A new media without animal component for sperm cryopreservation: motility and various attributes affecting paternal contribution of sperm

Akansha Tiwari¹ · Merih Tekcan² · Leyla Sati³ · William Murk¹ · Jill Stronk⁴ · Gabor Huszar¹

Received: 7 September 2016 / Accepted: 27 January 2017 / Published online: 9 March 2017
© Springer Science+Business Media New York 2017

Abstract

Purpose Our aim was the development of a safe sperm cryopreservation New Media (NM), composed of consistent and reproducible components devoid of any animal origin, and evaluation of NM in terms of its effect on sperm structure and function as compared to regularly used yolk media (TYM) (Irvine Scientific).

Methods We evaluated patient semen samples and cryopreserved them in duplicates in either NM or TYM. The samples were cryopreserved for either a short term of 1 week or long term of 1 month prior to thawing. The parameters investigated include sperm motility via computer-assisted semen analysis (CASA), sperm concentration, and sperm biomarkers that promote paternal contribution of spermatozoa to fertilization including hyaluronic acid binding, chromatin maturity, apoptotic markers, cytoplasmic retention, and sperm DNA integrity. **Results** As compared to TYM, NM was equally capable of sperm cryopreservation with both short-term and long-term storage in media, and after freeze-thaw and gradient processing of sperm. HA binding of sperm was comparable post thaw in both NM and yolk media. There are also no differences observed between the samples cryopreserved in NM or TYM in terms of their aniline blue staining, CK

immunocytochemistry, caspase 3 immunostaining, or DNA nick translation.

Conclusions NM has the advantage of being xeno-free, yet in preservation of sperm motility and other sperm attributes, the NM is as effective as the TYM.

Keywords Cryopreservation · Male fertility · Sperm maturity · Biochemical markers · Sperm motility · HA binding

Introduction

In 1953 at the University of Iowa, a milestone event occurred in fertility medicine, as the first case of a live birth to a female fertilized with cryopreserved and thawed spermatozoa was reported [1]. Since then, a new field in male infertility medicine called sperm banking was initiated. Over the years there has been a growing need and use of sperm banks to provide a source of sperm for infertile men with poor semen parameters. In addition, the increased incidence and prevalence of testicular and other cancers highlighted the need for sperm cryopreservation. Testicular cancer is the most common malignancy in the age group between 25- and 29-year-old males (19.4% incidence), and between 30- and 34-year-old males (19.6%). Testicular cancer patients have an excellent prognosis with treatment. Although male cancer survivors can become parents through other options such as adoption or semen donation [2], most individuals prefer to have their own biological offspring [3–5].

The maintenance of fertility after cancer treatment is variable and usually depends on the type of chemotherapy or radiation therapy used, the size of the radiation field, dose intensity, method of treatment administration, age of patient, and pretreatment fertility [6, 7]. The measurable effects of chemotherapy or radiation therapy applied include

✉ Gabor Huszar
gabor.huszar@yale.edu

¹ Sperm Physiology Laboratory, Yale University, 330 Cedar St, New Haven, CT 06519, USA

² Medisu Hospital IVF Laboratory, Antalya, Turkey

³ School of Medicine, Akdeniz University, 07070Campus, Antalya, Turkey

⁴ Yale University, 150 Sargent Dr, New Haven, CT 06511, USA

compromised spermatozoa concentration, motility, morphology, and/or DNA integrity. There are clear advantages to sperm cryopreservation prior to cancer treatment initiation as there is a viable option for reproduction even after years of sperm storage [8]. Sperm cryopreservation provides a link that connects sperm donors to infertility patients and cancer patients.

The main component of the original cryopreservation media was glycerol, a cryo-protectant most widely used for human semen preservation following processing as defined by Sherman [9]. The glycerol was later replaced by egg yolk citrate media (initially used widely as semen extender for cattle) [9] and egg yolk [10, 11] for human sperm cryopreservation. The common extender, egg yolk, acts as an excellent reservoir of phospholipids and cholesterol allowing adequate replenishment of lipids lost from the sperm during the freeze-thaw process [10]. The composition of egg yolk and BSA as cryoprotectants has potential risks of harboring infectious animal diseases. Such a contamination could result in avian influenza [12] and bovine spongiform encephalopathy [13]. In addition, animal components such as BSA and egg yolk are not of standard composition leading to inherent variations. Thus, the use of media containing no animal products would be advantageous due to the reliability of composition and the absence of potential morbidity.

The New Media (NM) is chemically defined and yields reproducible results in cryopreservation and thawing. This allows for reduction in variability of quality of preservation media and provides a more favorable outcome for sperm recovery, as determined by concentration, motility, and DNA integrity. Media containing animal products may cause infections such as salmonella from egg yolk or prions in bovine albumin with the danger of mad cow disease. The components of the NM are reproducible and are similar to those described in the 1983 by Mahadevan and Trounson, containing cations, glycerol, sucrose, glucose, and lipids, where the lipids are contributed as mixture of cholesterol and other lipids essential to the proper function of the cell membranes [14].

To evaluate the new media for its effect on sperm structure and function, we followed the conventional semen parameters and sperm biochemical markers used to determine adequate sperm fertilization capacity as compared to conventional control media. We hypothesize that sperm survival and functional preservation will be equal in both media types providing an animal product-free media with equivalent cryopreservation potential. The objective of this study is to determine the effects of a new media devoid of risk of animal components as compared to control media containing egg yolk, with a focus on preservation of sperm motility, sperm hyaluronic acid binding, and sperm biomarkers that promote paternal contribution of spermatozoa.

Materials and methods

Control total yolk media (TYM) Irvine Scientific Freezing Media with 12% v/v glycerol, 20% ultra-filtered egg yolk, and 10 µg/mL gentamicin sulfate.

New media (NM) Sodium chloride (100 mM), potassium chloride (5.4 mM), calcium chloride (2.7 mM), magnesium chloride (0.5 mM), sodium lactate (12.8 mM), sodium dihydrogen phosphate (0.32 mM), sodium hydrogen carbonate (31 mM), Hepes (20 mM), sucrose (50 mM), glycine (13.2 mM), glucose (5.51 mM), glycerol (15% v/v), lipids.

Semen collection Research participants were recruited from the Yale Sperm Physiology Laboratory. The sperm sample donors were patient population referred for semen analysis potentially requiring infertility treatment. The samples were produced by masturbation. The semen samples were collected in four sample sets originated in 9, 13, 11, and 18 patients (Fig. 1). The samples used were de-identified. Studies were approved by the Yale School of Medicine IRB.

Sperm freezing Semen was diluted with room temperature cryopreservation media within 1 h of liquefaction. Approximately 0.1 mL of semen was used for evaluation of sperm concentration, percent motility, and morphological analysis via computer-assisted semen analysis (CASA). Following the determination of sperm concentration and motility with CASA, the sperm samples were mixed either with NM or with the control ultra-filtered egg yolk media (Irvine Scientific) in a ratio of 1:1 and placed in cryopreservation vials. The vials were then held for 20 min in liquid nitrogen vapor and subsequently plunged into liquid nitrogen. Each semen sample set was cryopreserved in duplicate for short term (1 week) and long term (1 month) in TYM. Similarly, each semen sample set was also cryopreserved in NM for short and long term. The samples were also evaluated with respect to sperm motility before and after the cryopreservation and freeze/thaw process.

Sperm thawing The cryopreserved duplicate vials were warmed up in 37 °C water bath for 10 min and analyzed via CASA. Aliquots were also used for analysis of sperm morphology and sperm biomarkers including hyaluronic acid (HA) binding, chromatin maturity (aniline blue staining), apoptotic markers (caspase 3 immunocytochemistry), cytoplasmic retention and sperm developmental arrest (creatine kinase immunohistochemistry), and sperm DNA integrity (DNA nick translation). The samples allowed a comparison of NM and total egg yolk media (TYM) in terms of sperm motility and other biological markers. DNA integrity was assessed via HA binding score, which represents sperm zona pellucida binding.

* Data Shown

Fig. 1 The experimental design illustrates the assessment of sperm samples obtained from Yale Sperm Physiology Laboratory. Samples were collected in four sets (9, 13, 11, and 18) in duplicates. The sperm samples were first evaluated post liquefaction using computer-assisted semen analysis (CASA) for sperm motility, concentration, motility, and morphology. The samples were then diluted 1:1 in new media and subjected to freezing using liquid nitrogen vapor for 20 min followed by liquid nitrogen immersion. The samples were frozen for either short term (1 week) or long term (1 month) and then thawed at 37 °C for 10 min.

Sample smears were used to evaluate sperm biomarkers via aniline blue staining, creatine kinase immunohistochemistry, hyaluronic acid binding, and DNA nick translation. Sample sets 11 and 18 were used to determine post thaw sperm motility and concentration at 0 min, 1 h, and 4 h post thaw. The sample sets 11 and 18 were also subjected to gradient fractionation protocol similar to the one used for in vitro fertilization procedure. The samples were then evaluated for sperm motility and concentration at 0 min, 1 h, and 4 h post gradient. Data for sample set 11 is shown for all experiments

Sperm concentration and motility A CASA instrument (IVOS, Hamilton-Thorne Research, Beverly, MA) was used to determine sperm motility. After mixing the samples, 7 μ L of semen was placed in a 10- μ M Makler chamber. Four microscopic fields, originating in two drops of semen, were evaluated at 36.5 °C. The CASA settings were as follows: 30 frames per second acquired at 60 Hz; minimum cell size, 2.0 pixels; non-motile sperm; head size cutoff, 3.0 pixels; low VAP and low VSL cutoff, 5.0 μ m/s.

Sperm biomarkers Before and after the cryopreservation process in both media, sperm smears were studied for cytoplasmic retention with creatine kinase immunostaining for persistent histones, chromatin integrity with aniline blue staining, and for DNA degradation by DNA nick translation [15–17]. Following determination of sperm motility (CASA) and HA binding score with the sperm-HBA Makler test chamber (Origio MidAtlantic Devices, Mt Laurel, NJ), the

semen was combined 1:1 with NM or the control ultra-filtered TYM. The duplicate vials were cryopreserved for short term (1 week) and long term (1 month) storage by exposure first to liquid nitrogen (LN) vapor for 20 min, followed by LN immersion. The samples were thawed rapidly at 36 °C. Sperm motility in thawed semen-cryopreservation media mixture was assessed after time periods of 1 and 4 h. Another aliquot of both the NM and total egg yolk media and sperm mixtures were subjected to an in vitro fertilization sperm preparation protocol by gradient fractionation (45%/85% isolate gradient, centrifugation at 500 $\times$ g, 18 min, at room temperature). Following resuspension, sperm motility was determined after 1 and 4 h. Post-thaw assessment of the sperm biomarkers: sperm HA binding, sperm cytoplasmic retention by CK immunohistochemistry, chromatin maturity by aniline blue, apoptotic process by caspase 3 staining, and the degree of DNA chain fragmentation by DNA nick translation were also determined. The sperm staining patterns were graded as

“light” (normal) and “dark” (arrested development and increased DNA chain fragmentation).

CK immunohistochemistry The procedures used were described previously [18, 19]. After centrifugation at $500\times g$, the sperm pellet was resuspended and drops of the washed sperm were applied to polylysine-coated glass slides and fixed with 3.7% paraformaldehyde in phosphate buffer/sucrose (PB-suc) for 20 min at room temperature. The slides were then allowed to air dry. After three washing steps in PB-suc, the spermatozoa were exposed to a 3% bovine serum albumin solution in PB-suc at room temperature. After further washing, the sperm were overlaid with a 1:1000 dilution of polyclonal anti-CKB antiserum (Chemicon Co, Temecula, CA). Following further PB-suc washes, the slides were treated with biotinylated second antibody at 1:1000 dilution and were exposed to a Vector horseradish peroxidase/ABC (avidin-biotin-complex, Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. The ABC treated slides were further processed with diaminobenzidine and hydrogen peroxide (Sigma, St. Louis, MO). The developed brown color highlights sperm with various degrees of cytoplasmic retention. The specificity of the CK staining was established by using pre-immune serum in place of the first antibody or by applying the second antibody only. The data were quantified by the density of the immunocytochemistry (representing cytoplasmic retention). The staining patterns were designated as L (light) and D (dark), which indicate the respective staining intensities. This system was also used in the evaluation of persistent histones (aniline blue), DNA fragmentation, and chromatin maturity. Apoptosis was evaluated using a caspase immunostaining. The staining designations were light and dark.

Aniline blue staining of sperm chromatin Sperm samples were smeared on polylysine-coated slides, air-dried, and fixed for 30 min in 3% glutaraldehyde (Merck, Darmstadt, Germany). Slides were stained for 7 min in acid aniline blue pH 3.5 (Riedel de Haen, Seelze, Germany), washed in 100 mM PBS pH 7.2, and carefully dried with filter paper. Examination was performed under oil at a magnification of $\times 1000$ with Leitz Ortho-plan microscope (Leitz, Germany). Spermatozoa ($n = 400$) were evaluated and classified using the following criteria: unstained (0 = mature nucleus), weakly stained, and strongly stained. The latter patterns (weakly and strongly stained) were regarded as sperm forms of retarded chromatin maturity. Sperm smears were dried on glass and stained with a 5% aniline blue solution acidified to approximately pH 3.5 with acetic acid. The slides were washed and air-dried and a cover slip was applied before evaluation. Due to persistent histones, sperm with immature chromatin were stained to various intensities of blue [20–23].

Assessment of DNA integrity by nick translation All steps were carried out at room temperature. The procedure was adapted from Irvine Scientific [16]. Briefly, slides were fixed for 15 min with methanol-acetic acid (3:1) and dehydrated through an ethanol series of 70, 80, and 100%. After air-drying, the slides were treated with 10 mM DTT (Sigma) in 10 mM Tris-HCl (pH 8.0) for 30 min. Subsequently, the slides were exposed to 10 mM lithium diiodosalicylic acid in 10 mM Tris-HCl for 1 h. In order to block any endogenous biotin/avidin binding sites within sperm, the slides were subjected first to biotin and then to avidin solutions in phosphate-buffered saline for 20 min. In situ nick translation was performed with the DNA polymerase I (Boehringer Mannheim, Biochemical, Indianapolis, IN). The slides were flooded with a DNA polymerase cocktail containing biotin 16-dUTP, a mixture of dGTP, dCTP, dATP, and 0.025 U/mL DNA polymerase I, for 30 min. The incorporation of biotin-labeled nucleotides was detected by the avidin-biotin horseradish peroxidase method. In further steps, the slides were treated with an ABC kit (Vector Laboratories). Finally, diaminobenzidine was used for the color development [24]. Prior to counterstaining with 0.1% Coomassie Blue, the slides were air-dried and mounted with Permount. All reagents were from the Sigma Chemical Company.

In each phase-contrast image, two different investigators in a blinded manner scored sperm shape according to Tygerberg-Kruger criteria [25]. Subsequently, the results were averaged for each of the sperm nuclei categories. Sperm head shape, neck midpiece, and tail were evaluated. Sperm that were considered Kruger normal morphology displayed oval-shaped heads, symmetrical tail insertions, and no visible indications of neck or midpiece shape defects.

Statistical analysis In order to compare the sperm concentrations, motilities, shape attributes, and various darkness factors before and after cryopreservation, we used the computer-based SigmaStat program to perform paired *t* test analysis on normally distributed data and Wilcoxon signed rank test on data that was not normally distributed (SigmaStat version 2.0; Jandel Scientific Corporation, San Rafael, CA). In testing the various correlations, we used the Pearson correlation analysis of the SigmaStat program. All data are presented as mean $\pm$ SEM. Level of significance was selected as $p \leq 0.5$.

Results

A sample set of $N = 11$ was studied post-cryopreservation in duplicates in TYM or NM for short term (1 week) and long term (1 month). Sperm motility was assessed after the samples were frozen and thawed in NM and TYM media and after gradient centrifugation processing. There was a slight reduction in sperm motility due to the NM and TYM alone (post

thaw 0 min) (Fig. 2). The reduction in motility, however, was comparable between the two media types after both short- and long-term cryopreservation. Motility was also assessed via CASA at 1 h post thaw and 4 h post thaw in the NM and TYM. There was a decline observed in post-thaw motility; however, this decline was not different between NM and TYM. In comparison to TYM, a slight decline in sperm motility was noticed at 1 and 4 h post thaw when using NM, especially in the long-term incubation (Fig. 2b). This decline was however, recovered post gradient. Sperm motility was evaluated further by repeating the study in another sample sets of 18 samples (total $N = 29$). The data obtained was similar to the one represented in Fig. 2. The four sample sets showed no statistically significant difference in sperm motility between the NM and TYM, especially at 4 h post gradient. Furthermore, the entire experiment series was repeated for determination of sperm concentration as affected by NM and TYM in all four sample sets (Fig. 3). Sperm concentrations declined both post thaw and post gradient with both short-term and long-term incubation. However, the changes were comparable, and no statistically significant differences were detected between the two media.

The sperm samples were further analyzed for sperm aniline blue staining, creatine kinase immunocytochemistry, caspase 3 immunocytochemistry, and DNA nick translation. Aniline blue staining of sperm detects persistent histones in spermatozoa representing arrest in chromatin maturity [26]. An increased histone content indicates a decreased amount of intermediate protein and protamine. This affects the folding ability of DNA and the sensitivity of DNA chains for oxidative degradation. In order to confirm the data, the proportion of the spermatozoa stained light or dark was determined (Fig. 4). The freezing process lead to a decline in dark staining spermatozoa likely due to a lack of survival of dark underdeveloped spermatozoa post the cryopreservation and freeze/thaw process. The average percentage of dark sperm cells in all the samples before cryopreservation was 17%, which decreased to 4% after cryopreservation in both types of media. The NM sample set showed slightly improved results in the fertilizing capacity of the cryopreserved sperm after the short-term incubation in media. Using NM media for long-term cryopreservation, a decline in the dark cells occurred in both media samples (17% before cryopreservation/thawing process and 4.1% (NM) and 5.8% (TYM) after). The light stained sperm,

Fig. 2 Comparison of the effects of incubation in NM and TYM media on sperm motility post thaw and cryopreservation media for short term (1 week) or long term (1 month) post gradient (p value <0.05 , $N = 11$). **a** Sperm motility with short-term incubation. **b** Sperm motility with long-term incubation

Fig. 3 Comparison of the effects of incubation in NM and TYM media on sperm concentration post thaw and cryopreservation in media for short-term (1 week) or long-term (1 month) post gradient (p value <0.05 ; $N=11$). **a** Short-term incubation. **b** Long-term incubation

Fig. 4 The effects of short-term or long-term cryopreservation in NM or TYM media on the percentage of light and dark aniline blue stained sperm cells. **a** Short term, $N=11$. **b** Long term, $N=11$

however, increased in percentage from 76.1% before cryopreservation/thawing process to 90.4% (NM) and 87.5% (TYM) after. The NM media is somewhat better in preserving the fertilizing capacity of the frozen sperm than TYM (Fig. 3a). A comparison of the samples from both media types illustrated no significant difference between the percentage of aniline blue light staining sperm cells in NM and TYM after short-term or long-term cryopreservation.

Subsequently, the samples were analyzed for creatinine kinase immunocytochemistry to demonstrate the sperm cytoplasmic retention. A decline in the dark cells was observed in both NM and TYM with short-term cryopreservation from $19.7\% \pm 0.2$ before cryopreservation/thawing to $8.3\% \pm 1.10$ (NM) and $11.2\% \pm 0.69$ (TYM) after. The light mature sperm, however, increased in percentage from $67.4\% \pm 0.20$ before cryopreservation/thawing to $84.7\% \pm 1.10$ (NM) and $80.1\% \pm 0.69$ (TYM) after (Fig. 4a). Long term cryopreservation analysis of the samples with CK immunocytochemistry illustrated a decline in the dark staining sperm in both media types from $19.7\% \pm 0.2$ before cryopreservation/thawing to $7.2\% \pm 1.18$ (NM) and $11.0\% \pm 0.61$ (TYM) after. The light mature sperm, however, increased in percentage from $67.4\% \pm 0.20$ before cryopreservation/thawing to $86.1\% \pm 1.18$ (NM) and $81.6\% \pm 0.61$ (TYM) after in the long-term cryopreserved samples (Fig. 4b). When the effects of long-term and short-term cryopreservation using NM was investigated, the proportion of CK immunohistochemistry light staining sperm cells showed an increase post thaw, which was comparable or higher to TYM (Fig. 5).

HA binding of the sperm samples was assessed to determine the sperm maturity and functionality in zona pellucida binding. Figure 6 shows the HA binding capacity of spermatozoa in the NM or TYM after long-term or short-term cryopreservation. HA binding in both media increased post thaw; however, there was no difference between the NM media and TYM. This was apparent in all four sample sets. A comparison of all four sample sets from both media types demonstrated that there is no significant difference between the HA binding capacity of NM and TYM after short-term ($p=0.23$) or long-term ($p=0.38$) cryopreservation.

Furthermore, the sperm samples were analyzed for apoptosis (caspase 3 immunocytochemistry) to indicate the caspase activity and sperm cell survival pre- and post-processing. Cryopreservation did not affect the apoptosis since the dark and light sperm percentage was similar before and after freezing in both media types. Caspase 3 immunocytochemistry represents the apoptotic process. The proportion of dark staining sperm represents the spermatozoa with ongoing apoptotic process. The NM samples after short-term cryopreservation showed an increase in dark cells in both media from $9.6\% \pm 0.87$ before cryopreservation/thawing to $15.4\% \pm 0.64$ (NM) and $15.1\% \pm 0.81$ (TYM) after. The light mature sperm,

Fig. 5 The effects of short-term or long-term cryopreservation in NM or TYM media on the percentage of light and dark creatine kinase immunocytochemistry stained sperm cells. **a** Short term, $N=11$. **b** Long term, $N=11$

however, decreased in percentage from $90.4\% \pm 0.87$ before cryopreservation/thawing to $84.6\% \pm 0.64$ (NM) and $85.0\% \pm 0.81$ (TYM) after in the short-term cryopreserved samples (Fig. 7a).

Long-term analysis of the samples with caspase 3 immunocytochemistry also illustrated an increase in the dark staining sperm in both media types from $9.6\% \pm 0.87$ before cryopreservation/thawing to $14.1\% \pm 0.84$ (NM) and $13.5\% \pm 0.97$ (TYM) after (Fig. 7b). The light mature sperm, however, decreased in percentage from $90.4\% \pm 0.87$ before cryopreservation/thawing to $85.0\% \pm 0.84$ (NM) and $85.9\% \pm 0.97$ (TYM) after in the long-term cryopreserved samples.

Moreover, the samples were analyzed for DNA nick translation. DNA degradation causes a declined fertilization rate and also an increased likelihood of spontaneous abortion may occur. Cryopreservation decreases DNA integrity since the freezing process denatures DNA via unwinding it and lending it vulnerable to degradation. This biomarker is a substantial indicator of paternal sperm contribution and early embryo support via DNA integrity. The data obtained from our studies showed a decline in light staining sperm cells and an increase in the dark staining sperm cells. This indicates a decrease in the sperm that exhibited high DNA integrity from 48 to 17%. The data, however, was similar between the two types of cryopreservation media in long- or short-term independent of the duration of freeze (Fig. 8).

Fig. 6 The effects of short-term or long-term cryopreservation in NM or TYM media on HA binding capacity of sperm cells. p values are included in figure ($p < 0.01$). **a** Short term, $N=11$. **b** Long term, $N=11$

Fig. 7 The effects of short-term or long-term cryopreservation in NM or TYM media on the percentage of light and dark caspase 3 immunocytochemistry stained sperm cells. **a** Short term, $N=11$. **b** Long term, $N=11$

Fig. 8 The effects of short-term or long-term cryopreservation in NM or TYM media on the percentage of light and dark sperm cells post DNA nick translation. **a** Short term, $N=11$. **b** Long term, $N=11$

The samples were analyzed for DNA nick translation to indicate sperm DNA integrity. An increase in the dark cells was observed in both NM and TYM with short-term cryopreservation from $18.4\% \pm 0.97$ before cryopreservation/thawing to $49.2\% \pm 1.28$ (NM) and $50.1\% \pm 1.07$ (TYM) after. The light mature sperm, however, decreased in percentage from $46.18\% \pm 0.73$ before cryopreservation/thawing to $12.5\% \pm 0.76$ (NM) and $13.1\% \pm 0.73$ (TYM) after (Fig. 8a). Long-term analysis of the samples with DNA nick translation illustrated an increase in the dark staining sperm in both media types from $18.4\% \pm 0.97$ before cryopreservation/thawing to $50.0\% \pm 0.50$ (NM) and $45.8\% \pm 1.00$ (TYM) after (Fig. 8b). The light mature sperm, however, decreased in percentage from $46.2\% \pm 0.73$ before cryopreservation/thawing to $14.6\% \pm 0.60$ (NM) and $15.1\% \pm 0.54$ (TYM) after in the long-term cryopreserved samples.

Discussion

Sperm cryopreservation is an important technique that allows extended use of sperm. Aside from patients seeking assisted

reproduction, patients with testicular cancers or other non-malignant and autoimmune diseases can benefit from the cryopreservation of sperm prior to therapeutic interventions [27, 28]. The process of cryopreservation requires the sperm to be cooled, frozen, and thawed, which can lead to sperm damage [29]. Due to the high fluidity of sperm cell membrane and low water content, sperm cells are less susceptible to damage by freeze-thaw procedure compared to other cells types.

The mechanism of sperm damage is multifactorial and involves primarily oxidative stress, dehydration, osmotic stress, cold shock, as well as intracellular crystal formation [30–33]. These stressors can lead to sperm DNA fragmentation and single stranded DNA breaks. In addition, the freezing damage can lead to changed membrane lipid composition, acrosomal damage, and reduced sperm motility [34–39]. It has been also established that only about 50% of sperm in an ejaculate can survive post-thaw [40, 41]. The altered antioxidant defense mechanisms and the creation of reactive oxygen species via osmotic and cold stress lead to disruption of the mitochondrial and plasma membranes as well as chromosomal damage leading to detrimental effects of sperm capacitation, acrosomal reaction, and binding to the zona pellucida [42, 43].

Furthermore, cryopreservation reduces the rate of lipid peroxidation in human sperm caused in part by the loss of superoxide dismutase activity as a result of the freeze/thaw process. This results in the loss of essential components such as phosphatidylcholine and phosphatidylethanolamine, and to a lesser extent polyunsaturated lipid vesicles from the spermatogenic membrane [44, 45]. The fertilization and pregnancy rate with frozen/thawed spermatozoa are significantly lower than the spermatozoa from fresh human ejaculates due to a loss of sperm motility, viability, mitochondrial function, premature acrosomal reaction, ultra-structural changes, and increased DNA damage [10]. The sperm cell membrane glycocalyx is responsible for ion transport and metabolism, immunoprotection, acrosomal reaction, and early gamete interaction. Cryopreservation can have deleterious effects on the membrane glycocalyx [46]. Thus, the growing need for sperm cryopreservation demands the development of a safe cryopreservation media to enhance the sperm structural and functional integrity.

Cryoprotectants are low molecular weight and highly permeable chemicals used to protect spermatozoa from freeze damage by ice crystallization by decreasing the freezing temperature of the samples [47]. The most commonly used cryoprotectants are glycerol, ethylene glycol, dimethyl sulfoxide (DMSO), and 1,2-propanediol. Cryopreservation media with the addition of cholesterol has shown to improve sperm membrane hydrophobicity and integrity leading to decreased osmotic stress [48]. Glycerol is most widely used for human sperm cryopreservation due to its ability to stabilize the lipid bilayer and membrane surface proteins. Glycerol, however,

can lead to damage of acrosomal internal membrane and structure [49]. It can also lead to structural changes in cell membrane, nuclear disorganization of mitochondrial structure. In addition, egg yolk and bovine serum albumin (BSA) are regular components of cryopreservation media and allow resistance to hypoosmotic shock [50]. BSA increases sperm motility and longevity after long-term cryopreservation in low temperatures [51, 52]. However, BSA and egg yolk do carry the risk of infection with the cryopreserved sperm.

A reproducible sperm cryopreservation NM was developed using no animal components, and its cryopreservation capabilities were analyzed as compared to the control TYM that contains animal products such as BSA and egg yolk. The NM was evaluated against the control media containing animal components specifically to show the difference in sperm function when this isolated parameter is changed between media. The maintenance of sperm motility is important for IUI, IVF, or assisted reproduction. NM provided a motility recovery comparable to that of the TYM. It was found that the conventional sperm markers such as the amount of motile sperm per milliliter were comparable or higher with NM. As the samples were thawed, the motility and concentrations of sperm declined (Fig. 2). This was further observed in the post gradient phase. However, in both short-term and long-term exposure to media, recovery of motility was observed indicating that the sperm motility and hence potential for fertility was maintained both short and long term. In addition, sperm motility in NM was comparable to TYM specifically in the post gradient motility in both short- and long-term studies. This suggests that the long-term sperm recovery and motility are preserved in NM at the same rate as TYM, despite the intermediate decline in motility post thaw. Thus, the NM is comparable in preserving sperm function with the conventional yolk media without the potential for infection.

The NM was also effective in preserving sperm HA binding, which reflects the zona pellucida binding capacity of sperm. The HA binding score is a marker of actual zona binding and morphology of sperm. This is an important marker of sperm function as it is necessary component for adequate fertilization. The HA score of sperm preserved in NM was higher since the underdeveloped sperm did not survive the process of cryopreservation/thawing and therefore, DNA integrity and apoptosis remained improved. This form of evaluation of sperm function with a xeno-free media has not been performed previously. The HA score is an important functional marker that is regularly used in sperm banks for sperm testing. The patient population, for example those anticipating chemo- and radiation therapy for testicular or other cancers specifically requiring storage of sperm, has an improved chance of fertility in the future without the potential for infections.

In addition, we were able to demonstrate that the semen markers such as sperm morphology, sperm cytoplasmic retention (CK immunohistochemistry), and chromatin maturity

(aniline blue staining of persistent histones) were improved with NM media compared to total egg yolk media (TYM). Chromatin maturity affects DNA folding since persistent histones arrest the histone intermediate protamine development leading to inadequate DNA folding and increased vulnerability to DNA degradation. When analyzing sperm samples using CK immunohistochemistry and aniline blue staining, there was no difference between the NM and TYM in preserving the normally developed light stained spermatozoa. The dark staining spermatozoa with persistent histones indicates an arrest in the histone-transition sequence. The dark stained sperm representing a lack of maturation showed a limited survival post-cryopreservation process (Figs. 4 and 5). The proportion of light staining sperm increased in both freezing media compared to the pre-cryopreservation values in both CK immunohistochemistry and aniline blue staining experiments. This may be because of the degradation of the immature or developmentally arrested sperm cells during the freeze/thaw process leaving behind only the mature sperm. Moreover, the dark sperm cells decreased post the addition of both media types, possibly due to the higher sensitivity of the immature sperm cells to the freeze/thaw process, making them unable to survive the harsh cryopreservation process.

Furthermore, both apoptosis and sperm integrity remained improved and comparable between media, as sperm nuclear analysis was conducted via caspase 3 immunohistochemistry. The proportion of dark staining sperm represents the spermatozoa with ongoing apoptotic process. The data showed that apoptosis was not substantially increased in the cryopreserved and thawed sperm in either media type. In addition, the most important biochemical marker in terms of paternal contribution and support of the embryo is sperm DNA integrity. Also, potential infections harbored in TYM may further damage the spermatid DNA integrity. In our study, a decline in DNA integrity was observed post thawing, as expected. This decline, however, was comparable between the NM and TYM in both the short-term and long-term studies (Fig. 8). Both cancer treatment as well as cancer itself can affect spermatogenesis via autoimmune, endocrine, or systemic effects [53]. Thus, a media that maintains adequate sperm integrity and apoptotic process is essential for cryopreservation.

It can be concluded from the data that the new media was able to comparably maintain sperm morphology and function as media containing animal products does. Sperm motility, concentration, and biological markers that highlight sperm function illustrated similar results between the yolk media and new media. We conclude that the TYM is comparable to NM in all biological parameters and furthermore the NM is advantageous due to a lack of the animal components and infective agents. The most significant markers of sperm function and genetic stability are sperm motility, sperm DNA integrity, and hyaluronic acid binding ability of the sperm. The data shows that these important aspects of the sperm function

are comparable between the NM and TYM. Thus, NM can be safely replace to yolk media in sperm cryopreservation and additionally have the advantage of being free of animal products.

Conclusion

In summary, this study shows that in addition to preservation of sperm motility and improvement of sperm DNA integrity, the NM media is also free of any animal components thereby minimizing any adverse effects. Regarding attributes important for sperm paternal fertility contribution to the embryo, the sperm cryopreservation in NM or in TYM exhibited comparable preservation of the normally developed sperm fraction by measures of sperm motility, sperm concentration, chromatin structure, excess cytoplasm, apoptotic process, and most importantly, DNA chain integrity. Thus, the NM has the advantage of being xeno-free, yet in preservation of all sperm motility and other attributes the NM is as effective as the TYM.

Acknowledgements Funding support from VitroLife AB, Göteborg, Sweden

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest. Funding for the research was provided by VitroLife for evaluation of New Media.

Ethical approval The study was approved by the Yale University IRB.

Informed consent Informed consent was obtained from all individual participants included in the study. The patient sperm samples were de-identified prior to the initiation of study.

References

- Bunge RG, Sherman JK. Fertilizing capacity of frozen human spermatozoa. *Nature*. 1953;172(4382):767–8.
- Rosen A. Third-party reproduction and adoption in cancer patients. *J Natl Cancer Inst Monogr*. 2005;(34):91–3.
- Fosså SD, Aass N, Molne K. Is routine pre-treatment cryopreservation of semen worthwhile in the management of patients with testicular cancer? *Br J Urol*. 1989;64(5):524–9.
- Schover LR, Brey K, Lichtin A, Lipshultz LI, Jeha S. Knowledge and experience regarding cancer, infertility, and sperm banking in younger male survivors. *J Clin Oncol*. 2002;20(7):1880–9.
- Schover LR, Rybicki LA, Martin BA, Bringelsen KA. Having children after cancer. A pilot survey of survivors' attitudes and experiences. *Cancer*. 1999;86(4):697–709.
- Gandini L, Sgrò P, Lombardo F, Paoli D, Culasso F, Toselli L, et al. Effect of chemo- or radiotherapy on sperm parameters of testicular cancer patients. *Hum Reprod*. 2006;21(11):2882–9.
- Res U, Res P, Kastelic D, Stanovnik M, Kmetec A, Merlo A. Birth after treatment of a male with seminoma and azoospermia with cryopreserved-thawed testicular tissue. *Hum Reprod*. 2000;15(4):861–4.
- Horne G, Atkinson AD, Pease EH, Logue JP, Brison DR, Lieberman BA. Live birth with sperm cryopreserved for 21 years prior to cancer treatment: case report. *Hum Reprod*. 2004;19(6):1448–9.
- Sherman JK. Research on frozen human semen: past, present, and future. *Fertil Steril*. 1964;15:485–99.
- Graham EF, Crabo BG, Brown KI. Effect of some zwitter ion buffers on the freezing and storage of spermatozoa. *J Dairy Sci*. 1972;55(3):372–8.
- Jeyendran RS, Van der Ven HH, Kennedy W, Perez-Pelaez M, Zaneveld LJ. Comparison of glycerol and a zwitter ion buffer system as cryoprotective media for human spermatozoa. Effect on motility, penetration of zona-free hamster oocytes, and acrosin/proacrosin. *J Androl*. 1984;5(1):1–7.
- Abe Y, Lee DS, Sano H, Akiyama K, Yanagimoto-Ueta Y, Asano T, et al. Artificial insemination with canine spermatozoa frozen in a skim milk/glucose-based extender. *J Reprod Dev*. 2008;54(4):290–4.
- Mortimer D, Barratt CL. Is there a real risk of transmitting variant Creutzfeldt-Jakob disease by donor sperm insemination? *Reprod Biomed Online*. 2006;13(6):778–90.
- Mahadevan M, Trounson AO. Effect of cryoprotective media and dilution methods on the preservation of human spermatozoa. *Andrologia*. 1983;15(4):355–66.
- Cayli S, Sakkas D, Vigue L, Demir R, Huszar G. Cellular maturity and apoptosis in human sperm: creatine kinase, caspase-3 and Bcl-XL levels in mature and diminished maturity sperm. *Mol Hum Reprod*. 2004;10(5):365–72.
- Irvine DS, Twigg JP, Gordon EL, Fulton N, Milne PA, Aitken RJ. DNA integrity in human spermatozoa: relationships with semen quality. *J Androl*. 2000;21(1):33–44.
- Huszar G, Jakab A, Sakkas D, Ozenci CC, Cayli S, Delpiano E, et al. Fertility testing and ICSI sperm selection by hyaluronic acid binding: clinical and genetic aspects. *Reprod Biomed Online*. 2007;14(5):650–63. Review.
- Ergur AR, Dokras A, Giraldo JL, Habana A, Kovanci E, Huszar G. Sperm maturity and treatment choice of in vitro fertilization (IVF) or intracytoplasmic sperm injection: diminished sperm HspA2 chaperone levels predict IVF failure. *Fertil Steril*. 2002;77(5):910–8.
- Huszar G, Vigue L. Incomplete development of human spermatozoa is associated with increased creatine phosphokinase concentration and abnormal head morphology. *Mol Reprod Dev*. 1993;34(3):292–8.
- Dadoun JP, Mayaux MJ, Guihard-Moscato ML. Correlation between defects in chromatin condensation of human spermatozoa stained by aniline blue and semen characteristics. *Andrologia*. 1988;20(3):211–7.
- Hammadeh ME, Al-Hasani S, Stieber M, Rosenbaum P, Küpker D, Diedrich K, et al. The effect of chromatin condensation (aniline blue staining) and morphology (strict criteria) of human spermatozoa on fertilization, cleavage and pregnancy rates in an intracytoplasmic sperm injection programme. *Hum Reprod*. 1996;11(11):2468–71.
- Franken DR, Franken CJ, de la Guerre H, de Villiers A. Normal sperm morphology and chromatin packaging: comparison between aniline blue and chromomycin A3 staining. *Andrologia*. 1999;31(6):361–6.
- Huszar G, Ozenci CC, Cayli S, Zavaczki Z, Hansch E, Vigue L. Hyaluronic acid binding by human sperm indicates cellular maturity, viability, and unreacted acrosomal status. *Fertil Steril*. 2003;79 Suppl 3:1616–24.
- Huszar G, Celik-Ozenci C, Cayli S, Kovacs T, Vigue L, Kovanci E. Semen characteristics after overnight shipping: preservation of

- sperm concentrations, HspA2 ratios, CK activity, cytoplasmic retention, chromatin maturity, DNA integrity, and sperm shape. *J Androl.* 2004;25(4):593–604.
25. Menkveld R, Stander FS, Kotze TJ, Kruger TF, van Zyl JA. The evaluation of morphological characteristics of human spermatozoa according to stricter criteria. *Hum Reprod.* 1990;5(5):586–92.
26. Sati L, Huszar G. Methodology of aniline blue staining of chromatin and the assessment of the associated nuclear and cytoplasmic attributes in human sperm. *Methods Mol Biol.* 2013;927:425–36.
27. Jensen JR, Morbeck DE, Coddington III CC. Fertility preservation. *Mayo Clin Proc.* 2011;86(1):45–9.
28. Anger JT, Gilbert BR, Goldstein M. Cryopreservation of sperm: indications, methods and results. *J Urol.* 2003;170(4 Pt 1):1079–84.
29. Colas C, Junquera C, Perez-Pe R, Cebrian-Perez JA, Muino-Blanco T. Ultrastructural study of the ability of seminal plasma proteins to protect ram spermatozoa against cold-shock. *Microsc Res Tech.* 2009;72:566–72.
30. Chatterjee S, Gagnon C. Production of reactive oxygen species by spermatozoa undergoing cooling, freezing, and thawing. *Mol Reprod Dev.* 2001;59:451–8.
31. Wang AW, Zhang H, Ikemoto I, Anderson DJ, Loughlin KR. Reactive oxygen species generation by seminal cells during cryopreservation. *Urology.* 1997;49:921–5.
32. Watson PF. The causes of reduced fertility with cryopreserved semen. *Anim Reprod Sci.* 2000;60–61:481–92.
33. Stanic P, Tandara M, Sonicki Z, Simunic V, Radakovic B, Suchanek E. Comparison of protective media and freezing techniques for cryopreservation of human semen. *Eur J Obstet Gynecol Reprod Biol.* 2000;91(1):65–70.
34. de Paula TS, Bertolla RP, Spaine DM, Cunha MA, Schor N, Cedenho AP. Effect of cryopreservation on sperm apoptotic deoxyribonucleic acid fragmentation in patients with oligozoospermia. *Fertil Steril.* 2006;86:597–600.
35. Hammad M, Askari A, Georg T, Rosenbaum P, Schmidt W. Effect of freeze-thawing procedure on chromatin stability, morphological alteration and membrane integrity of human spermatozoa in fertile and subfertile men. *Int J Androl.* 1999;22:155–62.
36. Isachenko V, Isachenko E, Katkov II, Montag M, Dessole S, Nawroth F, et al. Cryoprotectant-free cryopreservation of human spermatozoa by vitrification and freezing in vapor: effect on motility, DNA integrity, and fertilization ability. *Biol Reprod.* 2004;71:1167–73.
37. Said TM, Gaglani A, Agarwal A. Implication of apoptosis in sperm cryoinjury. *Reprod Biomed Online.* 2010;21:456–62.
38. Thomson L, Fleming S, Aitken R, De Iuliis G, Zieschang J-A, Clark A. Cryopreservation-induced human sperm DNA damage is predominantly mediated by oxidative stress rather than apoptosis. *Hum Reprod.* 2009;24(9):2061–70. doi:10.1093/humrep/dep214.
39. Zribi N et al. Effect of freezing-thawing process and quercetin on human sperm survival and DNA integrity. *Cryobiology.* 2012;65:326–31.
40. Bailey JL, Lessard C, Jacques J, Brque C, Dobrinski I, Zeng W, et al. Cryopreservation of boar semen and its future importance to the industry. *Theriogenology.* 2008;70:1251–9.
41. Johnson L, Weitze K, Fiser P, Maxwell W. Storage of boar semen. *Anim Reprod Sci.* 2000;62:143–72.
42. Agarwal A, Sharma RK, Nallella KP, Thomas AJ, Alvarez JG, Sikka SC. Reactive oxygen species as an independent marker of male factor infertility. *Fertil Steril.* 2006;86:878–85.
43. Taylor K, Roberts P, Sanders K, Burton P. Effect of antioxidant supplementation of cryopreservation medium on post-thaw integrity of human spermatozoa. *Reprod Biomed Online.* 2009;18:184–9.
44. Alvarez JG, Storey BT. Evidence for increased lipid peroxidative damage and loss of superoxide dismutase activity as a mode of sublethal cryodamage to human sperm during cryopreservation. *J Androl.* 1992;13(3):232–41.
45. Alvarez JG, Storey BT. Role of glutathione peroxidase in protecting mammalian spermatozoa from loss of motility caused by spontaneous lipid peroxidation. *Gamete Res.* 1989;23:77–90.
46. Benoff S. Carbohydrates and fertilization: an overview. *Mol Hum Reprod.* 1997;3(7):599–637.
47. Royere D, Barthelemy C, Hamamah S, Lansac J. Cryopreservation of spermatozoa: a 1996 review. *Hum Reprod Update.* 1996;2(6):553–9.
48. Chakrabarty J, Banerjee D, Pal D, De J, Ghosh A, Majumder GC. Shedding off specific lipid constituents from sperm cell membrane during cryopreservation. *Cryobiology.* 2007;54:27–35.
49. Fabbri R, Porcu E, Lenzi A, Gandini L, Marsella T, Flamigni C. Follicular fluid and human granulosa cell cultures: influence on sperm kinetic parameters, hyperactivation, and acrosome reaction. *Fertil Steril.* 1998;69(1):112–7.
50. Amidi F, Farshad A, Khor AK. Effects of cholesterol loaded cyclodextrin during freezing step of cryopreservation with TCGY extender containing bovine serum albumin on quality of goat spermatozoa. *Cryobiology.* 2010;61:94–9.
51. Matsuoka T, Imai H, Kohno H, Fukui Y. Effects of bovine serum albumin and trehalose in semen diluents for improvement of frozen-thawed ram spermatozoa. *J Reprod Dev.* 2006;52:675–83.
52. Yoshimoto T, Nakamura S, Yamauchi S, Muto N, Nakada T, Ashizawa K, et al. Improvement of the postthaw qualities of Okinawan native pig spermatozoa frozen in an extender supplemented with ascorbic acid 2-O- α -glucoside. *Cryobiology.* 2008;57:30–6.
53. Agarwal A, Ong C, Durairajanayagam D. Contemporary and future insights into fertility preservation in male cancer patients. *Transl Androl Urol.* 2014;3(1):27–40.