.M. De Wire, A.J. Thomas, and A. Agarwal (1995). A method of human semen centrifugation to minimize the iatrogenic sperm injuries caused by reactive oxygen species. Europian Urology. 28:31–35. Available at: http://humrep.oxfordjournals.org/cgi/external_ref/access_num=8521891&link_type=MED. [Retrieved 20 May 2006].

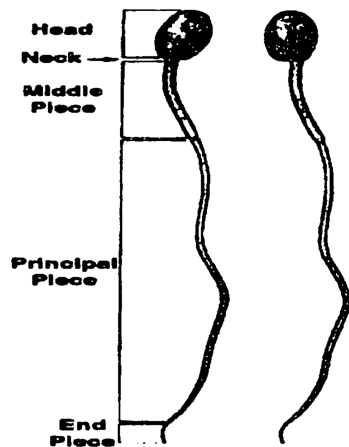
Sugulle, A. H., M. M. U. Bhuiyan and M. Shamsuddin (2006). Breeding soundness of bulls and the quality of their frozen semen used in cattle artificial insemination in Bangladesh. Livestock Research for Rural Development 2006. 18: 4. Available at : http://www.fao.org/waicent/faoinfo/agricult/aga/agap/frg/lrrd.htm. [Retrieved on 13 August 2006]

Ward, F., D. Rizos, M.P. Boland, P. Lonergan (2003). Effect of reducing sperm concentration during IVF on the ability to distinguish between bulls of high and low field fertility. Theriogenology (2003). 59:1575Availableat:http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&list_uids=12559462&dopt=Abstract [Retrieved 03 September 2006].

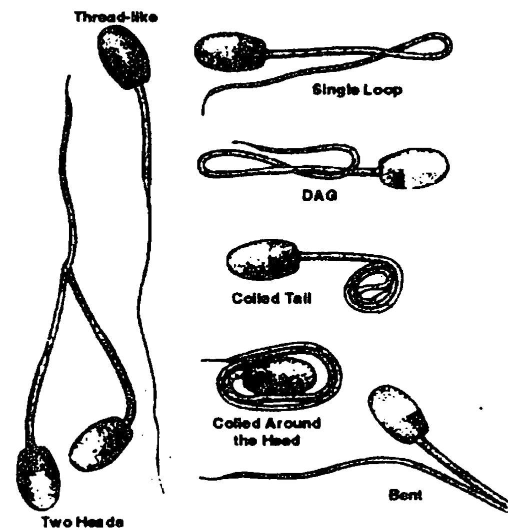
Younglai, E.V., D. Holt, P. Brown, A. Jurisicova and R.F. Casper (2001). Sperm swim-up techniques and DNA fragmentation. Journal of human reproduction, Ed. D.H.Barlow. Available at : http//:www.oxfordjournals.org/cgi .[Retrieved 25 August 2006].

Zavos, P.M., M.Abou-Abdallah, P. Aslanis, J.R. Correa and P.N. Zarmakoupis-Zavos (2000). Use of the Multi-ZSC one-step standardized swim-up method: recovery of high quality spermatozoa for intrauterine insemination or other forms of assisted reproductive technologies. Fertility and sterility. 74: 834–835. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=retrieve&db=pubmed&list_uids=11020536&dopt=Abstract[Retrieved 20 May 2006].

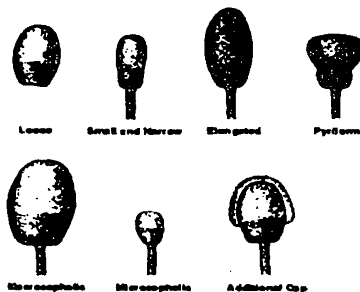
**Annexure 1: Sperm Abnormalities**



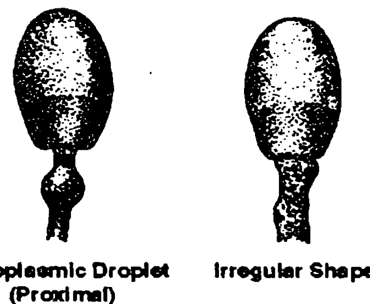

**Normal sperms**




**Tail abnormalities**



**Head abnormalities**




**Mid piece abnormalities**

**Annexure 2: Preparation of DM-H-SS Medium**

**DM-H Stock Solution (x10)**

The following compounds were accurately measured using a top loading balance (Gibbertini. Europe 500) and dissolved using a magnetic stirrer. The final volume was adjusted to 100ml in a volumetric flask.

| | |
|---|---|
| NaCl | 7.568g |
| KCl | 0.300g |
| $CaCl_2$=2H2O | 0.330g |
| $NaH_2PO4$=$H_2O$ | 0.115g |
| Mg $Cl_2$=$6H_2O$ | 0.106g |

**DM-H Working Solution**

10ml of the stock solution was added with following compounds with given amounts.

| | |
|---|---|
| $NaHCO_3$ | 0.035g |
| HEPES | 0.238g |
| Glucose | 0.045g |
| Pyruvic acid | 0.014g |
| Gentamycin sulfate | 0.005g |

They were dissolved using a magnetic stirrer and pH was adjusted to 7.4.

**DM-H-SS Preparation**

Sheep blood was obtained to sterilized vacuum tube and incubated for ½ h at 38°C. Then the blood was centrifuged at300g for 5minutes. The supernatant, the serum, was then subjected to heat at 56°C for 1h for heat inactivation. The heat-inactivated serum was stored in a freezer for future use. Prior to the each experiment to begin; DM-H-SS was prepared adding 20% of heat inactivated sheep serum to the DM-H working solution.