Standardisation of a novel sperm banking kit – NextGen® – to preserve sperm parameters during shipment

A. Agarwal¹, R. Sharma¹,², A. Singh¹,³, S. Gupta¹ & R. Sharma¹

¹ Andrology Center and Reproductive Tissue Bank, and American Center for Reproductive Medicine, Cleveland, OH, USA; ² Health Services Department, Saint Joseph University, Philadelphia, PA, USA; ³ The Ohio State University College of Medicine, Columbus, OH, USA

Keywords
Home banking—human spermatozoa—hypoosmotic swelling—motility—sperm vitality

Summary
Many male patients diagnosed with cancer are within their reproductive years. These men are advised to freeze their spermatozoa prior to the start of cancer treatment. Very often, sperm banking facilities may not be readily available and patients may be required to travel to distant sperm bank centres. Our objective was to design and standardise a remote home shipping sperm kit that allows patients to collect a semen sample at home and ship it overnight to a sperm bank. A total of 21 semen samples and two transport media (refrigeration media and human tubal fluid) and five different combinations of ice packs were tested for maintaining desired shipping temperature. Ten semen samples were assessed for pre- and post-shipment changes in sperm motility, membrane integrity, total motile spermatozoa and recovery of motile spermatozoa. Even though motility, membrane integrity and total motile spermatozoa declined both in samples examined under simulated shipped conditions and in overnight-shipped samples, the observed motility and total motile spermatozoa were adequate for use with assisted reproductive techniques. Using refrigeration media, cooling sleeve and ice packs, adequate sperm motility can be maintained utilising NextGen® kit and these spermatozoa can be used for procreation utilising ART techniques such as intracytoplasmic sperm injection.

Introduction
In the United States alone in 2010, approximately 8400 cases of testicular cancer were diagnosed among men between the ages of 15 and 44 years old (Rosen et al., 2011). According to the Surveillance Epidemiology and End Results (SEER) cancer statistics, it is estimated that one of every two men will be diagnosed with cancer in his lifetime, of which 4% are under the age of 35 (Howlader et al. 2015). Over the past quarter century, incidence of cancer in adolescents and young adults has been on the rise and several countries have reported improvement in the survival rates of adolescents and young adults (Daudin et al., 2015). Patients with cancer comprise about 44% of all referrals in the United States alone (Tomlinson, 2010). The American Society of Clinical Oncology (ASCO) advocates sperm cryopreservation as an effective method of fertility preservation in young men with cancer (Hourvitz et al., 2008; Loren et al., 2013). In a recently published report, it was suggested that all males within the reproductive age group and even men undergoing radical prostatectomy for prostate cancer should cryopreserve their spermatozoa (Salonia et al., 2013; Niederberger, 2014).

Cryopreservation of human spermatozoa has evolved as an important area in assisted reproductive technology (ART), oncology, men undergoing vasectomy (Gangrade, 2013) and in men engaging in potentially life-threatening activities, such as those in the military and those faced with conditions that are likely to impair their fertility, such as occupational exposure (Williams, 2010). Fertility preservation enhances the quality of life in cancer survivors with gonadal dysfunction brought about by cytotoxic agents and radiation (Williams, 2013; Robinson & Knudtson, 2014).

Patients with cancer are generally instructed to bank multiple specimens within a short period of time, prior to the start of cancer treatment. This is to have adequate numbers of spermatozoa for procreation utilising ART techniques such as intracytoplasmic sperm injection or ICSI. In this technique, a single viable, morphologically normal spermatozoon is injected into the oocyte.
Service land Clinic developed the NextGen Home Sperm Banking kit (Maxwell & Stojanov, 1996; Krzyzosiak et al., 2006). Unfortunately, however, sperm banking facilities are few and inadequate in number. Therefore, these patients who require sperm banking services often travel from other cities or neighbouring states to find a sperm bank and would have to take time off from work and incur significant travel expenses. These can also be an emotionally traumatic experience for these patients (Said et al., 2009). Development of a reliable method for shipment of semen specimens from remote areas to a specialised fertility preservation facility is a critical area of research. The ability to collect a sample in a private setting and directly ship it to a central laboratory may help alleviate these problems. Therefore, there is an increasing need to create practical and reliable methods for remote semen collection (Royster et al., 2000; Bjorndahl et al., 2000; Bjorndahl et al., 2000; Tournaye et al., 2013; Nangia et al., 2013; Pacey & Eiser, 2014; Tournaye et al., 2014).

In order for any sperm collection kit to successfully transport semen specimens from a remote collection site to a laboratory for andrological evaluation and clinical use, it must be able to maintain sperm viability and fertilising potential. With shipping, three factors are important: the transport media, the cooling components and transport time (Zavos et al., 1998). Transport time should optimally take no more than 24 h because in vitro fertility of the motile subpopulation of spermatozoa declines over time due to a decrease in their functional quality (Maxwell & Stojanov, 1996; Krzyzosiak et al., 2001).

To meet this need, the Andrology Center of the Cleveland Clinic developed the NextGen Home Sperm Banking Service®, which uses an at-home collection kit and overnight shipping to expand fertility preservation options for men throughout the United States. This service uses a special collection kit that comes with instructions and packaging for overnight mail delivery to Cleveland Clinic’s Andrology Laboratory for cryopreservation and long-term storage. The NextGen® sperm banking kit allows the patient privacy and a comfortable location to collect a sperm sample.

In this study, our objectives were to standardise the contents of the Home Sperm Banking kit called NextGen® and to examine whether the sperm quality, particularly the sperm motility and membrane integrity, was maintained during overnight shipment of this kit. Therefore, in this study, we describe the development process of the sperm banking kit including the initial stages of testing for kit components. A comparison of two transport media and the standardisation of the temperature in the kit are performed to determine the optimal NextGen® kit configuration, its feasibility and the effect of overnight shipping on sperm function.

Materials and methods
This study was approved by the institutional review board of the Cleveland Clinic. Twenty-one semen samples were collected from normospermic men by masturbation into a sterile wide mouth collection cup following a minimum of 48 to 72 h of sexual abstinence. The donors were healthy males, between the ages of 20 and 35 years old and whose semen samples fulfilled the criteria for normal semen parameters established by the World Health Organization, 2010 guidelines for semen analysis. The inclusion criteria for the donors were as follows: (1) normal semen parameters; (2) no sexually transmitted infections; (3) no recreational drug use; and (4) may or may not have initiated a pregnancy in the past. Following liquefaction at 37 °C for 20 min, semen specimens were evaluated for volume, sperm concentration, total cell count, motility and vitality according to the World Health Organization, 2010 guidelines. In this study, each of the 12 men collected a single sample at the Andrology Laboratory at Cleveland Clinic and each of the nine men collected a single sample at home.

Effect of various media on sperm motility, count and vitality
For the initial standardisation, each sample was divided into six equal aliquots. Three aliquots of each sample were supplemented with equal 1 : 1 volumes of refrigeration media (RM; Irvine Scientific, Santa Ana, CA, USA), while the remaining three aliquots were supplemented with equal 1 : 1 volumes of human tubal fluid (HTF) (Sharma et al., 1997). Each aliquot (one from RM-added aliquot and one from HTF-added aliquots) was incubated for 24 h at temperatures of 24 °C, 37 °C and 40 °C respectively. We chose 24 °C and 37 °C due to conflicting study reports on the optimal temperature for the maintenance of sperm motility and fertility (Appell & Evans, 1977; Appell et al., 1977; Esfandiar et al., 2002) and the ability of the spermatozoa to penetrate zona-free hamster eggs (Cohen et al., 1985). We chose 40 °C to determine the effects of high temperatures on seminal parameters that could arise during the actual shipping of samples, especially in extreme climates. Sperm motility, count, vitality by hypoosmotic swelling (HOS) test and percentage recovery of motile spermatozoa were assessed after a 24-h incubation period.

Measurement of sperm vitality
Each semen sample was examined for vitality using the HOS test (Esteves et al., 1996). One hundred microlitres of liquefied semen was mixed with 0.9 ml of HOS
solution and incubated at 37 °C for 1 h. After incubation, a 5 µl aliquot of the sample was examined for tail swelling using phase contrast illumination. A total of 200 hundred spermatozoa per sample were scored. Spermatozoa were classified as osmotically competent if tail swelling was observed after exposure to the hypoosmotic solution. Spermatozoa displaying >60% swollen tails were considered as normal with intact membrane.

Optimisation of overnight shipment temperature

The goal was to test the temperature of the ice sleeve surrounding the semen specimen collection container as well as the temperature of the ice packs surrounding the sleeve to determine its ability to maintain the temperature during shipment, melting of the ice bricks and to avoid exposure to extreme temperatures. To examine this, we assessed 5 different ice packs, both gels and ice bricks (Polar Tech Industries Inc., Genoa, IL, USA) in terms of their ability to maintain the desired temperature of the kit and its contents during shipping. Each of the five different kinds of ice packs was placed at the bottom and top of the shipping containers and was tested for their ability to resist temperature elevation when placed frozen inside the kit and incubated at 37 °C for 24 h. Ice packs were frozen for 24 h in a −18 °C freezer prior to usage as per the manufacturer’s instructions. We also assessed the effect of adding a cooling sleeve directly around the semen specimen container and placing ice packs around the cooling sleeve. Temperatures inside the kit were recorded using a digital thermometer – the probe was placed in the centre of the kit, surrounded by the appropriate ice packs. To simulate shipping conditions, the kit was then placed in an incubator to compare the ability of the five ice packs being tested to resist a temperature increase and to maintain the temperature of the kit. Temperature readings were taken by digital output provided by a thermometer unit placed outside the incubator in addition to comparisons between different ice packs.

Effect of 24-h incubation at 37 °C on sperm motility, count and vitality

Ten semen samples from 10 healthy donors were examined. Each sample was analysed pre-incubation for motility, concentration and vitality. Each sample was then supplemented with 5 ml of RM, placed in the kit with the freezing components and incubated for 24 h at 37 °C. Post-incubation samples were again assessed for motility, count, vitality and percentage recovery for motile spermatozoa.

Effect of overnight shipment in NextGen® kit on sperm motility, count, vitality and percentage recovery for motile spermatozoa

Once the transport media and cooling packs were chosen, we shipped nine kits (Figure 1) to seven different andrology laboratories in the United States. Semen samples were collected from normospermic men (n = 9) by masturbation into a sterile wide mouth collection cup following a minimum of 48–72 h of sexual abstinence from various andrology laboratories. We chose these laboratories located in cities based on its distance from Cleveland as well the variation in temperature/climate in each city (e.g. Washington, DC vs. Orlando, FL). The kits contained the necessary components as well as next-day air return envelope identical to the kind that would be used by a patient. Each sample was analysed pre-shipment for motility, count and vitality according to the WHO, 2010 criteria (World Health Organization, 2010). Each sample was then supplemented with 5 ml of RM, placed in a NextGen® kit and shipped overnight to Cleveland Clinic. Shipments were scheduled overnight such that the sample would be picked up around 5:00 pm at the origin city and would arrive at the Clinic around 10:30 am, marking an average 17.5-h transit time. Post-shipment samples were assessed for motility, count, vitality and percentage recovery for motile spermatozoa.

Statistical analysis

Summaries of quantitative variables are expressed using medians and ranges. Comparisons of RM and HTF were performed using Wilcoxon rank sum tests, in anticipation of non-normal distributions. Comparisons of pre- and post-incubation periods, or pre- and post-shipment, were
performed using the Wilcoxon signed rank test for paired data.

Percentage sperm recovery was calculated as:

\[
\text{Post-shipment total motile spermatozoa (TMS)} \times 100
\]

\[
\text{Pre-shipment TMS}
\]

Results

Effect of various transportation media on sperm motility, count and vitality

The differences between RM and HTF were assessed on their ability to sustain sperm motility, count and vitality after 24 h of incubation at three varying temperatures: 40 °C, 37 °C and 24 °C.

Samples with RM resulted in excessive clumping at 40 °C and 37 °C post-incubation that made assessment of concentration difficult. The percentage of motile sperm recovery was not different (P = 0.31) for RM (median 28.6%) compared with HTF (median 54.4%) Post-incubation membrane integrity measured by the HOS test was comparable (Table 1). The post-incubation motility was best preserved at 24 °C with both types of media. Higher temperatures of 37 °C or 40 °C resulted in complete loss of motility function.

Optimisation of overnight shipment temperature and assessment of temperature resistance of various ice packs

The gel-based ice packs were not as successful in maintaining temperature compared to the solid ice bricks (TechPack Bricks, Polar Tech Industries Inc. Genoa, IL, USA). Solid ice bricks were the most efficient at maintaining optimal temperature. The cooling sleeve allowed for a slower melting once the sleeve reached 5 °C. Thus, a cooling sleeve wrapped around the collection container and cushioned with two ice bricks (TechPack) provided the maximum temperature retention.

Effect of 24-h incubation at constant 37 °C on sperm motility, count and vitality

Following the selection of the components for the home shipping NextGen® kit and the appropriate supplement media, we examined the ability of the NextGen® kit to sustain motility and vitality in ten samples incubated at 37 °C for 24 h. After 24 h, there were large decreases in motility, vitality and TMC (Table 2).

Effect of overnight shipment in NextGen kit on sperm motility, count and vitality

Table 3 summarises the differences in sperm motility, concentration and membrane integrity before shipment of the nine kits and after its receipt. The median pre-shipment percentage motility of the samples was 50.0% and post-shipment motility decreased to a median of 31.0%. The percentage recovery in motility was 46.5%.

The average pre-shipment total motile spermatozoa (TMS) were 44.3 × 10⁶ and post-shipment TMS were 24.3 × 10⁶ million as shown in Table 3. Two of the 10 samples had bacterial contamination and very poor motility.

Discussion

Patients with cancer are encouraged to bank their semen specimens prior to the start of their cancer treatment. Due to the fact that the window of opportunity to bank semen samples and the start of the treatment are too narrow, patients are advised to provide multiple ejaculates in the time that is available before the initiation of the treatment. With the availability of assisted reproductive

Table 1 Difference in pre- and post-incubation sperm quality after addition of refrigeration medium or human tubal fluid

<table>
<thead>
<tr>
<th>Semen Parameters</th>
<th>Refrigeration medium n = 6</th>
<th>Human Tubal Fluid n = 6</th>
<th>Wilcoxon rank sum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motility (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-incubation</td>
<td>50.0 (30.3, 69.8)</td>
<td>50.0 (30.3, 69.8)</td>
<td>0.0</td>
</tr>
<tr>
<td>Post-incubation</td>
<td>0 (0, 57.1)</td>
<td>0 (0, 62.7)</td>
<td>1.0</td>
</tr>
<tr>
<td>Percentage</td>
<td>0 (0, 81.8)</td>
<td>0 (0, 89.8)</td>
<td>1.0</td>
</tr>
<tr>
<td>Recovery HOS (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-incubation</td>
<td>49.1 (36.8, 61.4)</td>
<td>49.1 (36.8, 61.4)</td>
<td>0.18</td>
</tr>
<tr>
<td>Post-incubation</td>
<td>11.3 (6.5, 67.5)</td>
<td>27.8 (17.0, 60.0)</td>
<td>0.0</td>
</tr>
<tr>
<td>Percentage</td>
<td>28.6 (10.6, 109.9)</td>
<td>54.4 (29.3, 118.4)</td>
<td>0.31</td>
</tr>
</tbody>
</table>

Values are median (min, max). P < 0.05 was considered significant.

Table 2 Pre- and post-incubation differences in sperm quality (n = 10)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pre-incubation</th>
<th>Post-incubation</th>
<th>Wilcoxon signed rank sum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motility (%)</td>
<td>45.9 (40.0, 60.4)</td>
<td>15.8 (3.0, 49.0)</td>
<td>0.005</td>
</tr>
<tr>
<td>HOS (%)</td>
<td>53.6 (37.4, 67.3)</td>
<td>39.0 (14.0, 63.0)</td>
<td>0.002</td>
</tr>
<tr>
<td>TMS (×10⁶ spermatozoa)</td>
<td>19.6 (5.5, 100.3)</td>
<td>5.2 (1.7, 22.1)</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Values are median (range). P < 0.05 was considered significant. HOS, Hypoosmotic swelling test; TMS, Total motile spermatozoa.
techniques such as ICSI, the only requirement is the availability of single, morphologically normal, motile spermatozoa. Therefore, the number of spermatozoa available for each bank is of less importance; rather, it is more important to have multiple banks so that the patient has the opportunity to pool the available samples they have banked for procreation at a later date.

Remote sperm collection at home has been studied by many researchers in the past (Young et al., 2003; Zavos et al., 2006), and these studies have found that sperm collection at home can be performed safely. It has been reported that semen collection at home generates better quality semen than in a laboratory (Elzanaty & Malm, 2008). However, Licht et al. and Song et al. have not reported any differences between semen parameters in collections made at home or in the laboratory (Song et al., 2007; Licht et al., 2008).

The goal of our study was to standardise the components of the kit, to establish that the semen sample temperature is maintained during the overnight shipment of the sample and that the sample quality is not compromised. To accomplish this, it is important that the surrounding temperature of the specimen collection cup is also maintained and not affected by external temperatures, in case of any unforeseen delay in the delivery of the package or exposure to extreme temperature. It is prudent that the ice packs surrounding the NextGen kit and the ice sleeve wrapped around the specimen cup do not melt. Therefore, we performed a quality control to see how long the ice would maintain its frozen state without melting at the temperatures tested.

In the earlier studies examining the effect of temperature on sperm motility (Appell & Evans, 1977), the researchers examined semen specimen from pre-vasectomy patients that were maintained at 4 °C, 20 °C and 37 °C and were evaluated at 3 h, 6 h, 12 h and 18 h after collection. They concluded that samples should be kept at room temperature (20 °C) and not at 37 °C as any increase in temperature will lower motility. In another study by Cohen et al. (1985), spermatozoa stored in refrigerator had significantly higher fertilisation rates compared to samples stored at room temperature. In a study by Esfandiari et al. (2002), samples stored at 37 °C maintained the highest motility at 37 °C. Therefore, in the standardisation of the NextGen kit, we chose to use 24 °C as an optimal ambient room temperature, and 37 °C and 40 °C were selected as temperatures that the kit may be exposed to during the actual shipping of samples, especially in extreme climates or weather conditions.

Refrigeration medium TYB from Irvine Scientific is designed for short-term storage of human spermatozoa at 2° to 5° C, and it contains TYB and gentamicin. It has been used for overnight storage and shipment of samples. It contains TES and Tris and heat-inactivated egg yolk. It is intended for refrigeration of semen for use in assisted reproduction. Human tubal fluid from Irvine Scientific is a synthetic solution for use as a culture media for processing of gametes. It also contains gentamicin as an antibiotic. In an earlier study (Sharma et al., 1997), we compared the quality of spermatozoa prepared in RM and HTF medium by examining the motility after thawing. Results from the use of both mediums were comparable. Similarly, in this study, we found comparable results in motility, percentage recovery and HOS testing of both pre- and post-incubation in samples transported in HTF and RM. Refrigeration medium has been suggested as a preferred medium for overnight shipment and storage of semen samples. Therefore, although both media appear to be equally good in preserving motility, we preferred to use RM for transportation of spermatozoa in the NextGen® home banking kit.

Furthermore, based on the tests on different kinds of ice packs, the cooling sleeve allows for a slower melting at 5 °C. Therefore, this was wrapped around the specimen collection cup. The cooling sleeve was cushioned between two solid ice bricks (TechPack). This combination was most effective in retaining the optimal temperature required for overnight shipment of the NextGen® kit.

To evaluate the effects of a potential shipping delay or accidental moderate warming of the samples, we investigated the effects of 37 °C and room temperature (24 °C) on spermatozoa attributes pre- and post-incubation. There was a significant decrease in post-incubation motility ($P = 0.004$) and post-incubation vitality ($P = 0.004$) at samples exposed to room temperature (24 °C). This decrease could be attributed to bacterial contamination inside two of the ten samples during incubation. A decrease in motility and increase in bacterial counts was reported in samples maintained at 37 °C (Appell &
Evans, 1977). Even though the refrigeration media contains gentamicin, which will help delay normal bacterial growth, bacterial growth in the two samples in our study may have been transmitted during semen collection due to urogenital infection. This is an important finding, indicating that if the patient were to have an infection during the time of collection, the quality of the semen sample could be affected (Rybar et al., 2012).

We also shipped nine NextGen® kits to seven different andrology laboratories in the United States to determine the effects of actual shipping on the sperm parameters. Results indicated that samples shipped from various distances and temperatures using the NextGen® kit were successful in sustaining sperm quality. Our results show that if the home shipping kit is assembled correctly and shipment is optimised for overnight delivery in a time-appropriate manner, then the sperm banking kit can provide a safe, reliable, efficient and most of all convenient manner for men to transport their samples to the Cleveland Clinic from all parts of the United States.

The home sperm banking kit (NextGen®) presents a potential strategy to decrease barriers to sperm banking such as privacy, discomfort and access to sperm banking facilities (Young et al., 2003). By standardising the kit components, we can provide a standard of care for fertility preservation to men with cancer and infertility issues. Producing a semen sample on site is the best option. However, the kit offers patients who cannot come to a sperm bank facility with an alternative to collect a semen sample in the privacy of their own home and ship the samples overnight to the andrology laboratory for further processing and storage. Collecting semen at home and transporting the sample overnight reduces emotional anxiety, the need to travel from geographically distant places (different cities/states), and is cost-effective in terms of travel, hotel, taking time off from work, etc.

To our knowledge, this is the first study that has determined the effects of remote collection and shipping on semen parameters. Studies on other similar remote collection kits have only simulated the overnight shipping conditions or have evaluated only post-shipping semen parameters and thus are lacking comparative results (Royster et al., 2000).

Shipping delays resulting in accidental moderate warming of the samples might induce chromosome/DNA damage after remote collection and overnight shipping of semen samples (Young et al., 2003). Poor quality samples generally do not freeze as well, and this method will decrease the initial quality. So while this method allows men to bank who would otherwise not bank, if possible, it should not be used as a substitute to on-site production of samples.

There were some limitations to our study. One of our study limitations was the small sample size. We will be conducting another follow-up study with a larger number of patients in the future. Furthermore, we did not examine the samples for oxidative stress, apoptosis or DNA fragmentation that can be detrimental to the fertilising potential of the spermatozoa. Future plans include offering the NextGen® kit to our patients requesting collection of spermatozoa at home and shipping the kit overnight to determine rates of sperm banking among patients at our centre and from other centres that are lacking in sperm banking services.

Conclusion

In conclusion, we have standardised and designed a specialised sperm collection and transport kit, NextGen® at the Andrology Center at the Cleveland Clinic. This is an innovative, first-of-its kind product that is evaluated in a clinical setting and is specially designed for men with cancer and other clinical diagnosis of infertility that requires sperm banking. Semen parameters were maintained by remote collection and shipping utilising the NextGen® kit. Patients can collect semen sample in the privacy of their own home and ship the samples overnight to the andrology laboratory for further processing and storage. Although sperm quality is reduced, an adequate number of good quality spermatozoa are available that can be used by ART, such as ICSI for procreation. Collecting semen at home and transporting the same overnight reduces barriers such as emotional anxiety and the need to travel from remote locations.

Acknowledgements

This study was supported by the American Center for Reproductive Medicine, Cleveland Clinic. The authors thank Amy Moore for her help with the editing of this manuscript.

References


