Impact of temperature and incubation in 6%CO_2 in air on testicular sperm motility. Part 1: TESA sperm

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Abstract

The objective of this investigation is to ascertain the usefulness of incubating overnight TESA spermatozoa (TS) in HEPES-buffered flushing medium (FM) at 37°C in the gaseous phase (6% CO_2 in air). After maceration in FM, the testicular tissue was divided into four equal portions for individual treatments (Tx). The suspension was held overnight at either room temperature (RT) or 37°C with or without incubation gas (6% CO_2 in air) as follows: (i) without CO_2@RT; (ii) without CO_2@37°C; (iii) with CO_2@RT; with CO_2@37°C. The tubes are capped tight with the incubation gases sealed within the tube, and kept at RT or 37°C (in the incubator) overnight. Statistical analyses performed were Chi-square, Pearson's correlation studies, paired-T test, and two-by-two tables. Significant proportion of TS (21.4%) became motile after the Tx's indicating both physiological temperature (37°C) and physiological pH (attained by overnight incubation in 6% CO_2 in air) induced motility in and retained the viability of the TS. The differences between Tx's were statistically highly significant ($p<0.001$) indicating the critical impact of both physiological temperature and physiological pH on TS viability and motility induction. There was significant strong interaction ($p=0.0000$) between Tx's and, positive correlations between the Tx's ($p<0.001$; highly significant). Physiologic temperature and pH appear critical for retaining the viability of and for initiating motility in TS. It is safer to induce motility with physiological temperature and pH than with potentially toxic pentoxyfylline or theophylline. When exposed to ambient temperature and air, the HEPES medium drifted toward the alkaline phase, making it less dependable for sustaining physiological pH levels between 7.3 and 7.4 for prolonged periods of time in absence CO_2 incubation gas. In conclusion, physiological pH and temperature is critical to maintain motility/viability of TS. HEPES medium must be equilibrated in 6% CO_2 in air >60mins or overnight to maintain pH at physiological levels.

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Introduction

Reports in the Embryo Mail (EM) and a previous report noted incubation of testicular sperm (TS) at 37°C initiated motility (Ali, 2006). Sperm motility is critical for selecting viable sperm during ICSI. Pentoxyfylline (PTX; Aparicio et al., 1980) and theofylline TFX; Dougherty et al.,1976) are used for sperm motility enhancement but are toxic (Ali,1999; Azimi 2022).

It is not clear whether incubation of TS requires incubation in CO_2 gas (for pH) and, 37°C is essential since spermatogenesis and sperm maturation in vivo occurs below 37°C.

The objectives of this investigations to determine the need and importance of incubation at 37°C in gaseous phase (6%CO_2 in air) are reported herein.

Materials and methods

TS were obtained during diagnostic TESA, macerated using two needles of 1ml syringe in Flushing medium (FM) and apportioned equally for individual treatments (Tx) shown in Tables 1&2. Origio FM buffered with HEPES was used. Ongoing routine quality management for pH of media using the OCTAX Log & Guard monitoring system (MTG Germany) in Labotect C200 incubator set at 6% CO_2 in air noted that physiological pH was rapidly attained within 1 to 2 hours for culture medium.

However, during TESA biopsy tissue preparation in the HEPES-buffered medium exposed to air turned pink to a pH in excess of 8.7 to >9 when checked, indicating need for pH adjustment. In this study the medium containing processed TESA tissue was exposed to incubator gases as follows: The room temperature (RT) group was gassed maximum 60 mins in tube, sealed airtight with its cap with the gases sealed inside the tube and placed at RT that retained physiological pH. The 37^{o}C group was left inside incubator overnight exposed at gaseous phase. This treatment retained physiological pH when checked after overnight incubation.

Statistical analyses used paired-T test, Pearson's correlation studies, Chi-square and 2 by 2 tables.

Results

Differences between Tx's were statistically highly significant ($p<0.001$) indicating the critical impact of both temperature (37^{o}C) and pH. There was significant strong interaction ($p=0.0000$) between Tx's and, positive correlations between Tx significant ($p<0.001$). Both physiological pH (7-3-7.4) CO_2 and temperature (37^{o}C) are essential to initiate motility in TS.

Table 1B shows the frequency of motility for each treatment. The proportion of frequency distribution of 0% sperm motility within the various treatments groups were 51.1% (24/53) in the 0hr control group but was 29.8% (14/53; $p=0.043$) after overnight incubation at room temperature, and at 37^{o}C after incubation or without gas mixture but in the group incubated at room temperature without incubation gas it was higher at 61.7% (29/53; $p=0.331$).

The % Frequency of distribution of motile sperm at 0hr (control) was 27.7% (13/53), for specimen incubated at room temperature without incubation gases 29.8% ($p>0.05$; 14/53), for specimen incubated at room temperature with incubation gases was 8.5% ($p=0.017$; 4/53), for specimen incubated at 37^{o}C without incubation gas mixture was 45% ($p=0.096$; 21/53), and for specimen incubated at 37^{o}C with incubation gas was 6.4 ($p=0.007$; 3/53).

Generally, for higher frequencies of %motility of 21 to 60%, treatments at room temperature and 37^{o}C with gas incubation registered higher % frequencies of motility respectively ($p=0.001$; 14/39 and $p=0.001$; 23/53) than the control group (1/53).

The highest %frequency motility of 31 to 60% was noted for the treatment with specimen incubated at 37^{o}C with gas incubation ($p=0.000$; 17/53) compared to the 0hr (control) group that had no cases (0/53) with these % frequencies of sperm %motility.

Discussion

Importantly only HEPES buffered medium containing low levels (4.0mM) of sodium bicarbonate (SB) shows pH stability at around pH7.5 (Walker, 1989) but commercial IVF HEPES buffered medium supposedly contains more (~15 to 25mM) so as to be in the vicinity or to mimic physiological levels of SB. Higher levels of SB above the level of 4.0mM (Walker, 1989) inevitably result in a pH drift towards the alkaline phase (Ali 1992; personal communication) with time while the TESA biopsy is being macerated and prepared for sperm extraction either for biopsy or therapeutic ICSI with testicular sperm. The unstable pH condition conferred by HEPES buffered IVF medium is appears unreliable for maintaining the physiological pH of 7.3-7.4 for extended periods of time. This is especially true when handling

TESA biopsies exposed to temperature and ambient air in the absence of CO_2 gas mixture.

Table 1A: The impact of incubation with and without for a fixed duration of 20 minutes of 6% carbon dioxide at room temperature and 37°C on the motility of TESA sperm

Description	% 0hr sperm motility (Control)	% sperm motility (≥18hrs Incubated @Room Temp)		% sperm motility (≥18hrs incubated @37°C)	
		Without CO_2	With 6% CO_2	Without CO_2	With 6% CO_2
Mean	5.3	3.2	14.3	7.4	21.4
±1SD	7	5.2	11.2	6.7	17.9
±1SE	1	0.8	1.6	0.98	2.6
Range (n=)	0-25 (47)	0-20 (47)	0-34 (47)	0-25 (47)	0-58 (47)
p Value *	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Correlation (p Value)**	<0.002	<0.002	<0.002	<0.002	<0.002

*P value between all treatments are highly significantly different ;
**Highly significant positive correlation was noted between all treatments of temperature & incubation gas (The r values are shown in the key to Table 1A below)

Key to Table 1A Correlations (Pearson's; with correlation "r" and "p" values)

Description	Without CO_2 @RT	Without CO_2 @37°C	With CO_2 @37°C	With CO_2 @RT
Without CO2@37C;r =	0.8386			
p-VALUE	0.0000			
With CO2@37C ; r=	0.4430	0.7239		
p-VALUE	0.0018	0.0000		
With CO2@RT; r=	0.5455	0.8257	0.9545	
p-VALUE	0.0001	0.0000	0.0000	
0hrs Control; r=	0.9177	0.9089	0.464	0.593
p-VALUE	0.0000	0.0000	0.001	0.0000

Table 1B: The frequency distribution of TESA sperm by per cent motility

Description (Tx)	% 0hr sperm motility (Control)				% sperm motility ≥18-24hrs incubation; @Room Temp								% sperm motility ≥18-24hrs incubation;@37oC							
					Incubation without CO2				Incubation with CO2				Incubation without CO2				Incubation with CO2			
% Motility	Fre	%	C Fre	C %	Fre	%	C Fre	C %	Fre	%	C Fre	C %	Fre	%	C Fre	C %	Fre	%	C Fre	C %
0	24	51.1	24	51.1	29	61.7	29	61.7	14	29.8	14	29.8	14	29.8	14	29.8	14	29.8	14	29.8
1-10	13	27.7	37	78.7	14	29.8	43	91.5	4	8.5	18	38.3	21	45	35	74.5	3	6.4	17	36.2
11-20	9	19.1	46	97.9	4	8.5	47	100	15	31.9	33	70.2	10	21	46	97.9	7	14.9	24	51.1
21-30	1	2.1	47	100	-	-	-	-	11	23.4	44	93.6	2	4.2	47	100	6	12.8	30	63.8
31-40	-	-	-	-	-	-	-	-	3	6.4	47	100	-	-	-	-	11	23.4	41	87.2
41-50	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4	8.5	45	95.7
51-60	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	4.2	47	100

Table 2: Impact of incubation of fixed durations of carbon dioxide incubation gas mixture at room temperature and 37°C on TESA sperm

Description (Tx)	% 0hr sperm motility (Control)	Testicular specimen exposed to 6% CO_2 gas and incubated at room temperature or 37°C					
		Exposed for 20 mins		Exposed for 30		Exposed for 60 mins	
	Room Temp	Room Temp	37°C	Room Temp	37°C	Room Temp	37°C
Mean	5.2	8.4	14.7	10.5	12.5	10.2	18.7
±1SD	7	6.9	11.8	8.6	9.8	7.9	15.3
±1SE	1	1	1.7	1.3	1.4	1.2	2.2
Range (n)	0-25	0-25	0-37	0-28	0-30	0-25	0-50
P value *	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001
Correlation**	P<0.0005	P<0.0005	P<0.0005	P<0.0005	P<0.0005	P<0.0005	P<0.0005
Median	0	10	15	10	15	10	20

* The p values between all treatments including within individual groups were highly significant (p=0.0000)
**Correlation values r are high and highly significant (p<0.0001) between all treatments in Key to Table 2

Key: Correlation (Pearson's) for Table 2

Description		$CO_2$20min37°C	$CO_2$30min37°C	$CO_2$60min37°C	0hrControl	$CO_2$20minRT	$CO_2$30minRT
$CO_2$30min37°C,	r=	0.9696					
p-VALUE		0.0000					
$CO_2$60min37°C,	r=	0.9807	0.9560				
p-VALUE		0.0000	0.0000				
0hrControl ,	r=	0.5784	0.5914	0.4985			
p-VALUE		0.0000	0.0000	0.0004			
$CO_2$20minRT,	r=	0.9210	0.9278	0.8900	0.7915		
p-VALUE		0.0000	0.0000	0.0000	0.0000		
$CO_2$30minRT,	r=	0.9595	0.9498	0.9351	0.7010	0.9699	
p-VALUE		0.0000	0.0000	0.0000	0.0000	0.0000	
$CO_2$60minRT,	r=	0.9602	0.9710	0.9338	0.6631	0.9546	0.9710
p-VALUE		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000

The practice of using HEPES-buffered media without pH indicator (phenol red) makes pH control even more difficult during testicular tissue preparation which could be harmful to sperm.

Significant proportion of TS became motile after Tx indicating the critical role of both physiological pH and temperature on sperm viability and for inducing motility in testicular sperm. Our findings indicate overnight exposure physiological pH alone appears effective while physiological temperature further enhances sperm motility

Both Pentoxyfylline (PTX; Aparicio et al., 1980) and theofylline TFX; Dougherty et al.,1976) are used for sperm motility enhancement. However, PTX was reported to be embryotoxic (Ali,1999) whereas therapeutic administration of TFX in the human induced sperm DNA fragmentation (Azimi 2022). It is therefore assumed safer to apply the present findings of physiological pH and temperature as an inducer of motility in the testicular sperm. Both these factors are nontoxic to sperm.

Indeed, when the testicular sperm suspension was incubated overnight at room temperature under physiological pH the proportion of non-motile sperm increased to a level higher than the control, or the mean %motility dropped from the control 5.3% to 3.2% when incubated without physiological pH and temperature but increased to 21.4% when incubated at physiological pH and temperature.

All parameters investigated show a similar trend. This is a clear indication of the harm that can occur to the testicular sperm if due consideration is not given to the pH and temperature of the suspension, in particular pH. It is also clear that the testicular-tissue suspension must be incubated at 6% carbon dioxide in air for more than 60 minutes or overnight for best outcome of high sperm viability and motility.

The practice of HEPES-buffered media without pH indicator makes pH control even more difficult which could be harmful to sperm (and embryo).

In conclusion, handling or incubating testicular specimen without physiological pH is detrimental to TS motility, the incubation of testicular preparations at both physiological pH and temperature enhances TS viability maximally. HEPES media must be equilibrated in 6%CO_2 gaseous incubation mixture for over 60 minutes or overnight to retain pH. This is probably true for embryos/oocytes.

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