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ORIGINAL ARTICLE ***Andrology***

Sex chromosome-dependent differential viability of human spermatozoa during prolonged incubation

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STUDY QUESTION: Are there significant differences in the ability of X chromosome-bearing (X) spermatozoa and Y chromosome-bearing (Y) spermatozoa to survive incubation under stressful conditions?

SUMMARY ANSWER: Y spermatozoa are more vulnerable to stress than their X counterparts depending on culture period and temperature, and show higher expression of apoptotic proteins.

WHAT IS KNOWN ALREADY: The primary sex ratio is determined by there being an equal number of spermatozoa carrying X and Y chromosomes. This balance can be skewed by exposure to stressful environmental conditions such as changes in pH, pollutants or endocrine disruptors. However, less is known about the ability of sperm carrying either sex chromosome to withstand environmental stress.

STUDY DESIGN, SIZE, DURATION: The difference in survival between X and Y spermatozoa was evaluated by measuring motility, viability and Y:X chromosome ratio during incubation for 5 days, at three temperatures (4, 22 and 37°C), and three pH conditions (6.5, 7.5 and 8.5). To identify the critical factors that determine the survival of X and Y bearing spermatozoa, we analysed the expression levels of apoptosis-related proteins (Bcl, Bax and Caspase-3), as well as the extent of DNA damage under a subset of conditions.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Semen samples were obtained by masturbation from normozoospermic donors after 3 days of sexual abstinence. Four samples with >60% motility from different donors were mixed to obtain sufficient semen and eliminate sampling-related bias. Data are presented as mean ± SD of three independent experiments. Mean age of donors was 28.7 ± 3.2 years.

MAIN RESULTS AND THE ROLE OF CHANCE: In total, 58 489 spermatozoa were scored. The viability of Y spermatozoa was lower after exposure to different temperatures and culture periods than that of X spermatozoa ($P < 0.05$). Increased expression of apoptotic proteins in live Y spermatozoa was observed, despite the addition of tocopherol to the culture medium ($P < 0.05$).

LIMITATIONS, REASONS FOR CAUTION: Spermatozoa were cultured *in vitro* during the treatment period. It is difficult to extrapolate the observed lifespan differences to spermatozoa survival *in vivo*. The experiments were replicated only three times.

WIDER IMPLICATIONS OF THE FINDINGS: The prolonged survival of X spermatozoa under stressful conditions might lead to shifts in the ratio of male-to-female births.

STUDY FUNDING/COMPETING INTEREST(S): This study was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (MEST) (no. NRF-2014R1A2A2A01002706). The authors declare no competing financial interests.

Key words: sperm longevity / sperm sex chromosome ratio / sperm apoptosis / oxidative stress / antioxidant

Introduction

The global male-to-female ratio of humans at birth is currently estimated at 107 boys to 100 girls (Kvist et al., 2012). The sex ratio can deviate from the expected ratio of 1:1 based on factors, such as length of inter-pregnancy interval (James, 1996), conception on different days of the menstrual cycle (Harlap, 1979), birth order (Biggar et al., 1999) and exposure to environmental toxins (Mocarelli et al., 2000). In mammals, the distortion in the sex ratio at birth can also result from a disproportionate loss of embryos after fertilization, increased male/female ratio of foetal deaths (Mizuno, 2000) or alterations in the sex chromosome ratio of spermatozoa with fertilization capacity (Diasio and Glass, 1971). Although differences between X and Y spermatozoa (i.e. head size, motile speed, DNA content and pH susceptibility) can affect the offspring sex ratio (Gledhill, 1988), the exact physiological causes of changes in the ratio of spermatozoa carrying X or Y chromosomes in response to a wide range of environmental factors are still unknown.

Spermatozoa are motile vehicles that can contain either a X or a Y chromosome, which determine the gender of the fertilized oocyte (MacLaughlin and Donahoe, 2004). Although in theory, the ratio of X to Y bearing spermatozoa in semen is 1:1, this ratio can be altered upon exposure to certain stressors, such as pH fluctuations or environmental toxicants (Diasio and Glass, 1971). Notably, spermatozoa may have to survive for approximately a week in the male and female reproductive tracts because insemination and ovulation are often not synchronized (Suarez and Pacey, 2006). During this prolonged period, spermatozoa suffer from a time-dependent loss of motility due to physico-chemical stressors, such as changes in temperature and pH after ejaculation (Aitken and Curry, 2011).

Sperm capacitation and time-dependent loss in motility are associated with spontaneous apoptosis-related events, including phosphatidylserine exteriorization, caspase activation and DNA damage (Amaral et al., 2013). During capacitation, spermatozoa generate reactive oxygen species (ROS) (Aitken and Baker, 2013). Excessive ROS production overwhelms antioxidant defences and induces oxidative stress in spermatozoa. Furthermore, components of the intrinsic apoptotic pathway in the sperm mid-piece can affect sperm DNA and motility, which in turn leads to poor sperm quality and lower fertilization capacity (Weng et al., 2002; Paasch et al., 2004a, b). However, there is a lack of available assays to determine simultaneously the apoptotic signalling cascades and sex chromosome of individual spermatozoa under specific conditions.

We hypothesized that sex chromosome-dependent viability in long-lived spermatozoa is due to a discrepancy in apoptosis-related signalling under fluctuating physiological and sperm-storage conditions. Hence, we investigated sperm motility, viability and Y:X chromosome ratios of live sperm under a wide range of *in vitro* culture conditions. We also examined sex chromosome-specific regulation of lifespan by measuring the extent of DNA damage and determining intrinsic apoptotic protein expression in individual live spermatozoa.

Materials and Methods

Ethical approval

Ethical approval was obtained from the Institutional Review Board (IRB) for Human Research at Chung-Ang University Hospital in Seoul, Korea [IRB No. 12007039(97)]. The study design and all experimental methods were approved and carried out in accordance with the guidelines and IRB regulations of Chung-Ang University Hospital. All participants were fully informed about the study and provided written informed consent.

Preparation of medium

Modified HEPES buffered Tyrod's-Albumin-Lactate-Pyruvate medium (mTALP) consisted of 131.0 mM of NaCl, 0.3 mM of NaH_2PO_4, 3.1 mM of KCl, 2.1 mM of $CaCl_2$, 0.4 mM of $MgCl_2$, 10.0 mM of sodium lactate, 2.0 mM of $NaHCO_3$, 0.2 mM of sodium pyruvate, 10.0 mM of HEPES and 10 μg/ml of gentamycin. mTALP supplemented with 0.3% w/v bovine albumin serum for sperm culture. The adjustment of medium pH was controlled by HCl and NaOH for pH 6.5, pH 7.5 and pH 8.5 mTALP medium. We performed pretest for stability of each pH medium up to 5 days in 4, 22 and 37°C air.

Semen collection and sperm culture

Semen samples were obtained by masturbation from normozoospermic donors after 3 days of sexual abstinence. Sperm count and motility were evaluated according to World Health Organization semen analysis criteria (1999) using a computer-assisted semen analyzer (CTS-60/200; Motion Analysis, Santa Rosa, CA, USA). After liquefaction, samples were added with mTALP medium and centrifuged at 400×*g* for 5 min. Supernatant was removed and then sperm pellet was resuspended in mTALP. Samples with <60% motility were excluded from the experiment. For each replicate, four samples with greater than 60% motility were mixed to obtain sufficient spermatozoa and eliminate sampling-related bias. Different donors were used for different replicates. Two additional washing steps were performed, and the final cell concentration was adjusted to 1×10^6/ml.

To determine the effects of various experimental conditions on sperm survival in culture, we incubated spermatozoa for 5 days at three different temperatures (4, 22 and 37°C) and three pH levels (6.5, 7.5 and 8.5) (You et al., 2009). To maintain stable culture conditions, spermatozoa were incubated in mTALP medium containing 0.3% w/v bovine serum albumin (BSA), and the pH was adjusted with HCl or NaOH. Experiments were independently performed thrice.

Hypo-osmotic swelling test and pretreatment of spermatozoa nuclei

We performed a hypo-osmotic swelling (HOS) test for sperm viability and 5×10^6 spermatozoa were cultured in 5 ml of hypo-osmotic solution pre-warmed to 37°C (distilled water: 0.9% w/v NaCl [1:1], 150 mOsm/kg). Hypo-osmotic solutions containing spermatozoa were incubated at 37°C in a water bath for 30 min (You et al., 2014). After HOS treatment, the resulting mixtures were centrifuged at 400×*g*, and the pellets were resuspended in 100–200 μl of hypo-osmotic solution. One drop of the resulting suspension was smeared and fixed on a slide. Air-dried slides were incubated at 37°C for 45 min in 2 mmol/l dithiothreitol (DTT) in PBS.

Immunolocalization of apoptotic proteins

Immunocytochemistry was performed to compare the expression of apoptotic genes in spermatozoa. We focused on sperm survival under physiological condition. Among pH conditions at 37°C on 3 days, there is significant difference of sperm viability in pH 8.5 (Supplementary data, Table SI). Sperm motility was around 30% in both conditions. Therefore, spermatozoa were cultured at 37°C and two pH levels, i.e. 7.5 and 8.5 for 3 days.

After decondensation with 2 mmol/l DTT, spermatozoa were fixed in 2% w/v paraformaldehyde, incubated at 4°C for 15 min, and permeabilized in 0.2% Triton X-100 in PBS (PBS-T) for 2 min at 4°C. To block non-specific binding sites, the slides were first incubated in PBS-T containing 5%

BSA for 1 h. Slides were then incubated overnight with polyclonal anti-Bcl2, anti-Bax and anti-Caspase-3 antibodies (Abcam, Cambridge, UK) diluted 1:100 in PBS-T containing 5% BSA for 1 h. After washing twice with each PBS-T and PBS, slides were incubated at room temperature in the dark for 2 h with anti-rabbit FITC-conjugated IgG (Abcam) at 1:3000 dilution. Slides were incubated for 2 h, washed with PBS, counter-stained with 1 mg/ml DAPI (1:3000 dilution) in PBS, and sealed with a coverslip.

TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labelling assay

TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labelling (TUNEL) assay was performed to assess sperm DNA damage. Spermatozoa were cultured at pH 7.5 (control) and 8.5 with and without 200 μM α-tocopherol added as an antioxidant to alleviate oxidative stress.

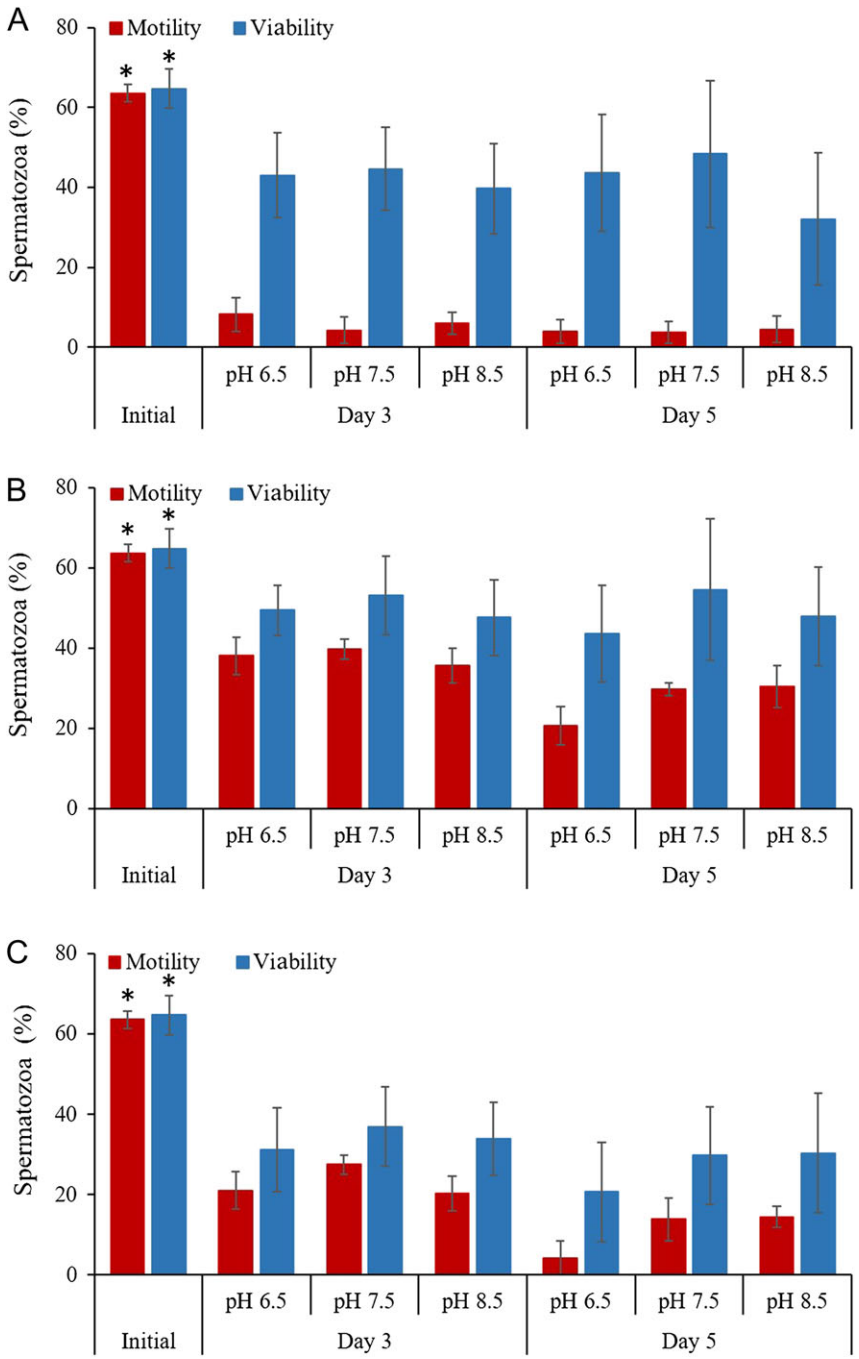

Figure 1 Changes in sperm motility and viability after 3 or 5 days of culture. Sperm motility and viability of sperm cultured at (**A**) 4°C, (**B**) 22°C and (**C**) 37°C. Asterisks indicate significant differences in motility and viability, respectively ($P < 0.05$). Data are presented as mean ± SD of three independent experiments.

After decondensation with 2 mmol/l DTT, spermatozoa were fixed in 2% paraformaldehyde, incubated at 4°C for 15 min, and permeabilized in 0.2% Triton X-100 in PBS for 2 min at 4°C. TUNEL assay was performed using an In Situ Cell Death Detection Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. Swelling patterns, the presence of sex chromosome and DNA damage in each individual spermatozoon were simultaneously observed with a Nikon TS-1000 microscope using NIS Elements imaging software (Nikon, Tokyo, Japan).

Fluorescence *in situ* hybridization

For fluorescence *in situ* hybridization (FISH), slides were denatured in 70% formamide/2× saline-sodium citrate solution at 80°C for 5 min. Multicolour FISH was performed using directly labelled DNA probes specific for chromosomes 18, X and Y (Kreatech, Amsterdam, the Netherlands) on the prepared slides containing HOS-treated spermatozoa following a previously described method (Pang *et al.*, 2010). To eliminate subjectivity, slides were analysed by an independent investigator. Swelling patterns and chromosome abnormalities were also noted for each individual spermatozoon.

Statistical analysis

We analysed the effect of medium pH, temperature and culture period on sperm motility, viability and sex chromosome ratio using a generalized linear model. Differences in the viability of X and Y spermatozoa were compared using multivariate analysis of variance. Statistically significant results ($P < 0.05$) were subjected to Tukey's honest significant difference test to evaluate differences between the experimental groups using SPSS software (v. 12.0; Chicago, IL, USA). Paired *t*-tests were used to analyse differences between the Y and X chromosome-bearing spermatozoa. Data are presented as mean ± SD of three independent experiments.

Results

Sperm motility, viability and Y:X chromosome ratios during 5 days incubation *in vitro*

Figure 1 shows the characteristics of spermatozoa cultured at 4, 22 and 37°C and pH conditions of 6.5, 7.5 and 8.5 in culture medium for 5 days. Sperm motility and viability were observed to decrease significantly over 5 days in all culture conditions ($P < 0.05$, Fig. 1). Likewise, sperm Y:X chromosome ratios were observed to decrease significantly in a time-dependent manner ($P < 0.05$, Fig. 2). However, this ratio at pH 7.5 on Day 5 raised up to initial ratio. Results were obtained based on the scoring of 58 489 spermatozoa with different swelling patterns and chromosomes contents (Fig. 3 and Supplementary data, Table SI). In univariate analysis, the observed changes over the culture period were influenced by temperature conditions (Table I). Culture period and temperature were found to significantly affect the functional viabilities of X and Y spermatozoa by multivariate analysis of variance ($P < 0.05$, Table II).

Differential expression of apoptotic proteins associated with sex-specific longevity of human spermatozoa

To identify the critical factors that lead to distorted sex chromosome ratios in live spermatozoa, we analysed the expression levels of apoptosis-related proteins and the numbers of sperm bearing Y or X chromosomes on Day 3 of culture under pH 7.5 and 8.5 conditions at 37°C relative to initial levels (Fig. 4). Scoring of 36 673 spermatozoa with different swelling patterns and chromosome contents revealed that culture at pH 8.5 caused a significant decrease in sperm viability and Y:X chromosome ratio and resulted in the upregulation of apoptotic proteins ($P < 0.05$; Fig. 4 and Supplementary data, Table SII). Surprisingly, live Y spermatozoa cultured for 3 days at pH 8.5 exhibited significantly greater downregulation of Bcl2 and upregulation of Bax and Caspase-3 relative to live X spermatozoa ($P < 0.05$; Fig. 4C–E). Moreover, the number of TUNEL-positive Y spermatozoa was significantly higher than that of live X spermatozoa on Day 3 at both pH 7.8 and 8.5 ($P < 0.05$; Fig. 4F). Thus, culture at 37°C and pH 8.5 for 3 days significantly affects sex chromosome ratio and the extent of apoptosis in spermatozoa ($P < 0.05$).

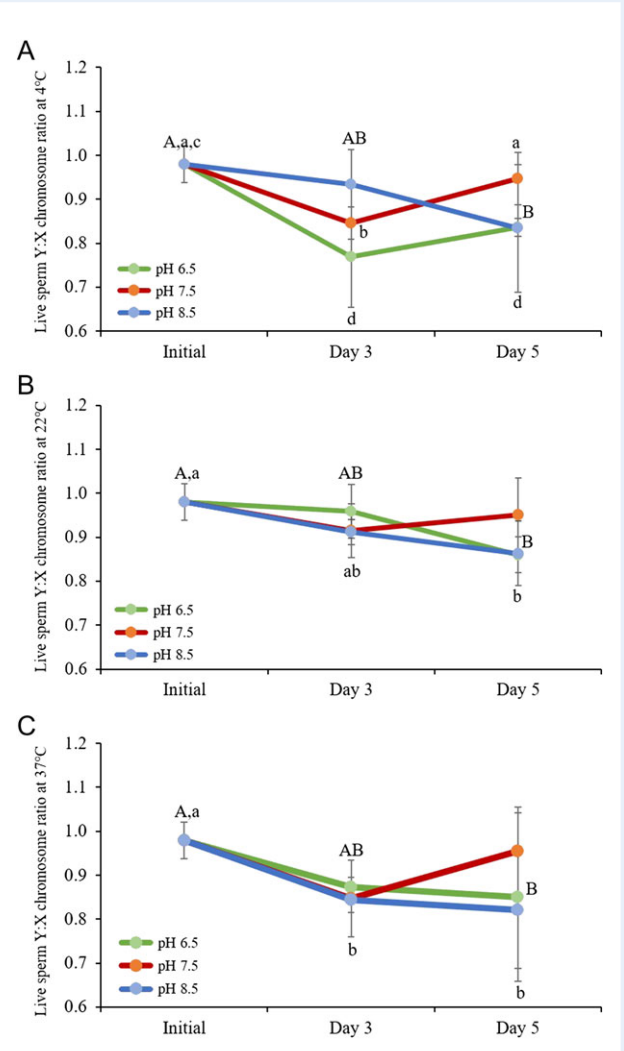

Figure 2 Changes in Y:X chromosome ratio of live sperm after 5 days of culture. Y:X chromosome ratio of live sperm cultured at (**A**) 4°C, (**B**) 22°C and (**C**) 37°C. Superscript uppercase and lowercase letters indicate significant differences in each pH condition, respectively ($P < 0.05$). Data are presented as mean ± SD of three independent experiments.

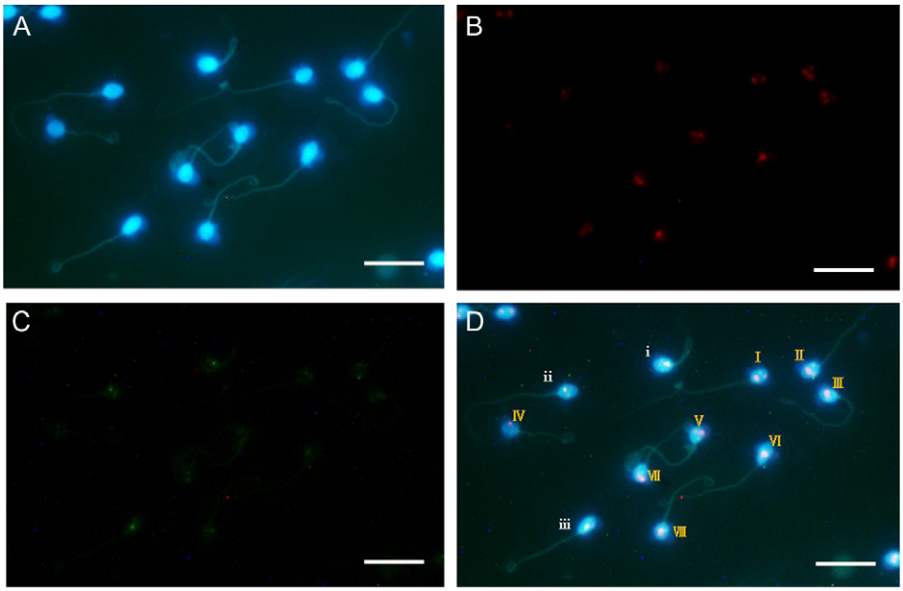

Figure 3 FISH in HOS-tested spermatozoa. (**A**) DAPI staining of spermatozoa after HOS test. (**B**) Red signals represent X chromosomes. (**C**) Green signals represent Y chromosomes. (**D**) Merged signals in spermatozoa. I and II, dead X spermatozoa; III–VIII, live X spermatozoa; i and iii, live Y spermatozoa; ii, dead Y spermatozoon. Bar = 20 μm. HOS, hypo-osmotic swelling; FISH, fluorescence *in situ* hybridization.

Table I Effect of culture conditions on sex chromosome and sperm characteristics analysed using univariate analysis.

Sperm characteristics	Factor	df	*F*-value	*P*-value
Motility	pH	2	0.500	0.609
	Temperature	2	49.585	<0.001
	Culture period	2	57.548	<0.001
	Error	76		
Viability	Sex chromosome	1	4.014	0.041
	pH	2	2.138	0.121
	Temperature	2	16.142	<0.001
	Culture period	2	79.390	<0.001
	Error	152		
Y:X chromosome ratio	pH	2	1.624	0.204
	Temperature	2	1.422	0.248
	Culture period	2	15.916	<0.001
	Error	75		

P-values were estimated using a generalized linear model. There were no significant interactions between factors.

Association between oxidative stress and Y:X chromosome ratio in live sperm

Spermatozoa were cultured with or without an antioxidant to investigate whether ROS levels affect sperm apoptosis and Y:X chromosome ratios. Sperm viability and chromosome contents were evaluated, and TUNEL assay was performed (Fig. 5). Sperm viability was significantly lower upon culturing at pH 8.5 compared to culturing at pH 7.5 after 3 days. Interestingly, addition of α-tocopherol maintained the viability of X spermatozoa in culture medium at pH 8.5 at a similar level to that observed at pH 7.5 (Fig. 5A). But did not protect Y spermatozoa to the same extent. Thus, the resulting Y:X chromosome ratio in live spermatozoa cultured at pH 8.5 with added tocopherol was still skewed despite a substantial increase in the viability of Y spermatozoa ($P < 0.05$; Fig. 5B and C).

Discussion

Our findings suggest that X spermatozoa survive longer than Y spermatozoa under some sperm-storage conditions, in other words that Y spermatozoa are more vulnerable to stress than X spermatozoa. Furthermore, Y spermatozoa exhibited upregulation of apoptotic proteins, which could explain their poorer survival. The greater survival of X spermatozoa might allow them to achieve higher oocyte fertilization success under stressful conditions.

When semen is ejaculated into the female reproductive tract, spermatozoa may be initially challenged by low pH levels in the vagina despite the buffering capacity of seminal fluid (Diasio and Glass, 1971; Arienti *et al.*, 1997). Spermatozoa swim in diverse physico-chemical environments and are subjected to alterations in temperature, pH and ROS levels for up to 1 week prior to fertilization (Suarez and Pacey, 2006). In addition, spermatozoa can preserve motility and viability in a wide range of atmospheric conditions such as refrigerator and room temperature (Appell and Evans, 1977; Cohen *et al.*, 1985). In this study, we used a culture technique that preserves sperm functional

Table II Comparison of functional viabilities of X and Y spermatozoa under various culture conditions.

Effect	Group	Level	N	Mean	SD	MANOVA P-value	ANOVA P-value	Tukey's HSD
pH	X sperm	pH 6.5	27	49.6	16.8	0.342	0.673	ND
		pH 7.5	27	52.8	16.0			
		pH 8.5	26	49.1	17.3			
	Y sperm	pH 6.5	27	45.2	17.5		0.569	ND
		pH 7.5	27	49.8	17.1			
		pH 8.5	26	45.6	18.6			
Temperature	X sperm	4°C	27	51.8	15.7	0.006	0.002	22°C > 37°C
		22°C	26	56.5	10.3			
		37°C	27	43.9	19.9			
	Y sperm	4°C	27	47.4	17.1		0.002	22°C > 37°C
		22°C	26	53.1	11.9			
		37°C	27	40.3	20.8			
Culture period	X sperm	Day 0	27	65.3	3.0	<0.001	<0.001	Day 0 > Day 3
		Day 3	26	44.8	10.8			Day 0 > Day 5
		Day 5	27	41.2	19.0			
	Y sperm	Day 0	27	64.1	5.1		<0.001	Day 0 > Day 3
		Day 3	26	39.3	10.3			Day 0 > Day 5
		Day 5	27	36.9	18.6			

MANOVA *P*-value, Wilks-Lamda probability; HSD, honest significant difference; ND, no significant difference.

viability (You *et al.*, 2009). Using this method, we evaluated sperm physiology in a wide range of environmental conditions and specifically tested the effects of medium pH and temperature on differential viability between X and Y spermatozoa cultured *in vitro* under normal atmospheric conditions for an extended period.

To assess the changes in Y:X chromosome ratios of live spermatozoa over time, we simultaneously analysed sperm viability and chromosome contents using the HOS test and FISH (Pang *et al.*, 2010; You *et al.*, 2014). Ratios of live sperm bearing Y:X chromosome significantly decreased after 3 days of culture, suggesting that more X spermatozoa can remain viable during extended culture periods. These findings are consistent with the observation that the length of the inter-coital interval affects the gender of offspring (Kleegman, 1954). Shettles (1970) reported that X spermatozoa are more resistant to acidity and other harmful conditions than Y spermatozoa. However, the present results show that X spermatozoa are more resistant to a wider range of pH conditions at 37°C during culture from 3 to 5 days. Thus, differential survivability between X and Y spermatozoa may be crucial in situations where the interval between coitus and fertilization is long.

Apoptosis affects sperm function and is involved in the removal of DNA-damaged spermatozoa in the male and female reproductive tracts (Ramalho-Santos *et al.*, 2009; Aitken and Koppers, 2011). This DNA damage process is driven by oxidative stress in mitochondria rather than endonuclease activity and ultimately results in sperm cell death (De Iuliis *et al.*, 2009; Aitken and Baker, 2013). Furthermore, sperm apoptosis may result from a number of conditions, including inadequate antioxidant protection within the male or female reproductive tract (Palmer *et al.*, 2012), cryostorage (Thomson *et al.*, 2009), lifestyle factors (Fraga *et al.*, 1996; Linschooten *et al.*, 2011), exposure to medicinal compounds (Ghosh *et al.* 2002) and prolonged incubation *in vitro* or *in vivo* (Balasuriya *et al.*, 2014). Such factors all lead to oxidative stress within the spermatozoa (Aitken and Baker, 2013).

In this study, spermatozoa showed time-dependent increases in apoptotic signalling based on DNA damage, Bax upregulation and Bcl downregulation under various stressful conditions. Although sperm apoptosis was delayed by the addition of an antioxidant, the sperm Y:X chromosome ratio continued to decrease relative to the initial ratio. These results suggest that X spermatozoa are more resistant to apoptosis and thus might have higher fertilization success and be more likely to produce live offspring than Y spermatozoa. Grunewald *et al.* (2008) reported that the activation of apoptosis-related signalling cascades in spermatozoa was negatively associated with oocyte penetration. Additionally, Burruel *et al.* (2014) reported that exposure of spermatozoa to oxidative stress affects early embryo development and results in lower success rates in reaching the blastocyst stage and further development. Some studies attribute the observed differences in lifespan between male and female to the upregulation of antioxidant genes and lower oxidative damage in female mitochondria (Borrás *et al.*, 2003; Tower, 2006; Marotti *et al.*, 2010). Thus, we suggest that X spermatozoa may have stronger defence systems against oxidative stress than Y spermatozoa. Moreover, exposure of the more vulnerable Y spermatozoa to stressful conditions leads to differences in the number of surviving sperm that are capable of fertilizing oocytes, which in turn results in skewed gender ratios.

Recently, many studies reported that high-level exposure to environmental toxins, such as endocrine disruptors and pollutants, is associated

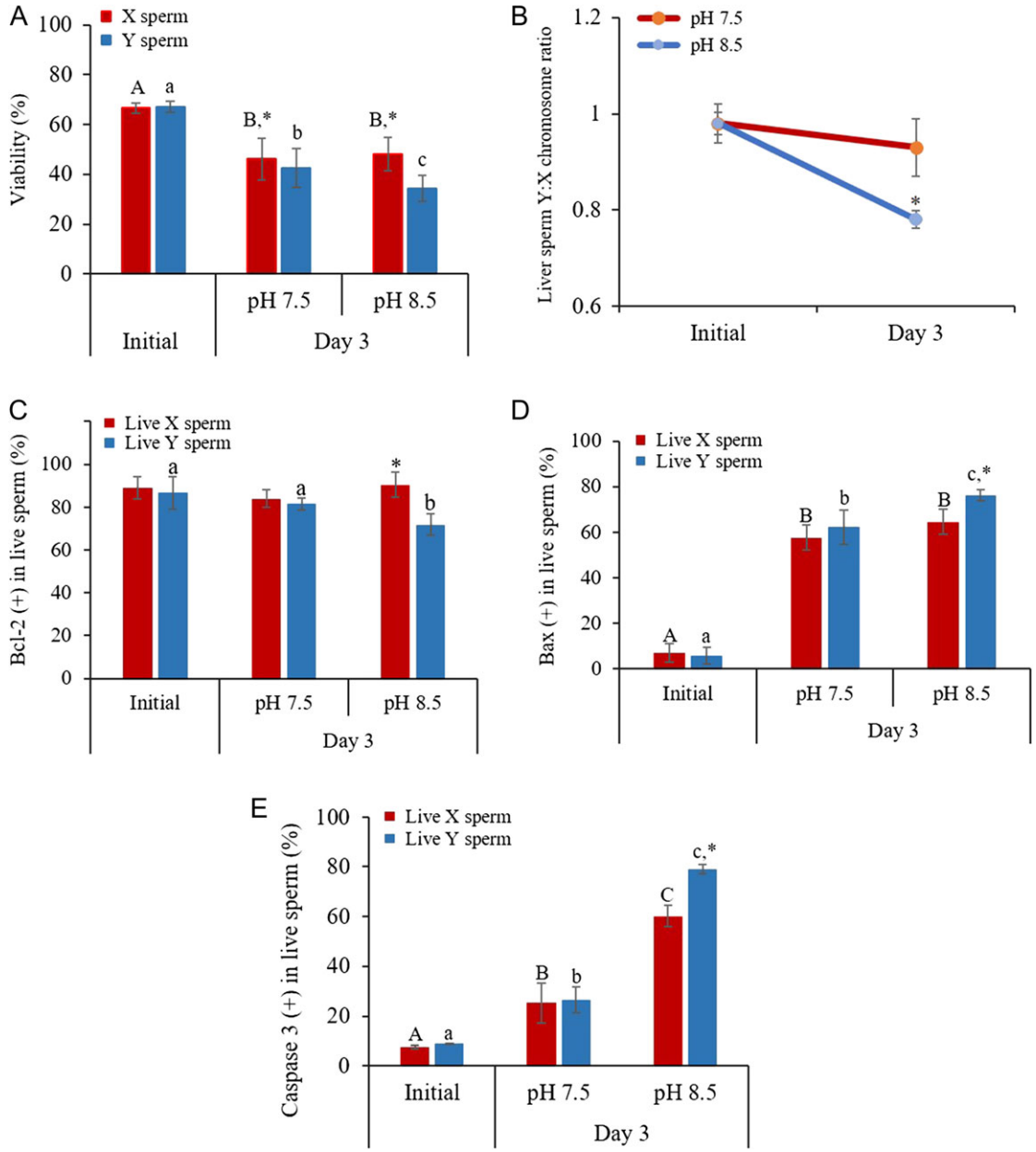

Figure 4 Comparison of apoptotic protein expression between live X and Y spermatozoa. (**A**) Changes in sperm viability after 3 days. (**B**) Comparison of live sperm sex chromosome ratio after 3 days. Graphs show the expression levels of (**C**) Bcl2, (**D**) Bax and (**E**) Caspase-3 in live spermatozoa. Asterisks indicate significant differences in protein expression between X and Y spermatozoa ($P < 0.05$). Superscript uppercase and lowercase letters indicate significant differences in X and Y spermatozoa, respectively ($P < 0.05$). Data are presented as mean ± SD of three independent experiments.

with distorted sex ratios at birth and sperm Y:X chromosome ratios (Robbins *et al.*, 2007; Kvist *et al.*, 2012, 2014). Interestingly, in the present study, both temperature and culture period were found to influence the Y:X chromosome ratios of live spermatozoa. Thus, it is tempting to speculate that exposure of sperm to stressful conditions also leads to deviations in sex ratio at birth.

Taken together, our results showed that Y spermatozoa are more vulnerable to fluctuations in physiological and sperm-storage conditions than X spermatozoa. Live Y spermatozoa were found to be more susceptible to apoptosis than X spermatozoa, which implies faster decline in fertilization capacity. Moreover, the longer survival of X spermatozoa under stressful conditions leads to higher probability of oocyte fertilization, suggesting that this phenomenon can be responsible for the observed shift in ratios of male-to-female births in humans. Further studies and experimentation using large sample sizes are required to elucidate the mechanisms behind the observed sex

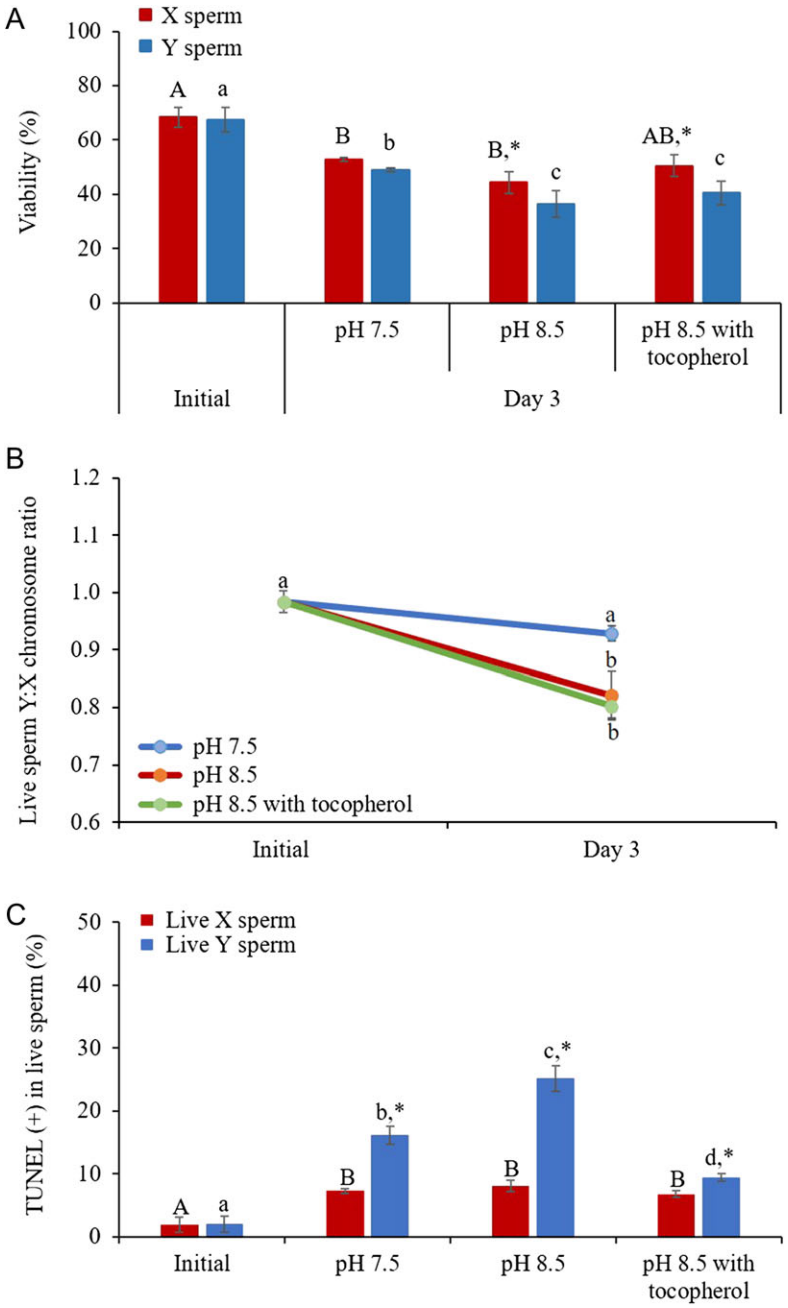

Figure 5 Effect of an antioxidant (tocopherol) on sperm characteristics and extent of DNA damage. (**A**) Sperm viability. (**B**) Live sperm Y:X chromosome ratio. (**C**) Extent of sperm DNA damage based on TUNEL assay. Asterisk indicates significant differences in protein expression between X and Y spermatozoa ($P < 0.05$). Superscript uppercase and lowercase letters indicate significant differences in X and Y spermatozoa, respectively ($P < 0.05$). Data are presented as mean (SD) of three independent experiments. TUNEL, TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labelling.

chromosome-dependent differential expression of apoptosis-related proteins.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Authors' roles

Designed the study: Y.A.Y., Y.J.K. and M.G.P. Collected samples: W.S.K., M.S.R. and Y.J.P. Performed experiments: Y.A.Y., W.S.K., M.S.R. and Y.J.P. Analysed the data: Y.A.Y., W.S.K. and M.G.P. Generated figures and tables: Y.A.Y. and Y.J.P. Wrote the manuscript: Y.A.Y. and M.G.P. All authors carefully reviewed the manuscript.

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Conflict of interest

None declared.

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