

Impact of temperature and incubation in 6%CO₂ in air on testicular sperm motility. Part 2: microTESA sperm

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Abstract

The objective of this investigation is to ascertain the usefulness of incubating overnight microTESA spermatozoa (mTS) in HEPES-buffered flushing medium (FM) at 37°C exposed to incubation gases (6% CO₂ in air). After maceration in FM, the testicular tissue was divided into four equal portions for individual treatments (Tx). The suspension is held overnight at either room temperature (RT) or 37°C with or without incubation gas (6% CO₂ in air) as follows: (i) without CO₂@RT; (ii) without CO₂@37°C; (iii) with CO₂@RT; with CO₂@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 mTS (11.4% vs 3.3%, $p < 0.0001$) became motile after the Tx's indicating both physiological pH (attained by ≥ 60 mins incubation in 6%CO₂ in air) and temperature (37°C) induced motility in and retained the viability of the mTS. The differences between Tx's were statistically highly significant ($p < 0.0001$) indicating the critical impact of both physiological pH and temperature on mTS viability and motility induction. There was significant strong interaction ($p = 0.0000$) between Tx's and, positive correlations between the Tx's ($p < 0.001$). Physiological temperature and pH appear critical (latter more impactful than the former) for retaining the viability of and for initiating motility in mTS. It is safer to induce motility with physiological temperature and pH than with potentially toxic pentoxifylline 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, particularly in absence of CO₂ incubation gas. In conclusion, physiological pH and 37°C is critical to maintain viability of mTS. HEPES medium must be equilibrated in 6%CO₂ in air for >60 mins or overnight to retain physiological pH.

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Introduction

This report on microTESA sperm (mTS) is very similar to that of TESA sperm by the authors (Sharif Hussain et al., 2025a) published in this journal previously. A previous report (Ali, 2006) and that of the authors (Sharif Hussain et al., 2025a) noted incubation of TESA sperm (mTS) at 37°C and under conditions that conferred physiological pH to the HEPES medium initiated motility which was in an order that was statistically significant. Sperm motility is critical

for selecting viable sperm during ICSI. The use of pentoxifylline (PTX; Aparicio et al., 1980) and theophylline TFX; Dougherty et al., 1976) to initiate sperm motility in non-motile spermatozoa, and in particular, immotile testicular sperm is thought to be hazardous as these agents are toxic (Ali, 1999; Azimi 2022).

The authors have demonstrated that mTS requires incubation in 6% CO₂ gas in air (for retaining physiological pH) and, under

physiological temperature of 37°C in vitro allowed initiation of sperm maturation leading to motility in a significant proportion of testicular sperm. The previous findings of the authors on TESA sperm (TS) noted that impact of physiological pH and temperature appear essential for initiation of motility contrary to the assumption that room temperature would be sufficient, since spermatogenesis and sperm maturation in vivo occurs below 37°C.

The authors repeated their TS study with mTS to determine similarities and or differences between the TS and mTS when subjected to the same in vitro culture conditions (Sharif Hussain et al., 2025a).

Materials and methods

mTS were obtained during diagnostic microTESA (mTESA) and treated in the same manner as described by the authors in their previous study on TS. Briefly the biopsied tissue was macerated using two needles of 1ml syringe in Flushing medium (FM) and apportioned equally for individual treatments (Tx) shown in Tables 1 & 2. HEPES buffered FM (Origio, Denmark) 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% CO₂ in air noted that physiological pH was rapidly attained within 1 to 2 hours for culture medium.

In this study, as in the previous investigation the medium containing processed mTESA tissue was exposed to incubator gases. The suspension is held overnight at either room temperature (RT) or 37°C with or without incubation gas (6% CO₂ in air) as follows: (i) without CO₂@RT; (ii) without CO₂@37°C; (iii) with CO₂@RT; (iv) with CO₂@37°C. The room temperature (RT) group was gassed ≥60 mins in tube, sealed airtight with its cap with its incubation gases sealed inside the tube and placed at RT. This enabled the retention of physiological pH in the tissue suspension in the tube. The 37°C group was left inside incubator overnight exposed at gaseous phase. This treatment retained physiological pH when checked after overnight incubation.

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

Results

The differences between all Tx's (except between control versus RT20; P>0.05) were statistically highly significant (p<0.001) indicating the critical impact of both temperature (37°C) and pH (conferred by incubation gases). There was significant strong interaction (p=0.0000) between Tx's and, positive correlations between Tx significant (p<0.001). Both physiological pH (7-7.4) obtained following incubation in CO₂ gas and temperature (37°C) are essential to initiate motility in TS. The impact of pH appears more pronounced and critical than temperature.

As shown in the authors' previous study on TS (Sharif Hussain et al., 2025a), physiological pH and temperature (37°C) are essential to initiate motility in mTS. Significant proportion of mTS (10.3 to 11.4%; Tables 1A and 2) became motile after Tx. Table 1B shows the %frequency distribution for motility for each treatment. The %frequency of immotile sperm in the fresh untreated (control at zero hour) specimen was about 62.3% (33/53), however the remaining specimen (37.7%; 20/53) had motile sperms. The %frequency motility distribution of specimen incubated at room temperature overnight without incubation in the incubation gas mixture, were immotile at levels marginally lower (56.6%; 30/53) compared to the control level (62.3%; 33/53); About 43.4% (23/53) of the specimen demonstrated marginally improved motility overnight compared to control values (37.7%; 20/53). Lack of 6%CO₂ gas and physiological temperature appear to be detrimental to sperm motility.

However, if the specimen was incubated in the gas mixture at the room temperature overnight, the %frequency distribution of motile sperm increased. About 60.4% (32/53) of the specimen demonstrated motility. Only 21 of 53 (39.6%) of the specimen did not show motility. Motility was marginally enhanced above the control levels (37.7%; 20/53). When the specimen was incubated overnight at 37°C but without the incubation gas mixture, the %frequency of motile forms was 58.5% (31/53) while 41.5% (21/53) did not show motility. If the specimen was incubated

overnight at 37°C with the incubation gas mixture the %frequency of motile forms increased to 62.4% (33/53) while 39.6% (21/53) did not show motility.

Discussion

Only HEPES buffered medium containing low levels (4.0mM) of sodium bicarbonate (SB) shows pH stability at around pH7.5 (Walker, 1989) but some commercial IVF HEPES buffered medium supposedly contains more (~15 - 25mM) of SB so as to be in the vicinity or to mimic physiological levels of SB.

In our previous communication (Sharif Hussain et al., 2025a) higher levels of SB above the level of 4.0mM (Walker, 1989) inevitably

resulted in a pH drift towards the alkaline phase with time while the mTESA biopsy is being macerated exposed to ambient environmental conditions and prepared for sperm extraction either for diagnostic purposes or therapeutic ICSI with testicular sperm.

The unstable pH provided by HEPES buffered IVF medium is unreliable for maintaining the physiological pH of 7.3-7.4 for extended periods of time. This is especially true when handling biopsies exposed to ambient air temperature in the absence of CO₂ gas mixture. Handling of specimen during processing and short-term storage must take into consideration the need to control pH of the specimen suspension. These two measures will prevent damage and enhance motility of mTS.

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	3.3	4.0	8.0	6.3	11.4
±1SD	4.9	5.4	7.5	6.6	10.0
±1SE	0.6	0.7	1.0	0.9	1.4
Range (n=)	0-17 (53)	0-20 (53)	0-25 (53)	0-24 (53)	0-28 (53)
p Value *	<0.009 ⁺	<0.0001	<0.0001	<0.0001	<0.0001
p value of “r” ^{***}	<0.002	<0.002	<0.002	<0.002	<0.002

*P values between all treatments are highly significantly different @ p<0.0001 and p<0.009

⁺Control vs Without CO₂@RT. ^{***}Highly significant correlation noted between all treatments (see Table 1A)

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

Description	Control	Without CO ₂ @RT	Without CO ₂ @37°C	With CO ₂ @RT
Without CO ₂ @RT; r =	0.9498			
p-VALUE	0.0000			
Without CO ₂ @37°C ; r=	0.8674	0.9173		
p-VALUE	0.0000	0.0000		
With CO ₂ @RT; r=	0.7956	0.8366	0.9342	
p-VALUE	0.0000	0.0000	0.0000	
With CO ₂ @37°C; r=	0.6627	0.7081	0.8697	0.9452
p-VALUE	0.0000	0.0000	0.0000	0.0000

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

Description /Treatment	% 0hr sperm motility (Control)				% sperm motility ≥18-24hrs incubation; @Room Temp								% sperm motility ≥18-24hrs incubation; @37°C							
					Incubation without CO ₂				Incubation with CO ₂				Incubation without CO ₂				Incubation with CO ₂			
% Motility	Fre	%	C Fre	C %	Fre	%	C Fre	C %	Fre	%	C Fre	C %	Fre	%	C Fre	C %	Fre	%	C Fre	C %
0	33	62.3	24	62.3	30	56.6	30	56.6	21	39.6	21	39.6	22	41.5	22	41.5	21	39.6	21	39.6
1-10	14	26.4	47	88.7	18	34.0	48	90.6	14	26.4	35	66.0	20	37.7	42	79.2	3	3.8	23	43.4
11-20	6	11.3	53	100	5	9.4	53	100	16	30.2	49	92.5	10	18.9	52	98.1	20	37.8	43	81.1
21-30	0	0	0	0	0	0	0	0	2	3.8	53	100	1	1.9	53	100	10	18.8	53	100
31-40	-	-	-	-	-	-	-	-	0	0	0	0	0	0	0	0	0	0	0	0
41-50	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
51-60	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

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	37°C	Room Temp	37°C	Room Temp	37°C
Mean	3.3	4.1	5.6	5.8	8.4	7.0	10.3
±1SD	4.9	5.3	6.2	5.8	7.6	6.6	9.0
±1SE	0.7	0.7	0.8	0.8	1.0	0.9	1.2
Range (n)	0-17	0-18	0-20	0-20	0-23	0-22	0-25
P value *	P>0.05vs RT20	P<0.0005	P<0.0005	P<0.0005	P<0.0005	P<0.0005	P<0.0005
Correlation**	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001
Median	0	0	10	5	10	8	12

* The p values are significantly different between all treatments (p<0.0005) except between Control vs CO₂ 20mins@RT (p>0.05)

**Correlation values r is high and highly significant (p<0.0000) between all treatments in Key to Table 2

Key: Correlation (Pearson's) for Table 2

Description	0hrControl	CO ₂ 20minRT	CO ₂ 30minRT	CO ₂ 60minRT	CO ₂ 20min37C	CO ₂ 30min37C
CO ₂ 30min37C, r=	0.9665					
p-VALUE	0.0000					
CO ₂ 60min37C, r=	0.8944	0.9350				
p-VALUE	0.0000	0.0000				
0hrControl, r=	0.8208	0.8846	0.9758			
p-VALUE	0.0000	0.0000	0.0000			
CO ₂ 20minRT, r=	0.8867	0.9417	0.9386	0.9187		
p-VALUE	0.0000	0.0000	0.0000	0.0000		
CO ₂ 30minRT, r=	0.7542	0.8244	0.9435	0.9698	0.9157	
p-VALUE	0.0000	0.0000	0.0000	0.0000	0.0000	
CO ₂ 60minRT, r=	0.6658	0.7477	0.8957	0.9437	0.8555	0.9838
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 monitoring pH even more difficult which could be detrimental for sperm viability.

Significant proportion of mTS became motile after Tx indicating the critical role of both physiological pH and temperature on sperm viability and for inducing motility in testicular sperm which could impact testicular sperm selection for ICSI positively.

Of considerable importance is that both pentoxifylline (PTX; Aparicio et al., 1980) and theophylline TFX; Dougherty et al., 1976) are used for sperm motility enhancement. However, pentoxifylline was reported to be embryotoxic (Ali, 1999) whereas therapeutic administration of theophylline 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 physiological pH and temperature, the %frequency distribution of motile sperm increased to 61.7% which was higher than the control (37.7%). The mean % motility increased significantly when incubated at physiological pH and temperature compared to the untreated control (3.3 vs 11.4; $p < 0.0001$).

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 the macerated tissue suspension, and including physiological temperature for enhanced motility. It is also clear that the testicular-tissue suspension must be incubated at 6% carbon dioxide in air for ≥ 60 minutes or overnight for best outcome of high sperm viability and motility.

In conclusion, physiological temperature without physiological pH is detrimental to mTS motility and to embryos and oocytes. HEPES media must be equilibrated in 6%CO₂ gaseous incubation mixture >60mins or overnight to retain pH. This is probably true for embryos/oocytes.

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