THE EFFECT OF DIFFERENT TALP-COMPONENTS ON THE PROGRESSIVE MOTILITY OF BOVINE SPERMATOZOA DURING THE CENTRIFUGATION AND INCUBATION STAGES AS A PREREQUISITE FOR IN VITRO FERTILISATION

BY

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SEPTEMBER, 1988

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CONTENTS **Page**

ACKNOWLEDGEMENTS

I wish to express my gratitude to Professor N. Gorman for giving me the opportunity to carry out this work in his department. I also wish to express my thanks to Dr. J. Renton, Dr. M. Harvey and Mr E. Cameron for their advice throughout the course of my studies. I would also like to thank Miss S. Harker, Mis M. Waterston and Mr. P. Toner for their technical assistance.

I would like to express my thanks to the Greek Government who supported the work throughout.

SUMMARY

In the present study the effect of different Modified Tyrode's media-TALP and different sperm concentrations on the progressive motility of the spermatozoa was examined.

TALP medium containing high levels of lactate, $NaHCO_3$ and Hepes provided the best environment for the spermatozoa during the <<swim-up>>, first and second centrifugation stages. However the medium,in which lactate was the primary nutritional source, gave the best results during CO_2 incubation. Treatments of spermatozoa in an air environment gave much poor results than treatments in a CO_2 environment.

Sperm motility was optimal when the sperm concentration was around $20x10^6$ sperm/ml or less when incubated in vitro (5% CO^2).

INTRODUCTION

The fertilisation of the mammalian egg outside the body leading to the production of viable offspring has been a target of biological science for more than a century. It was first achieved in laboratory animals such as rabbits (Chang, 1959), golden hamsters (Yanagimachi and Chang, 1963) and mice (Iwamatsu and Chang, 1969), while the first human baby resulting from In Vitro Fertilisation was born in 1978 (Steptoe and Edwards, 1978).

The number of established pregnancies and viable offspring produced in farm animals after In Vitro Fertilisation is very small. The most progressive work has been done in cattle. The first pregnancy in cattle using this technique was achieved in 1981 followed by the birth of a live calf in 1982 (Brackett et al., 1982). This calf was born alive, without any obvious abnormalities and was the result of In Vitro Fertilisation of an oocyte matured in vivo, subsequently fertilised in vitro and then transferred to a synchronised recipient. Recent workers have used matured oocytes recovered by laparoscopy (Brackett et al., 1984, Greve et al., 1984, Sirard and Lambert, 1986) or immature oocytes recovered from small follicles at the slaughterhouse (Iritani et al., 1984, Greve et al.,1987, Lu et al.,1987).

FACTORS INVOLVED IN THE SUCCESS OF IN VITRO FERTILISATION

a) The source of the spermatozoa and oocytes.

Until now epididymal, ejaculated or frozen-thawed spermatozoa have been used (Sirard et al., 1984, Parrish et al., 1985, 1986). Oocytes from follicles at different stages of development have been investigated and it appears that oocytes from small follicles, 1-7mm in diameter, are more successful (Ball et al., 1983, Xu et al., 1986).

b)The choice of the culture medium

The culture medium is also very important for successful In Vitro Fertilisation. Various media have been tried in an attempt to improve the viability and motility of the spermatozoa and the maturation procedure of the oocytes. These include: DM-medium (Brackett et al., 1982), Ham's F-10 medium (Xu et al., 1986), TALP (Lenz et al., 1983, Parrish et al., 1985, Davis and Foote, 1987), Krebs-Ringer-Bicarbonate solution (Iritani et al., 1984) or M199 (Hunter and Moor, 1987, Lu et al., 1987, 1988, Fukui and Ono, 1988). Unfortunately to date a completely satisfactory medium has still not been identified for both gametes.

c)Sperm quality

The progressive motility of the spermatozoa appears to be a very important factor associated with the penetration of the zona pellucida of the ovum. In addition, prior to fertilisation, spermatozoa must undergo capacitation, this includes an acrosome reaction and the subsequent release of acrosomal enzymes (Bedford, 1970, 1974, Hunter, 1980).

d)Cumulus cells

The cumulus cells surrounding the egg apparently play an important role in ensuring sperm penetration. Several researchers suggest that the ovum is only able to be penetrated after partial removal of the expanded cumulus cells (Lu et al., 1987, 1988). However it is believed that it is beneficial to leave as many of the separated cumulus cells in the culture medium in close apposition to the partially denuded ovum. It is suggested that bovine cumulus cells secrete certain hormones, which may well be important in achieving fertilisation. A similar role of a cumulus complex of hormones has been put forward in the human (Laufer et al., 1985, Tapanainen et al., 1987, Lobb and Dorrington, 1987).

e)The benefit of extra additives

The benefit of the addition of a variety of additives such as Bovine Serum Albumin, Oestrous Cow Serum, Fetal Cow Serum and gonadotrophins to culture media has been investigated (Hensleigh and Hunter, 1985, Sanbuissho and Threlfall, 1985, Xu et al.,1987, Sirard et al., 1988). The results are promising and suggest that the effect of some of the additives is beneficial.

SPERM CELL MORPHOLOGY

Spermatozoa are distinctly organised into head and tail segments. The head consists of a condensed nucleus, which is covered by the overlying acrosome. The tail includes the axoneme, mitochondria and other structural elements and is responsible for cell movement. The whole sperm cell is covered by the plasma membrane (Fawcett, 1970, Dym, 1977).

CAPACITATION AND ACROSOME REACTION

The procedure for preparing sperm capable of fertilising the ovum is composed of at least two sequential phases, an initial alteration of the sperm membrane, called capacitation, which allows the spermatozoa to continue to the second phase, called the acrosome reaction, during which a fusion of the sperm plasma membrane and outer acrosomal membrane occurs (Austin, 1952, Bedford, 1970).

MATERIALS AND METHODS

MATERIALS

Spermatozoa

Semen extended in milk-glycerol was supplied by the Scottish Milk Market Board in 0.25ml straws (IMF, France) stored at -196^{o}C in liquid nitrogen.

Reagents and Media

Modified Tyrode's media-TALP (Preparations are given in Appendix).

METHOD 1.

The method used is a modification of that first described by Parrish et al. (1985) and contains the following steps:

1.Straws were removed from the liquid nitrogen and thawed separately by placing in a water bath at 35^{0}C for 30 seconds.

2. After thawing, one drop of the milk-sperm mixture was added to 1ml of warm 0.85% saline in order to examine the progressive motility. After shaking the mixture, one drop of the sperm-extender-saline was placed on a warm slide (BDH Co., UK) and examined on a microscope at magnification x100. The residual milk-sperm mixture was gently transferred to the bottom of a 62x10mm plastic tube (BDH Co.), which contained 1ml of the TALP-medium. A number of modifications of this medium were used in the study. The tubes were then incubated in a water bath at 39^{0}C for 1 hour.

3. At the end of this time, 0.85 ml of the supernatant was removed with a 1ml pipette (Volac Co.,UK) and placed into a pre-warmed 10ml sterile centrifuge tube (Sterling Co. UK) to avoid cold shock. The tube was inverted gently several times to mix the suspension.

4. The spermatozoa were pelleted by centrifuging at 300xg for about 7 minutes. The supernatant was discarded and the pellet was resuspended in 3-5ml of TALP.

5. This mixture was again centrifuged at the same speed and time. The supernatant was discarded and the pellet was finally suspended in TALP to give a final

concentration of 19-21x10^6 sperm/ml by using a haemocytometer (Weber Ltd.,UK).

6. 50µl of the sperm suspension were dispensed into each of 2 wells of two sterile 96-well culture plates (NUNC Co.,Denmark) and 200µl of the same suspension into four 10ml centrifuge tubes. 50µl paraffin was then added to one of the two wells of both the 96-well culture plates, whereas 200µl paraffin was added to two of the tubes. One 96-well culture plate was incubated at 39^{0}C in a humidified incubator containing 5% CO_2, 95% air and the other one incubated at 38^{0}C (100% air).Paraffin-covered and uncovered tubes were put in the same incubators. The caps of the tubes in the CO_2 incubator were loosed to allow continous supply of CO_2, while the tubes in the incubator containing 100% air were tightly sealed.

The average progressive motility of the spermatozoa was assessed visually on a light microscope at magnification 100x after the swim-up (2nd step), the first and second centrifugations and after the 2nd, 4th, 6th, 9th and 12th hour of incubation. Also the pH of some media in the paraffin-uncovered tubes, in both CO_2 and air incubators, was measured after approximately 12 hours of incubation.

METHOD 2.

The steps of experiment 1 were repeated using only 10ml tubes, no paraffin, the TALP-medium 6 for the preincubation sperm treatment and TALP-medium 1 for incubation (5% CO_2, 95% air). The sperm concentration was adjusted immediately after the 2nd centrifugation to 1, 10, 20, 30, 40, 50 or 60x10^6 sperm/ml with TALP-medium 1.

Sperm motility was assessed using a light microscope at 100x magnification at the 0, 2nd, 4th, 6th, 9th and 12th hour of incubation.

RESULTS

There were 18 replicates of the experiments 1 and 2.

The effect of CO_2 environment on the sperm motility

There were no great variations in sperm progressive motility among the samples in the paraffin-covered and uncovered wells of the 96-well culture plate and the paraffin-covered and uncovered plastic tubes at a given incubation time, when incubated at 39^0C ($5\%CO_2$, 95%air). Because of the similarity of these results in the CO_2 incubator, only the sperm motility results from the paraffin-uncovered tubes are presented in Table 1 comparing the variety of the TALP-media used. Also in Table 1 and Figure 1 comparison of the sperm motility before and after 12 hours of incubation is shown for every TALP-medium. In addition, it was observed that the pH of the media in the paraffin-uncovered tubes was similar to that before incubation (7.4) in the CO_2 incubator.

Medium 6 was far more effective in sustaining sperm motility than any other medium during swim-up, first and second centrifugations. Apart from medium 6, medium 4 also gave satisfactory results and it could be a secondary choice at the same stages. Medium 1 proved to be the best culture system for the motility of the spermatozoa during incubation in CO_2 environment. However it was a much poorer culture system for the spermatozoa than the media 6, 4 and 3 during the sperm pre-incubation treatment. The combination of medium-6 for the swim-up, first and second centrifugations and the medium-1 for incubation gave the best results after approximately 12 hours of incubation (42%). The advantage of the combination 6 and 1 media is illustrated graphically in figure 2. On the other hand, medium-9, which was supplemented with high level of D-glucose, showed a dramatical reduction in sperm motility during incubation ($5\%CO_2$, 95%air), after approximately 9 hours the motility was 0.

The effect of air incubation on the sperm motility

During air incubation at 38^0C, there were no variations in the sperm motility in paraffin-covered wells and tubes. Because of the similarity of the figures, only the results of the tubes are given in Table 2. However there were great variations between the paraffin-covered and uncovered sperm samples. Also there were differences in the motility between the paraffin-uncovered wells and tubes. The results of the sperm motility in air incubation are given in Tables 2, 3 and 4. In addition, it was found that the pH was increased from 7.4, before incubation, to 8.1 at the 12th hour of incubation.

Motility in paraffin-covered wells and tubes with TALP-medium 1 was slighly better than that in any other culture system during air incubation, however the results were much worse than those in the same medium during CO_2 incubation (Tables 1 and 2). Even the combination of the TALP-media 6 and 1 gave disappointing results by comparison with those in CO_2 incubation. The reduction of the sperm motility in media 6, 1 and the combination 6/1 in the air incubator is given graphically in figure 3 and it can be compared with the motility results in the same medium during CO_2 incubation in figure 2. Also it is obvious in Tables 2, 3 and 4 that the sperm motility in the paraffin-uncovered wells and tubes declined faster than that in the paraffin-covered wells and tubes in air incubation. In addition, a considerable reduction of the sperm motility occured in the paraffin-uncovered wells compared to that of the paraffin-uncovered tubes (Tables 3 and 4).

The effect of the sperm concentration on sperm motility

As is shown in Table 5, there were no differences in the sperm progressive motility of the samples with concentration 1, 10 or $20x10^6$ sperm/ml at any examination time during incubation (5%CO_2, 95%air). However, the sperm motility was influenced variously by higher sperm

concentrations. Table 5 and figure 4 indicate that the highest sperm concentration, beyond $20x10^6$ sperm/ml, showed the lowest sperm motility, especially after the 9th hour of incubation.

Table 1: The average sperm progressive motility (%) in paraffin uncovered tubes throughout the preincubation and incubation time (hours) in the various TALP-media (5% CO2, 95% air).

(a).The reduction in average sperm motility between the 3rd and 8th step of incubation.

± .The standard deviation.

TALP MEDIA

	Steps	1	2	3	4	5	6	7	8	9	10	$^{4}/_{1}$	$^{4}/_{7}$	$^{6}/_{1}$	$^{6}/_{7}$
SPERM MOTILITY (%)	1 - Swim-up	85 ±2.4	78 ±2.1	83 ±2.1	85 ±3.4	75 ±2.5	85 ±2.5	85 ±2.4	82 ±3.4	80 ±2.5	85 ±2.4	85 ±3.4	85 ±3.4	85 ±2.5	85 ±2.5
	2 - 1st Centrifugation	73 ±3.8	62 ±2.4	78 ±1.5	80 ±2.4	60 ±2.5	82 ±2.4	68 ±2.1	63 ±1.5	74 ±2.5	77 ±3.4	80 ±2.4	80 ±2.4	82 ±2.4	82 ±2.4
	3 - 2nd Centrifugation	60 ±2.5	51 ±2.1	62 ±2.4	65 ±2.5	43 ±2.9	74 ±2.5	57 ±2.4	53 ±2.1	56 ±3.2	61 ±2.1	65 ±2.5	65 ±2.5	74 ±2.5	74 ±2.5
	4 - 2h	58 ±2.1	38 ±1.5	57 ±3.4	53 ±2.1	36 ±3.2	69 ±2.5	53 ±2.1	49 ±3.4	47 ±2.4	54 ±2.5	62 ±3.4	62 ±2.4	71 ±2.1	70 ±2.5
	5 - 4h	54 ±2.5	27 ±3.4	51 ±2.1	43 ±2.1	28 ±3.2	62 ±2.4	48 ±2.9	45 ±2.5	23 ±2.9	42 ±3.4	58 ±2.1	57 ±3.4	66 ±2.1	64 ±2.5
	6 - 6h	50 ±2.5	21 ±3.2	45 ±2.5	32 ±3.4	14 ±2.5	54 ±2.5	43 ±3.2	39 ±3.4	17 ±2.4	29 ±2.5	53 ±3.2	52 ±2.4	60 ±2.5	57 ±2.4
	7 - 9h	45 ±2.5	16 ±3.2	37 ±3.4	17 ±2.4	6 ±2.9	44 ±2.5	37 ±3.4	31 ±2.1	0 ±0	16 ±2.1	46 ±2.1	44 ±2.5	52 ±2.4	48 ±2.1
	8 - 12h	36 ±2.1	9 ±2.5	27 ±2.4	5 ±2.5	1 ±0.9	32 ±2.4	31 ±1.5	22 ±3.4	0 ±0	7 ±0	38 ±3.2	35 ±2.5	42 ±2.4	36 ±1.5
	a	24	42	35	60	42	42	26	31	56	54				

TALP MEDIA

	Steps	1	2	3	4	5	6	7	8	9	10	$^{4}/_{1}$	$^{4}/_{7}$	$^{6}/_{1}$	$^{6}/_{7}$
SPERM MOTILITY (%)	1 - Before Incubation	60 ±2.5	51 ±2.1	62 ±2.4	65 ±2.5	43 ±2.9	74 ±2.5	57 ±2.4	53 ±2.1	56 ±3.2	61 ±2.1	65 ±2.5	65 ±2.5	74 ±2.5	74 ±2.5
	2 - 2h	48 ±2.1	32 ±3.4	46 ±1.5	48 ±2.1	26 ±2.9	60 ±2.5	44 ±2.5	41 ±2.1	33 ±3.1	35 ±2.5	49 ±2.5	46 ±2.1	56 ±1.5	54 ±2.5
	3 - 4h	36 ±1.5	15 ±3.4	30 ±2.5	33 ±2.1	11 ±2.0	42 ±2.4	30 ±2.5	28 ±2.9	14 ±2.5	19 ±3.4	38 ±2.1	34 ±2.5	39 ±2.5	33 ±1.5
	4 - 6h	19 ±2.5	0 ±0	12 ±2.4	14 ±2.5	0 ±0	23 ±2.1	17 ±2.4	12 ±3.4	0 ±0	3 ±2.1	19 ±2.5	18 ±1.5	24 ±2.5	12 ±2.4
	5 - 9h	5 ±2.5	0 ±0	0 ±0	0 ±0	0 ±0	1 ±0.9	3 ±2.1	0 ±0	0 ±0	0 ±0	4 ±2.5	1 ±0.9	5 ±2.5	2 ±0
	6 - 12h	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0

Table 2: Results of the average sperm motility (%) in paraffin covered tubes throughout the incubation time (hours) in the various TALP-media **(100%air)**.
± .The standard deviation.

TALP MEDIA

	Steps	1	2	3	4	5	6	7	8	9	10	4/1	4/7	6/1	6/7
SPERM MOTILITY (%)	1 - Before Incubation	60 ±2.5	51 ±2.1	62 ±2.4	65 ±2.5	43 ±2.9	74 ±2.5	57 ±2.4	53 ±2.1	56 ±3.2	61 ±2.1	65 ±2.5	65 ±2.5	74 ±2.5	74 ±2.5
	2 - 2h	32 ±2.4	17 ±3.4	31 ±2.1	33 ±1.7	18 ±2.1	39 ±2.5	31 ±2.1	27 ±3.4	19 ±3.4	24 ±2.4	36 ±2.1	31 ±1.5	37 ±2.4	35 ±2.5
	3 - 4h	9 ±2.4	0 ±0	4 ±2.5	8 ±1.5	0 ±0	8 ±2.9	5 ±2.5	2 ±0	0 ±0	1 ±0.9	8 ±1.5	5 ±2.5	8 ±1.5	7 ±2.4
	4 - 6h	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0
	5 - 9h	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0
	6 - 12h	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0

Table 3: The reduction in average sperm progressive motility (%) in paraffin uncovered tubes during incubation time (hours) in the various TALP-media **(100% air)**.

± .The standard deviation.

TALP MEDIA

SPERM MOTILITY (%) Steps	1	2	3	4	5	6	7	8	9	10	4/1	4/7	6/1	6/7
1 - Before Incubation	60 ±2.5	51 ±2.1	62 ±2.4	65 ±2.5	43 ±2.9	74 ±2.5	57 ±2.4	53 ±2.1	56 ±3.2	61 ±2.1	65 ±2.5	65 ±2.5	74 ±2.5	74 ±2.5
2 - 2h	25 ±2.5	12 ±3.4	25 ±3.5	28 ±2.1	11 ±1.5	32 ±2.4	26 ±1.5	22 ±2.4	7 ±2.4	15 ±3.4	30 ±2.5	22 ±2.4	29 ±2.5	24 ±2.5
3 - 4h	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0
4 - 6h	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0
5 - 9h	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0
6 - 12h	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0

Table 4: Results of the average sperm motility (%) in paraffin uncovered wells during incubation time (hours) in the various TALP-media **(100% air)**.
± .The standard deviation.

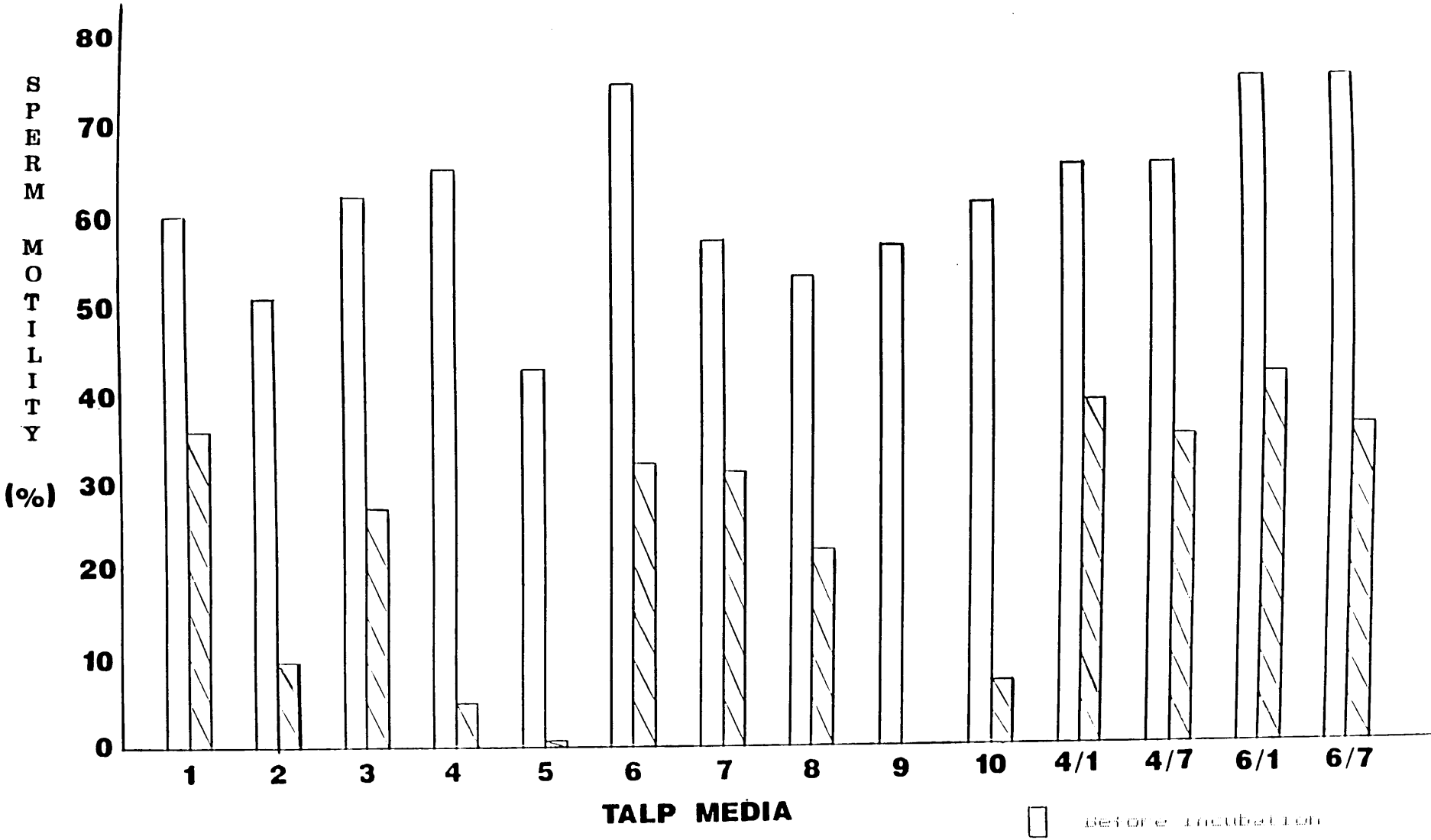

Figure 1: The average sperm progressive motility (%) in the various TALP-media in the paraffin uncovered tubes before incubation and at the 12th hour of incubation (5% CO2, 95% air).

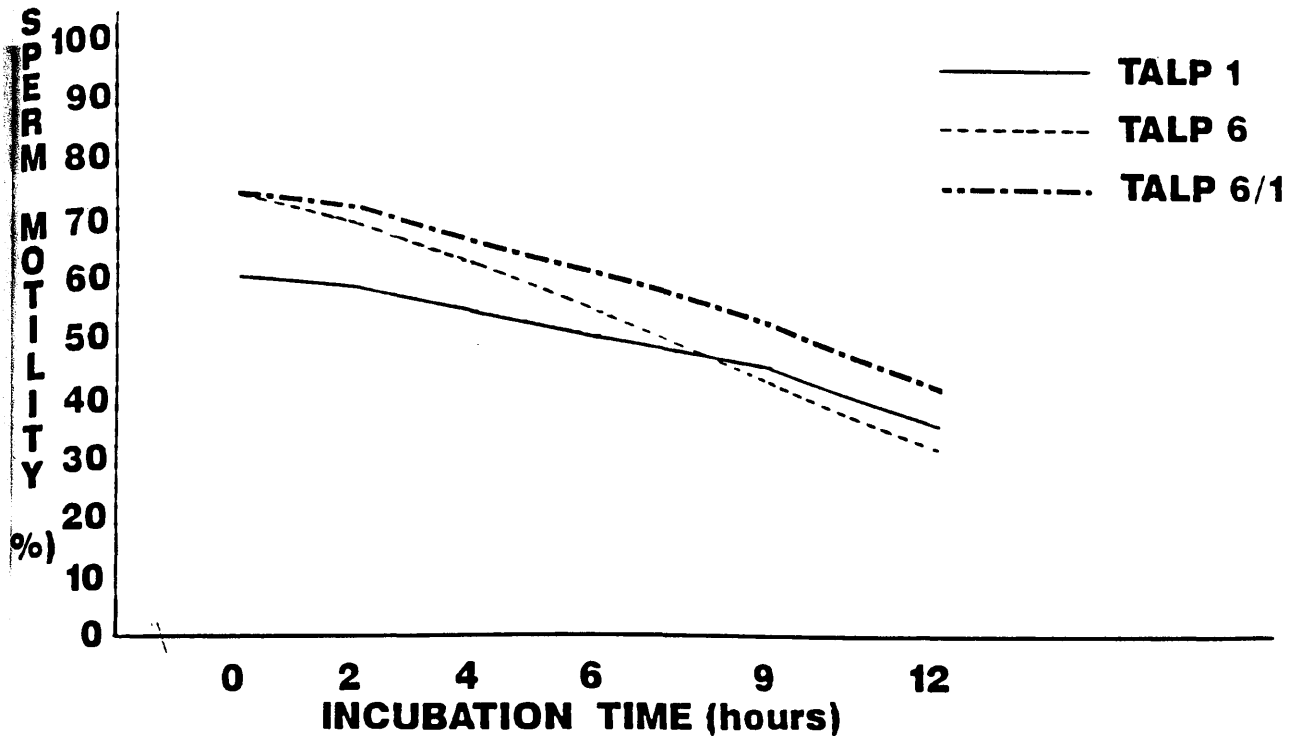

Figure 2: The reduction of the average sperm motility (%) in the TALP-media 1, 6 and 6/1 in paraffin uncovered tubes during incubation time (hours) (5% CO_2, 95% air).

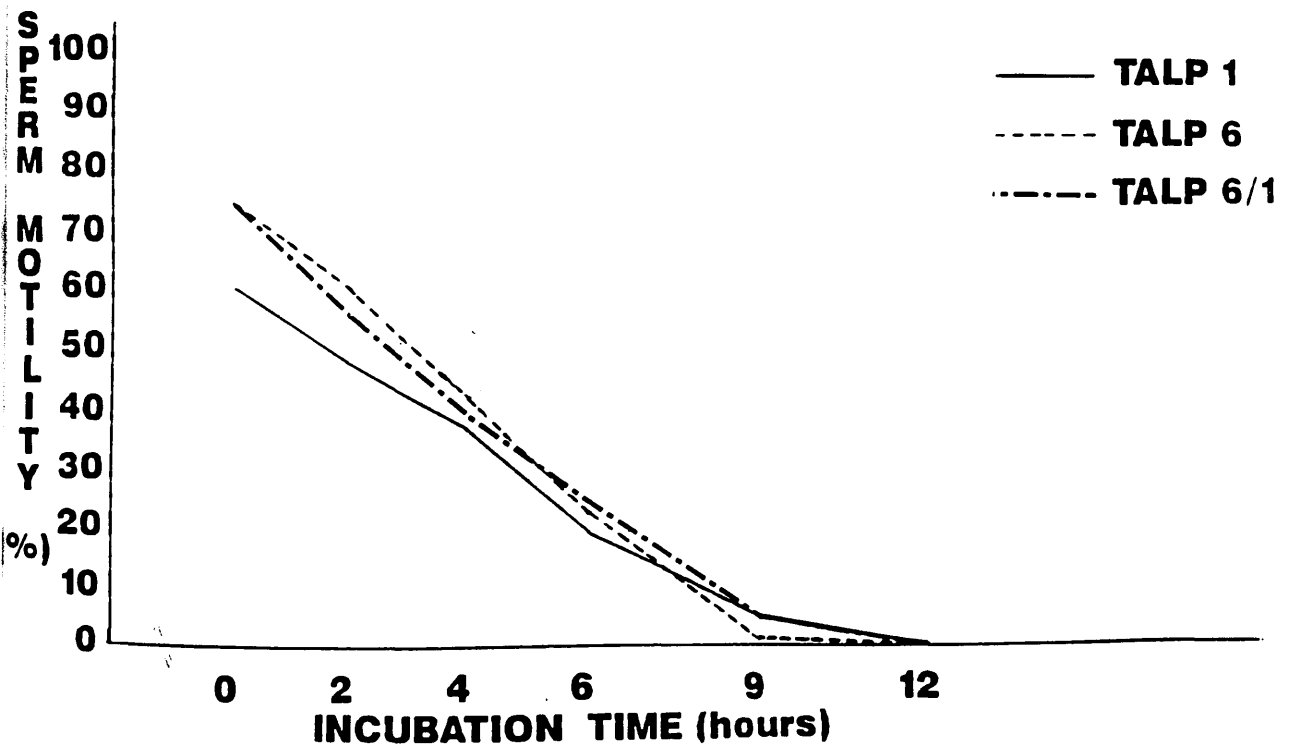

Figure 3: The reduction of the average sperm motility (%) in the TALP-media 1, 6 and 6/1 in paraffin covered tubes during incubation time (hours) (100% air).

SPERM CONCENTRATION (x 10^6 sperm/ml)

SPERM MOTILITY (%) Steps	1	10	20	30	40	50	60
1 - Before Incubation	73 ±2.1	73 ±2.1	73 ±2.1	73 ±2.1	73 ±2.1	73 ±2.1	73 ±2.1
2 - 2h	70 ±2.5	70 ±2.5	71 ±2.8	68 ±2.1	66 ±1.5	66 ±2.1	65 ±2.5
3 - 4h	67 ±2.4	66 ±1.5	67 ±1.7	64 ±2.5	63 ±2.3	61 ±1.5	60 ±2.9
4 - 6h	60 ±2.5	58 ±1.5	59 ±2.5	57 ±1.5	56 ±2.1	52 ±2.4	52 ±1.7
5 - 9h	53 ±1.5	51 ±1.2	53 ±3.0	48 ±2.1	44 ±2.5	41 ±2.5	39 ±2.5
6 - 12h	43 ±1.5	42 ±2.9	42 ±2.0	37 ±2.9	35 ±3.0	29 ±2.5	26 ±3.3

Table 5: The effect of the sperm concentration (x10^6 sperm/ml) on the sperm motility (%) throughout the incubation time (hours) **(5% CO2, 95% air)**.
± .The standard deviation.

Before incubation, the motility was assessed in the

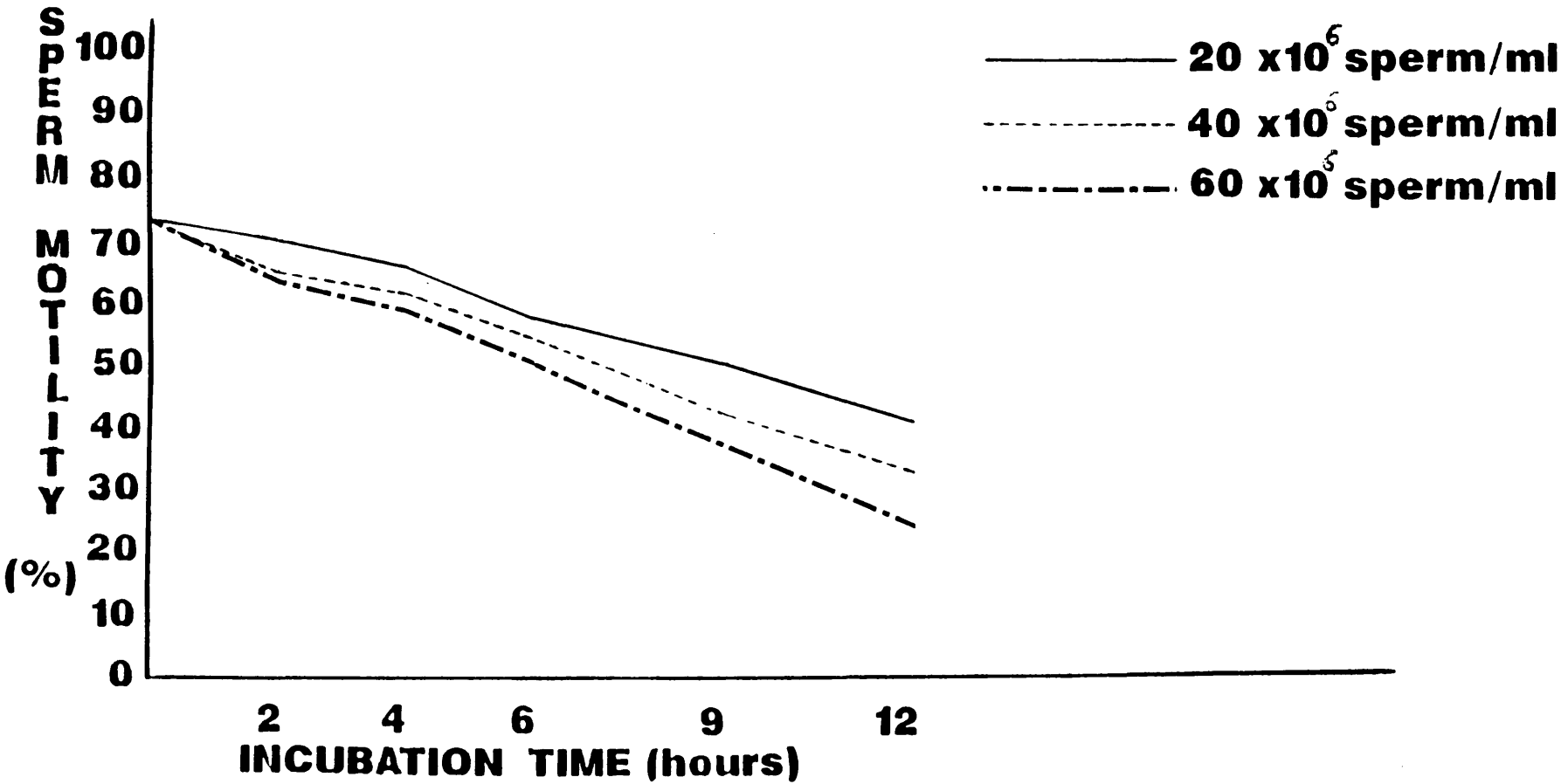

Figure 4: The reduction of the average sperm motility (%) at the sperm concentration 20, 40 and 60 ($x10^6$ sperm/ml) during incubation time (hours) (5% CO2, 95% air).

DISCUSSION

Sperm motility in CO_2 environment

Lactate, pyruvate, $NaHCO_3$, Hepes and glucose are nutritional sources for the spermatozoa, however a specific balance was necessary during these experiments to provide the best sperm energy metabolism, which occurs in the mitochondria as it does in most cells (Storey and Kayne, 1978, Darnell et al., 1986).

Many workers put forward the view that in the mitochondrial sheath of the spermatozoon both aerobic and anaerobic glycolysis take place (Peterson and Freund, 1973, Garbers et al., 1973, Storey and Kayne, 1977). The direct products of the aerobic glycolysis is firstly pyruvate and then CO_2 and water, whereas during anaerobic glycolysis, glucose is first metabolised to pyruvate and then to lactate or to ethanol (Lehninger, 1982, Stryer, 1988). Both aerobic and anaerobic glycolysis produce the ATP, which is necessary for maintaining sperm function and integrity (Garbers et al., 1973, Tash and Means,1983). Storey and Kayne (1977) reported that mammalian spermatozoa utilise both extracellular glucose and lactate aerobically as energy sources, while pyruvate interferes in the anaerobic-Krebs cycle for oxidation in the mitochondria. Meanwhile, there is disagreement among the researchers about the role of glucose in the presence of lactate and pyruvate. Some of them suggested that glucose provides the necessary energy balance for the spermatozoa and that it is utilised for the production of lactate (Hammerstedt, 1975, Hoskins, 1975). However, other investigators have found that the use of glucose as a sperm energy source is limited and that lactate and pyruvate are the main nutritional substrates, which support sperm viability (Bavister and Yanagimachi, 1977, Mita and Hall, 1982, Nakamura et al., 1984). Also Rogers and Yanagimachi (1975) observed that guinea pig spermatozoa undergo an acrosome reaction in vitro much more rapidly in culture systems containing lactate and pyruvate than in another one containing glucose as the sole energy source, while Susko-Parrish et al. (1985)

noticed the inhibitory effect of the high concentration of glucose on the induction of acrosome reaction to the bovine spermatozoa in vitro. In addition, glucose and other similar intermediates failed to support growth of the two-cell embryos (Whitten, 1957, Wales, 1987).

In the present study the role of glucose appeared to be of much lower significance for the spermatozoa than that of lactate. The results were in agreement with the suggestion that glucose is not an important nutritional factor when the spermatozoa are in a culture system. It is doubtful if glucose provided any energy, either during the pre-incubation or incubation sperm treatment. The culture media in this experiment were supplemented with various concentrations of lactate and pyruvate and none of them had glucose as the sole energy source. The factors which are involved in that partial or complete inhibition of glucose metabolism through the glycolysis process are unknown. Therefore, it would seem that these factors have to play an important role mainly during anaerobic glycolysis, which includes the metabolism of glucose firstly to pyruvate and then to lactate. Possibly glucose is unable to be metabolised anaerobically in vitro, so that there is no energy production-ATP for the spermatozoa. Although in the present experiment ATP was not measured, the suggestion is in agreement with the results of Mita and Hall (1982), who observed in rat spermatids in vitro that there was no extra production of ATP when the culture medium Phosphate Buffered Saline was supplemented with glucose. From the present experiment, it can be concluded that glucose is of rather a lower significance and perhaps completely unused nutritional source from the frozen-thawed bovine spermatozoa in vitro. Possibly glucose plays a significant role for the sperm metabolism immediately after ejaculation as other authors suggested (Hammerstedt, 1975, Hiipakkaand Hammerstedt, 1978) and may be a very important sperm nutritional factor during the movement of the spermatozoa through the female genital tract. However, the low concentration of glucose in the oviduct, about 1mM

(Hiipakka and Hammerstedt,1978) may be an indication of the low significance of glucose prior to fertilisation in vivo.

Bavister and Yanagimachi (1977), using hamster spermatozoa and different culture media, showed that pyruvate is necessary for the oxidative metabolism of the spermatozoa, whereas lactate should play an important role in maintaining a sufficient amount of pyruvate in the medium and/or in the sperm themselves. They suggested that lactate could play its supporting role in maintaining the level of pyruvate through a mechanism, during which an inhibiting reduction of pyruvate could effect the supporting action of lactate. Contrary to this, Nakamura et al.(1984) put forward the opposite view using rat spermatocytes. They observed that the conversion of pyruvate to lactate was greater than the reverse reaction.

In these experiments with bovine frozen-thawed spermatozoa, the role of pyruvate was not considered important during the pre-incubation sperm treatment, but it improved the sperm viability during CO_2 incubation. Although pyruvate and lactate are convertible and both of them are nutritional sources for the spermatozoa, it is unknown why the presence of high concentration of pyruvate in the TALP-medium 4 gave worse results for the sperm motility by comparison with the TALP-medium 6 during the pre-incubation treatment. Possibly high levels of pyruvate in the culture system may inhibit the production of ATP from lactate as happens with rat spermatids incubated in vitro. It was observed in rat spermatids that high concentrations of pyruvate (>1mM) inhibits the action of the enzyme lactate dehydrogenase, which is a necessary oxidizer of the exogenous lactate in the culture system (Mita and Hall,1982). Possibly the decreased ATP production from lactate caused by pyruvate is associated with decreased oxidation of lactate.

In the present study the observations suggest that energy production by bovine spermatozoa in vitro is most efficient when the level of lactate is high and the

concentration of pyruvate low at the pre-incubation treatment. However after incubation the sperm motility was better in culture medium 1, which was supplemented with high concentration of pyruvate. The sperm motility in medium 7, which was similar to the medium 1, except in that of pyruvate concentration, was slightly lower than that in medium 1. It can be concluded that pyruvate is a factor which provides the best energy source for the spermatozoa during incubation, however it cannot be considered as a crucial nutritional source.

Bavister and Yanagimachi (1977) in their experiments suggested that pyruvate provides the principal energy source for sperm metabolism. In the present study, lactate, Hepes and $NaHCO_3$ were considered the most important nutritional factors for sperm metabolism during the pre-incubation sperm treatment and only lactate for the incubation treatment in the presence of low level of glucose. Therefore the sperm nutritional requirements are higher during the swim-up, first and second centrifugations than those during incubation.

Effect of PH and Osmolarity on Sperm Motility in air incubator

Hepes and $NaHCO_3$, apart from being nutritional sources, are also buffers having the ability to protect the culture systems from any significant changes of pH. They allowed pH to be maintained in a CO_2 incubator at a level comparable to that before incubation (7.4), however they failed to achieve this when incubation took place in the air incubator (8.1 after approximately 12 hours of incubation). However, other authors have reported that the motility of epididymal or ejaculated spermatozoa is not strongly affected by pH within the range 5.5 to 8.0 for short-term incubation (Acott and Carr, 1984).

In the present study the increase of pH from 7.4 to 8.1 in air incubator was considered as one of the most important factors contributing to the rapid death of the spermatozoa in the air incubator. The present results were in agreement with those of Peterson and Freund

(1973), who incubated human spermatozoa for 60-75 minutes in media of different pH. A reduction of ATP production was observed after the completion of incubation in their media with pH 7.8 and 8.2. It can be presumed that the ATP reduction after 6, 9 or 12 hours of incubation has to be much higher by comparison with the results of Peterson and Freund. Possibly the alkaline environment may have an inhibitory effect on sperm metabolic activity thereby decreasing ATP production from exogenous substrates. Apart from the harmful effect of alkalinisation on the spermatozoa, there is similar evidence concerning maturing oocytes. Bagger et al. (1987) noticed that alkalinisation beyond pH 7.45 results in an increase of oocyte degeneration and a reduction of germinal vesicle breakdown and polar body formation. Conclusively maintaining the pH at 7.4 appears to be an imperative prerequisite for the spermatozoa during long term incubation.

Apart from pH, evaporation of the medium is considered to be another factor responsible for the variation in results concerning the sperm motility of the samples during air incubation.This evaporation induces an increase in the osmolarity of the media, which finally cause the death of the spermatozoa. This suggestion is in agreement with the results of Miller and Hunter (1986), who observed in bovine ejaculate spermatozoa that the motility was rapidly decreased when the osmolarity of the medium was higher than 295 mmOsmol/Kg. In the present experiments, evaporation influenced to a greater degree the sperm motility of the paraffin-uncovered wells compared to the uncovered-tubes. Possible due to the different ratios of volume:surface area between the tubes and the wells, the degree of evaporation and subsequently of osmolarity differs.

Concentration and Sperm Motility

The viability of the spermatozoa is affected by sperm concentrations higher than $20x10^6$ sperm/ml. The present results are in agreement with those of Dentch et

al.(1985), who used human spermatozoa diluted in seminal plasma or media. They observed in both cases that the sperm oxygen consumption is increased when the sperm concentration is lower than $25x10^6$ sperm/ml. These authors also noticed that above this concentration there was an inverse relationship between respiration and concentration. However the factors which effect these concentration-dependent changes in sperm oxygen consumption are unknown. Some workers have suggested that these factors may be respiratory inhibitors, which act on the membranes of the spermatozoa (Eliasson, 1971, Kelly, 1977, Deutch et al., 1985). Possibly these factors may be by-products produced during the sperm metabolism. Therefore sperm viability may be affected by high levels of these inhibitors caused by a high sperm concentration in the medium.

Another hypothesis that could be proposed is that the supplemented nutritional sources in the medium are consumed faster when the sperm concentration is higher than $20x10^6$ sperm/ml. However if this is correct then all the spermatozoa should die at the same time. Probably there is a different metabolic activity among spermatozoa and the most vigorous spermatozoa are able to survive for a longer time using low amount of energy, while the weak spermatozoa die very soon after initiation of the reduction of the energy sources. In addition, it has been put forward by Dresdner and Katz (1981) that there is a hydrodynamic interaction among mammalian spermatozoa when they are in any kind of diluent, even in the seminal plasma. They observed, and presented with mathematic means, that there are great variations of the manner of sperm movement at different sperm concentrations. Dentch et al.(1985) later suggested that there may be a <<hydrodynamic communication>> among spermatozoa, which becomes weaker when the sperm concentration is low. However there is no evidence how this hydrodynamic process can influence the sperm respiration and viability. Possibly at low sperm concentrations a specific hydrodynamic movement may allow maximal benefit

of the nutritional sources by the spermatozoa. Obviously the interaction between concentration and viability of the bovine frozen-thawed spermatozoa needs further investigation, while the influence of the individual bull on the results has to be investigated further.

CONCLUSION

The long term (10-14 hours) motility of the spermatozoa is one of the major factors for sustaining the fertilisation of the oocytes in vitro. In the present study, it was proved that the sperm motility-viability was achieved at a higher rate when the spermatozoa were treated, during the preincubation treatment, in the TALP-medium 6, which was supplemented with high levels of lactate, $NaHCO_3$ and Hepes. However TALP-medium 1, which had lactate as the principal nutritional source, was shown to sustain the sperm motility better than the other media.

The concentration of the spermatozoa in the medium is another crucial factor, which can affect the success of the In Vitro Fertilisation. In this work, a sperm concentration of $20x10^6$ sperm/ml or less provided the best environment for the spermatozoa resulting in the best sperm progressive motility.

APPENDIX

Reagents

NaCl (SIGMA Co., UK), KCl (BDH Co.,UK), $MgCl{\cdot}6H_2O$ (BDH Co.), $NaHCO_3$ (FORMACHEM Co., UK), Sodium Lactate (SIGMA Co.), Sodium Pyruvate (SIGMA Co.), D-Glucose (BDH Co.), Hepes (SIGMA Co.) and Bovine Serum Albumin-BSA, Fraction V (SIGMA Co.).

Preparation of the modified Tyrode's-TALP media

The ingredients were weighed on a balance in 35x10mm plastic Petri dishes(NUNC Co., Denmark). They were then placed into measuring beakers and de-ionised water was added. After stirring by a spatula, the pH of the mixture was checked using a standard pH-meter and adjusted to 7.35 to 7.4. The osmolarity of the media was 280-295 mmOsmol/Kg. The culture media were then filtered with a 0.22-Millipore filter under a sterilised hood into sterilised plastic bottles (NUNC Co.,Denmark) and stored in the fridge. The TALP-media were freshly prepared after every 20 days to avoid any chance of contamination. The required amount of the media was taken out under a sterilised hood from the stock, while the rest was again restored at 4^0C.

MODIFIED TYRODE'S MEDIA-TALP

Ingredients	1	2	3	4	5	6	7	8	9	10
	mM	mM	mM	mM	mM	mM	mM	mM	mM	mM
NaCl	109.61	109.61	109.61	109.61	109.61	109.61	109.61	109.61	109.61	109.61
KCl	2.95	2.95	2.95	2.95	2.95	2.95	2.95	2.95	2.95	2.95
$MgCl_2.6H_2O$	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40
Glucose	4.99	4.99	4.99	4.99	4.99	4.99	4.99	4.99	13.87	8.90
Sodium Pyruvate	1.00	1.00	1.00	1.00	0.20	0.20	0.20	0.20	1.00	1.00
Sodium Lactate	21.59	21.59	21.59	21.59	21.59	21.59	21.59	21.59	10.00	14.00
$NaHCO_3$	12.09	24.99	12.09	24.99	24.99	24.99	12.09	12.09	24.99	24.99
Hepes	-	-	10.00	10.00	-	10.00	-	10.00	10.00	10.00
BSA	6 mg/ml	6 mg/ml	6 mg/ml	6 mg/ml	6 mg/ml	6 mg/ml	6 mg/ml	6 mg/ml	6 mg/ml	6 mg/ml

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