Sperm parameters that play a major role in the assessment of semen quality after cryopreservation

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Received: 1 March 2017 / Accepted: 7 June 2017 / Published online: 17 June 2017
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Semen cryopreservation is the only available method to preserve the fertility in young and adult men. Semen freezing is the first line option for the group of patients in which fertility preservation is required. Conventional slow freezing of spermatozoa is commonly used for cryopreservation of both ejaculated and surgically retrieved spermatozoa for preservation of fertility before cancer treatment, in severe male factor infertility (obstructive azoospermia) and for establishment of donor banks. Cryopreservation of spermatozoa is therefore an important part of a successful assisted reproductive technology program.

Sperm cryopreservation should be considered in cases of azoospermia so repeated surgical sperm retrieval techniques are avoided; also, regarding male patients who present impaired semen parameters, sperm cryopreservation is indicated to prevent the risk of azoospermia [1]. In addition, fertility preservation is a major concern for male cancer patients who are undergoing chemotherapy or radiotherapy because these gonadotoxic agents threaten their reproductive potential [2]. Moreover, some studies suggest that not only malignancies but also other non-malignant diseases including autoimmune disorders affect male fertility. Nowadays, there is an increasing interest in fertility preservation of patients with cancer or systematic diseases, since survival rates are increasing due to the new therapeutic options [3, 4]. Therefore, as long-term quality of life after cancer treatment has become a significant fact, semen cryopreservation before chemotherapy is seen as a

real possibility of maintaining male reproductive potential after cancer treatments.

Unfortunately, semen cryopreservation has a great impact on the quality of thawed samples. According to several studies in this field, viability of semen samples after freezing and thawing process only raise 50% [5]. The drastic decrease in viability of thawed spermatozoa is due to cell damage caused by intracellular ice formation, and osmotic and oxidative stress during the freezing process [6–8]. During the freezing process, freezing rates and concentration of cryoprotectant agents have to be balanced, to minimize mechanical and osmotic damage. Despite all the research done to date, there is no standardized freezing protocol leading consistently to higher viability rates. Moreover, in the literature, there are a wide range of sperm parameters that are thought to be affected by sperm freezing, but only a few are really useful in clinical practice.

Cryopreservation and cryoprotectant agents

Low temperatures and osmotic stress during freezing cause stress-induced damage to sperm cells. The temperature decrease leads to lipid peroxidation, causing reactive oxygen species (ROS) formation [7]. Osmotic stress is a result of extra-cellular ice crystal formation. The extra-cellular ice crystals increase solute concentration in the extra-cellular media, causing cellular dehydration in order to maintain osmotic balance. Even though sperm cell dehydration avoids intracellular ice crystal formation, if the cell shrinkage exceeds the osmotic tolerance, the consequences could be lethal. Cellular dehydration and osmotic cryoinjury occur when low cooling rates are used. In contrast, at high cooling rates, cellular dehydration is not completed enough, so there is still liquid water inside sperm cells causing intracellular ice crystal formation [9]. Freezing-induced damage is caused by osmotic

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and mechanical stress during temperature decrease, but once sperm cells are frozen in liquid nitrogen, the period of time does not increase the damage extent. The study of Tiwari and collaborators reflect this fact.

Cryoprotectant agents are needed during the freezing process since they play a protective role. Permeating cryoprotectants can pass through sperm cell plasma membrane and replace water molecules. Non-permeating cryoprotectants cannot pass through the plasma membrane and promote cellular dehydration by increasing extracellular solute concentration. However, high concentrations of cryoprotectants contribute to osmotic damage. To minimize toxic effects of cryoprotectants, it is important to do the addition and removal method properly. Both, addition and removal must be stepwise processed in order to minimize osmotic shock [9].

The most commonly used cryoprotectant agent is glycerol, and the most common extender is egg yolk. Egg yolk contains phospholipids and cholesterol that aid to increase the cryotolerance of the sperm cell plasma membrane. However, egg yolk and other extenders as bovine serum albumin (BSA) derive from animal origin [10]; hence, these compounds could be a potential source of infectious animal diseases. Therefore, more research is needed to find new animal-free extenders that contribute to standardize the sperm freezing process. The recent article of Tiwari is a precedent in this issue.

However, there are some points regarding sperm cryopreservation that deserves special attention in the study of Tiwari and collaborators [11] in order to avoid biased conclusions. Firstly, cryoprotectant agents are required for cryopreservation, but they are also toxic for sperm cells. After thawing, the cryoprotectants must be rapidly removed by centrifugation. Tiwari and collaborators [11], in their study, did not remove cryoprotectant agent until 4 hours after thawing. Therefore, during this period of time, there is a decrease in sperm motility, as a response to cytotoxic effects of cryoprotectants. Secondly, there is a high variability in the cryoresistance within sperm samples. Therefore, to compare the effect of different cryoprotectant agents, an aliquot of the same semen sample must be used. Even though Tiwari and collaborators report that long and short-term cryopreservation, it was performed from a duplicate of the same sample [11]; it is not clarified whether aliquots from the same semen sample were used in order to test both cryoprotectants included in their study. Also, it should be noted that, as we could guessed, only normozoospermic patients were included in the study, so in future studies would be recommended to include male factor patients because sperm cryopreservation is indicated specially in patients with impaired sperm parameters. Moreover, sample availability is often limited in this group of patients, so it is even more important to achieve the best semen quality in thawed samples. Moreover, the sample size is still small, only results from 11 patients are published.

Seminal parameters affected by sperm freezing

In the literature, a wide range of seminal parameters are found to be affected by cryopreservation, but it is important to take into account that not all of them are useful enough to be implemented in the clinical routine.

Sperm DNA integrity

The sperm DNA integrity is one parameter that some authors include in their studies. The production of ROS is the main mechanism by which DNA integrity is affected during cryopreservation of semen samples [8, 12]. The addition of antioxidants to semen samples improves cryopreservation results, in terms of motility and viability [12, 13]. So, these studies demonstrate that the oxidative stress is the main cause of the increased DNA fragmentation after thawing. Ribas-Mayou also showed that after semen cryopreservation was observed, there were single-strand DNA breakages; these sorts of DNA lesions are related to oxidative stress-induced damage [13]. However, the effect of cryopreservation on sperm DNA integrity is still controversial. Different conclusions about the relationship between sperm DNA integrity and sperm freezing can be due to different cryoresistance, different freezing protocols, and different methods used to assess DNA integrity. In addition, sperm DNA integrity prior to the freezing process is also a relevant factor. In regards of sperm DNA integrity, *in situ* DNA nick translation was the method chosen in order to evaluate sperm DNA integrity in the study of Tiwari and collaborators [11]. Tiwari reports a decrease in normal sperm cells (light-stained cells after immunocytochemistry detection), as well as, an increase in sperm cells presenting DNA chain fragmentation (dark-stained cells) after freezing and thawing, but it must be noted that no statistical significance value is reported. Even though DNA nick translation is an elegant method to assess DNA integrity, the level of DNA fragmentation and reproductive outcomes is not strongly correlated. While it is demonstrated that cryopreservation increases DNA fragmentation, as it is also suggested in the research performed by Tiwari and collaborators [11], there is no strong evidence of decreased fertilization rates or worse reproductive outcome in cycles in which frozen semen is used. The fertilization rate and early embryo development not only depend on paternal DNA contribution, but also it is strongly correlated with oocyte quality. It is thought that oocyte has the machinery needed to repair damaged paternal DNA [8].

Maturity status biomarkers

Currently, some studies have described a group of biomarkers related to spermatozoa maturity status. High cytoplasmic levels of creatinine kinase (CK) are related to spermatozoa immaturity, indicating a defect in cytoplasm extrusion during

the last steps of spermatogenesis. Another sperm maturity biomarker, in combination with a low CK level, is a high expression of the chaperone HspA2 [14].

Moreover, in mature spermatozoa have been characterized, at least, three hyaluronic acid binding proteins. These membrane receptors are involved in several relevant physiological processes as acrosomic reaction, the action of hyaluronidase and the binding to zona pellucida. A kit based on the spermatozoa binding ability to hyaluronic acid is available [15]. The hyaluronic acid binding assay (HBA assay) selects those mature spermatozoa that bind to hyaluronic acid. Even though this assay has been used in several research works, including the recent study of Tiwari, there are some studies that do not observe any correlation between spermatozoa hyaluronic acid binding capacity and survival rate after thawing [16, 17]. This fact shows that hyaluronic acid binding sites are not affected by the freezing/thawing protocols. So, HBA assay is not useful enough to be used as freezability marker.

Tiwari and collaborators, in their recent research work [11], have studied in-depth on how sperm maturity status is affected by the freezing process. HA binding, CK levels, and arrested chromatin maturity are maturity markers included in this study. Tiwari and collaborators suggest that in thawed samples were observed higher percentages of immature spermatozoa. Unfortunately, this fact is not showed clearly in their results since any reference value of statistical significance is reported. In addition, attending to the graphs representing the results for CK immunochemistry, there is no significant difference in the percentage of light (mature spermatozoa) and stained cells (immature spermatozoa) between fresh and thawed samples. Furthermore, analyzing the results from aniline blue immunohistochemistry, in spite of the lack of a statistical significance value, the graphs show a slight decline in dark-stained sperm cells after freezing. Aniline blue staining represents an arrest in chromatin maturity; hence, dark cells indicate arrested development while light cells correspond to mature cells. Therefore, the slight decrease in the percentage of dark cells in thawed samples reveals that after freezing; there are less immature spermatozoa. Anyway, there is a lack of evidence in the data reported to conclude that maturity status is affected by the freezing process.

Apoptosis

It is demonstrated that sperm cryopreservation induces apoptosis. Apoptosis is a programmed cell death mechanism that involves a large number of cell proteins. It is well-documented that caspase family proteins play an important role in apoptosis cascade [18]. Tiwari and collaborators did not observe any difference in apoptotic (status between fresh and frozen/thawed samples, as it is showed in caspase 3 immunochemistry

results [11]. The lack of differences in the apoptotic status can be due to the low sample size (11 samples) considered in this study.

Sperm motility, viability, and total motile sperm count

Spermatozoa can exhibit three types of motility, which are grouped in two main categories: progressive (types a and b) and non-progressive motility (type c). Total motility (TM) includes progressive and non-progressive motility (PM and NPM). Routine semen diagnosis is based mainly in sperm progressive motility due to the difficulty to detect non-progressive spermatozoa in the traditional Mackler chamber. However, it is a tendency towards higher objectivity of semen analysis. Therefore, computer-assisted semen analyzer (CASA) systems allow more objective semen analysis. Moreover, CASA systems provide information about sperm concentration and also several kinetic parameters of spermatozoa movement. So, three types of sperm motility and sperm concentration can easily be evaluated by using a CASA system. Even though Tiwari and collaborators [11] used a CASA system, it is not specified which type of motility is considered in their work. From our own experience, progressive motility does not consider all viable spermatozoa in a sample whereas total motility fits more accurately to sperm viability.

Sperm viability is the main relevant parameter in order to evaluate cryopreservation results. Viability of a semen sample can be evaluated by HOS test or eosin test. These tests focus on spermatozoa membrane integrity, so non-motile but viable spermatozoa can be detected by these methods [19]. Several studies agree that after cryopreservation, there is a decrease in the number of viable spermatozoa because of the detrimental effects of cryopreservation process on the sperm membrane [19–22]. Eosin staining does not allow the use of stained sperm sample in following artificial reproduction techniques. In contrast, HOS test is based on the swelling capability of spermatozoa when they are exposed to hypo-osmotic environment, in order to maintain osmotic balance. After HOS test, the spermatozoa remain viable so, the sample could be used in the following ICSI. However, some studies suggest that the spontaneously developed tail swellings (SDTS) occur in some cases. SDTS increase the percentage of false positives because the tail swelling is not a response to the exposure to hypo-osmotic solution. Hossain also reports that SDTS increases in thawed samples [21]. Therefore, it is important to develop other viability tests that avoid this phenomenon.

Our preliminary results (data not published yet) show a strong correlation between TM and HOS test results; hence, TM could be used in order to assess sperm viability in thawed samples. That is why it is crucial to specify which type of

motility is considered, while TM gives an approximate value of viability, PM is dismissing a part of viable spermatozoa.

Moreover, together with total motility, sperm count is another relevant factor to take into account, especially when sample availability is limited. Total motile sperm count (TMSC) is a parameter that combines sperm total motility and sperm concentration. Recent studies in testicular cancer patients' cryopreservation [23], in ongoing pregnancy [24] and in ICSI cycles [25] suggest the relevance of TMSC in achieving better reproductive outcomes. TMSC gains importance when a subfertile men population is considered due to these patients often present impaired sperm quality parameters as sperm count or motility. In this group of patients, it is vital to obtain the maximum number of motile (or viable) spermatozoa. In the study of Tiwari and collaborators, it is not highlighted enough the importance of these parameters [11].

Role of seminal plasma and sperm selection techniques in cryopreservation

Drawing attention to components in the ejaculate that prevent cryodamage; seminal plasma plays a protective role during sperm freezing. Seminal plasma is a secretion of several glands of the male reproductive system that contains factors that prevent acrosomic reaction and other physiological changes that spermatozoa must suffer to acquire their fertilization potential. Also, in the seminal plasma, there are antioxidants found, lipids, proteins, sodium, calcium, sugars (fructose and sucrose), as well as other products derived from sugar metabolism as lactic acid and pyruvate. Glutathione peroxidase, superoxide dismutase, and other enzymatic compounds (E vitamin or carotenoids) are the antioxidant systems that prevent ROS damage during freezing. Moreover, polyunsaturated fatty acids (PUFAs) present in seminal plasma increases plasma membrane fluidity, improving the resistance to low temperatures [26]. Some proteins in seminal plasma, as heparin binding proteins (HBPs), prevent thermal shock and lipid peroxidation [27]. Physical adsorption of certain proteins to spermatozoa surface could also prevent the thermal shock [28]. Hence, the potential protective role of seminal plasma during the freezing process cannot be dismissed. So freezing fresh ejaculate before sperm preparation or selection could take advantage of the protective role of seminal plasma. However, some studies suggest that semen preparation, by swim-up or density gradients, prior to the freezing process results in higher progressive motility after thawing.

From our experience (data not published yet), freezing fresh ejaculate before sperm selection technique improves both progressive and total motility as well as TMSC. Donnelly also reported higher PM percentages when freezing prior to sperm selection by swim-up (45% PM vs 23% PM) [29]. In addition, this study demonstrated the beneficial effect

of seminal plasma during cryopreservation. Donnelly and collaborators found that swim-up preparation prior freezing in presence of seminal plasma leads to PM percentages similar to those achieved by freezing fresh ejaculate before swim-up selection (50% PM and 45% PM, respectively). However, Donnelly did consider neither TM nor TMSC.

In the study of Tiwari and collaborators, sperm selection by density gradient is performed after thawing [11]. Their results do not show a great increase in motility percentages because sperm selection by density gradients is performed after 1 and 4 h post-thawing. As it has been commented in this study, cryoprotectant agents are not retired immediately after thawing; hence, cytotoxic effects of cryoprotectants are showed in motility decrease observed at 1 and 4 h after thawing. This could be the reason why motility recovery after density gradients is not as high as the results published by other authors. A decrease in sperm concentration is shown after density gradients selection due to sperm selection techniques (as swim-up or density gradients centrifugation) select a sub-population of higher quality spermatozoa. Motile sperm count would be recommended in this case, because it shows more clearly the amount of viable spermatozoa in the sample. In addition, according to Donnelly, swim-up selection raises higher PM percentages than Percoll density gradients.

In contrast, other studies suggest that sperm selection prior to freezing leads to higher motility percentages. Two studies done by Petyim [30] and Esteves [31] suggest an improvement in motion characteristics when sperm preparation by swim-up is performed prior to cryopreservation. Regarding the study of Petyim and collaborators, their results reported a slight improvement in sperm motility after freezing when semen sample is prepared by swim-up (99.5% PM vs 93.9% PM). Despite the light difference in progressive motility percentage between two cryopreservation protocols, it raises a high level of significance, possibly because of the low variability in motility values among the patients studied (from 41.9 to 64.2%) [30]. It would have been interesting to compare these results with those obtained from a subpopulation of patients with asthenozoospermia, where, undeniably, the motility values will vary among the patients. The results published by Esteves and collaborators show the same tendency [31]. Even though in the cited research work is reported with a slight improvement in sperm motility after freezing when semen sample is prepared by swim-up (30.1% PM vs 28% PM), it does not reach statistical significance, as it is clearly shown in the result table provided by the authors. Therefore, the improvement in sperm motility is not clearly established.

However, according to the number of spermatozoa obtained, in both studies not only with the total sperm count, but also with the total motile sperm count are higher when fresh ejaculate is frozen prior to sperm selection by swim-up [30, 31]. Despite its high level of significance, this result is not detailed in-depth in the results or discussion.

As TMSC provides information about the number of viable spermatozoa available, it should not be dismissed because it could guide to an inaccurate vision of the research performed.

Another relevant fact to remark is that these studies only include healthy donors. TMSC gains importance when a subfertile men population is considered due to these patients often present impaired sperm quality parameters as sperm count or motility. In this group of patients, it is vital to obtain the maximum number of motile spermatozoa.

A more recent study published by Brugnon in 2013 [32] studied a population of oligoasthenoteratozoospermia male patients, but in this case, the study design is not as accurate as it could be since each study group include different patients, so it is not used with the same semen samples from different patient were used in each protocol. The conclusions are very close to what Esteves and Petyim suggested [30, 31].

As a summary, even though some studies suggest an improvement in sperm motility when sperm selection is performed prior to cryopreservation, when TMSC is considered, there is a change in the tendency observed, so higher TMSC are achieved when the fresh semen samples are frozen before sperm selection.

Conclusions

In conclusion, some important facts regarding human semen cryopreservation must be reviewed in the study of Tiwari and collaborators [11]. First of all, a suitable experimental design is crucial to reach accurate conclusions. In the study of Tiwari and collaborators, aliquots of the same semen samples must have been used to compare the effect of the two cryoprotectant agents studied. Then, only results from 11 semen samples were published, so in further studies, sample size should be increased. Moreover, a reference value of statistical significance must be included in the study.

In regards of the parameters studied, some of the biomarkers used as CK, aniline blue staining, or HA binding capacity lack of significance whereas other semen parameters that could provide more useful information (as PM, TM, or TMSC) are dismissed. From our experience, TMSC gives information related to the amount of viable spermatozoa in an objective and simple manner, using a CASA system.

The relevance of using TMSC must be highlighted even more in the cases of semen sample availability that is limited, for examples in oncologic patients or in cases of pseudoazoospermia.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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