Case report: the use of annexin V coupled with magnetic activated cell sorting in cryopreserved spermatozoa from a male cancer survivor: healthy twin newborns after two previous ICSI failures

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Abstract

Purpose The aim of the study is to report successful outcome (live births) after sperm sorting with annexin V-MACS on cryopreserved spermatozoa with high level of sperm DNA fragmentation from a cancer patient survivor.

Methods Cryopreserved spermatozoa were sorted with annexin V-MACS prior to ICSI. Sperm DNA fragmentation was evaluated by SCSA[®] and TUNEL.

Results The couple had two previous IVF/ICSI cycles failures using sperm cryopreserved before cancer treatment. On third ICSI cycle attempt results were as follow: pre-annexin V-MACS sperm quality: 10×10^6 /ml, 3.3 % progressive motility, 1 % normal forms, TUNEL: 72.5 % positive cells, SCSA[®]: 76.6 % DFI. Post-annexin V-MACS sperm quality: 2.8×10^6 /ml, 10 % progressive motility, TUNEL: 58.8 % positive cells. Eight metaphase II oocytes were collected, 4 fertilized, 2 embryos were transferred on day 3 and healthy twins were born (1 boy, 1 girl).

Capsule Sperm cryopreservation is an effective method to preserve fertility. However, sperm DNA integrity could be impaired. The use of annexin V-MACS, to reduce the number of apoptotic sperm in a male cancer survivor, improve sperm quality that in turn led to better embryo quality. This is the first report of a successful live birth after using annexin V-MACS on pre-chemotherapy cryopreserved spermatozoa.

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Conclusions Annexin V-MACS technique could be a potential tool to improve sperm quality on cryopreserved spermatozoa of cancer patient and improve ICSI outcome.

Keywords Cancer survivor · Cryopreserved sperm · Annexin V-MACS

Introduction

With the advances in cancer therapy particularly with combination chemotherapy regimens, survival rates from many common cancers affecting men at reproductive age such as testis cancer, Hodgkin's and non-Hodgkin's lymphoma have improved significantly [21]. Since cytotoxic anti-cancer therapies for these malignancies can affect male reproductive health, an important step in oncofertility counselling for young men with newly diagnosed cancer is sperm cryopreservation. Cryopreserved sperm can be used for assisted reproduction techniques (ART) such as intrauterine insemination (IUI), in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI).

Though cryopreservation is currently the only established method to preserve male fertility, it has significant limitations. Current freezing-thawing protocols of sperm cause a decrease in sperm motility and morphology as well as DNA quality [8]. In particular, the effect of cryopreservation and thawing on DNA integrity has been shown to be greater in infertile men than in fertile men [1,8]. More importantly, we have recently shown that in some young men with testis cancer and lymphoma, at the time of sperm cryopreservation before beginning chemotherapy, sperm DNA integrity and compaction were significantly impaired compared to age-matched healthy individuals [17]. As DNA fragmentation has been shown to be negatively correlated with embryo quality, implantation rates and miscarriages, ideally spermatozoa containing fragmented DNA should be avoided when using ART [2,4,33,34].

In two separate reports [22, 23], it was demonstrated that the proportion of spermatozoa showing apoptotic features can be reduced by the use of annexin V coupled to magnetic activated cell sorting (MACS). Indeed, within the early events of apoptosis, spermatozoa will externalize phosphatidylserine in the outer leaflet of the plasma membrane. Annexin V has specific affinity to phosphatidylserine, therefore it can bind to the apoptotic cells. When annexin V is coupled to superparamagnetic beads and incubated with spermatozoa, the fraction that is annexin V-positive can be separated and removed from the rest of the sperm sample [14,31]. The fraction enriched with non-apoptotic spermatozoa can then be used for ICSI. This method of sperm enrichment has been shown to increase the oocyte penetration potential and the sperm chromatin decondensation [13]. Two case reports described successful pregnancies [19] and healthy newborn [20] using MACS before ICSI. In both reports fresh ejaculates from infertile patients with a history of varicocele were used.

In this report we present a couple in which the 34 year-old male partner was a survivor of non-Hodgkin's lymphoma with a history of recurrent IVF/ICSI failure using sperm cryopreserved prior to chemotherapy. We describe the use of annexin V-MACS selection on cryopreserved-thawed spermatozoa prior to ICSI and the various reproductive outcomes measured, including the semen parameters before and after MACS, the embryological quality and the obstetric outcomes.

Materials and methods

Subject and clinical findings

In November 2005, a 28 year-old previously healthy man was diagnosed with stage IV non-Hodgkin's lymphoma and was referred to our center for pre-chemotherapy fertility preservation. His semen profile at the time of cryopreservation was 63×10^6 /ml, 26 % progressive motility (A + B) and 32 % normal forms [32]. In view of the advance stage of his disease and the urgency of starting chemotherapy, only one semen sample (4.5 ml) was obtainable prior to chemotherapy. Subsequently, he underwent salvage chemotherapy with etoposide, cytarabine, cisplatin and methylprednisolone (ESHAP) for relapse followed by total body irradiation therapy with bone marrow transplantation. Upon completion of his chemotherapy, he was followed on a regular basis every 6 months. Although he was in remission with respect to his lymphoma, all of his semen analyses revealed azoospermia and his serum total testosterone was 320 ng/dl and FSH 22 IU/L.

Results

In January 2010, the cancer survivor patient presented with his 35 year-old female partner to receive fertility treatment. Fertility evaluation of the female partner revealed normal uterus and

ovaries and an antral follicle count (AFC) of 20 on early follicular phase ultrasound. Serum day 3 values were: FSH: 7.9 IU/L; LH: 3.9 IU/L; and estradiol: 169 pmol/L. The couple delayed their fertility treatment with ICSI using cryopreserved sperm until January 2011 since they wanted to take advantage of the provincial coverage of the fertility treatment program (which began in August 2010). At that time, an aliquot of the cryopreserved sperm was thawed and parameters were 60×10^6 /ml, 1 % motility and 1 % morphology before density gradient centrifugation (DGC) [32]. The female patient underwent an antagonist cycle receiving 10 days of 150 IU Gonadl F® and 75 IU Luveris® and Cetrotide 0.25 mg from day 6 of stimulation. Three mature follicles yielded 3 oocytes of which two were metaphase II (M II) oocytes and were fertilized by ICSI. On day 2, a 5-cell/grade 2 embryo was transferred. However, the female partner failed to conceive. In April 2011, the female patient underwent a low dose GnRH agonist long protocol cycle with Marvellon® followed by Buserelin® for suppression and 225 IU Gonal F® and 75 IU Luveris® for stimulation for 9 days. In this second attempt, 17 oocytes were collected, of which 9 were MII and of which 5 fertilized following ICSI. On day 2, there were one 4-cell embryo, and four 2-cell embryos. On day 3, one 7-cell/grade 2 embryo was transferred along with a 5-cell/grade 3 embryo but the female partner again failed to achieve pregnancy. Based on the poor quality of embryos, further evaluation of the frozen semen sample was performed, which involved sperm DNA fragmentation assessment by TUNEL and Sperm Chromatin Structure Assay (SCSA®) [6,9]. In spermatozoa selected after DGC, the SCSA® assay revealed 76.6 % DFI and 2.19 % HDS, whereas TUNEL was 72.5 % positive cells (Table 1). Under the provincial fertility-treatment coverage, the couple is eligible for a third (and last) ICSI cycle. The couple were once again counselled for various options including the use of donor sperm and attempt of microsurgical testicular sperm extraction [5]. The couple consented to participate in an experimental procedure, as approved by the institutional ethics board, to use annexin V coupled to MACS in an attempt to evaluate if the chromatin structure of the cryopreserved sperm could be improved. The experimental nature and the potential risks of using the enriched sperm for ICSI were explained to the couple and they opted to use the enriched sperm for their last covered ICSI cycle. The cryopreserved sperm were thawed, motile spermatozoa were selected by DGC and subjected to annexin V-MACS [23] before ICSI. After sperm enrichment, 2.8×10^6 /ml sperm were recovered with 10 % progressive motility. The percentage of sperm that were positive for TUNEL decreased (Table 1). The quantity of sperm was too low for SCSA® reassessment. Sperm with grade A motility and normal morphology were selected for ICSI. The female patient underwent the same low-dose GnRH agonist long protocol with stimulation using 225 IU Gonal F® and 75 IU Luveris®. A total of 15 oocytes were collected, of which 8 were MII oocytes and of which 4 fertilized with ICSI. On day 2, 3 embryos were 4-cell and one was a 2-cell embryo. On transfer day (day 3), one 8-cell/grade

Table 1 Sperm parameters and DNA fragmentation during MACS procedure in cycle 3

	DGC/preMACS	postMACS
Concentration (M/ml)	10	2.8*
Progressive motility (%)	3.3	10
Vitality (%)	5.5	5.88
TUNEL (% positive)	72.5	58.8
SCSA (%DFI)	76.6	(*)
SCSA (%HDS)	2.19	(*)

(*) A 50 μ l aliquot was obtained with a sperm concentration of 2.8×10^6 /ml after performing MACS

2 and 6-cell/grade 3 embryos were transferred (Table 2). Sixteen days after oocyte collection, a positive chemical pregnancy was confirmed with serum β -hCG level at 681 IU/L. The female partner was confirmed to have clinical twin pregnancy from the two embryos transferred.

The pre-natal course of the twin pregnancy was uneventful. Serum screening and detailed anomaly ultrasound at 20 weeks were normal. Third trimester monitoring was normal. Presentation was cephalic for both twins. At 38+ weeks, labour commenced spontaneously and both twins were delivered spontaneously vaginally without complication. There was a 6 min inter-twin interval. The first twin was a baby boy – birth weight 2,410 g and cord pH 7.13. The second twin was a baby girl – birth weight 2,460 g and cord pH 7.26. Both babies were normal at birth and remained with their mother during the immediate post-partum period. Neonatal assessment the following day revealed no demonstrable congenital abnormality in either twin.

Table 2 Description of embryological aspects

	# oocytes collected	MII	2PN	Day 2	Day 3
Cycle 1	3	2	2	5 cell/grade 3 (ET) 1 cell	
Cycle 2	17	9	5	4 cell 2 cell 2 cell 2 cell 2 cell	7 cell/grade 2 (ET) 5 cell/grade 3 (ET) 2 cell/grade 4 2 cell/grade 4 2 cell/grade 4
Cycle 3	15	8	4	4 cell 4 cell 4 cell 2 cell	8 cell/grade 2 (ET) 6 cell/grade 3 (ET) 6 cell/grade 4 2 cell/grade 4

(ET) = embryo transferred

Bi-monthly review since delivery has failed to identify any abnormality or developmental issues in either child.

Discussion

In the recent years, oncofertility counselling has a growing importance for patients with newly diagnosed cancer as fertility management options appear to be a major cancer survivorship concern, particularly for those who had cancer diagnosed at reproductive age [12]. For male cancer survivors who either became permanently azoospermic or have sperm with poor quantity and quality, the use of cryopreserved sperm obtained before cancer treatment may be their only resort for fathering genetic children. The challenge in our case was that the cryopreserved sperm have poor sperm chromatin quality even before receiving chemotherapy, consistent with what we previously reported in young men with other cancers [17]. This might explain why this couple failed to achieve live birth with previous trials of assisted reproduction. With the use of annexin V-MACS, we were able to enrich sperm with more intact chromatin structure, and therefore able to improve the assisted reproductive outcomes as measured by sperm quality, embryo quality and live-birth. To the best of our knowledge, this is the first report in the literature of a successful live birth after using annexin V-MACS on pre-chemotherapy cryopreserved spermatozoa with significant sperm chromatin damage as determined by TUNEL and SCSA[®] from a cancer survivor patient. In the present study, the cryopreserved spermatozoa possessed a high level of DNA fragmentation as measured by TUNEL, which was reduced after annexin V-MACS.

The notion on the correlation between sperm DNA integrity and fertilization is still controversial. Several studies suggested that sperm with DNA damage are still capable of fertilization and defects may not become evident until the embryo has divided [3,11,26]. Other studies showed a significant inverse relationship between fertilization and the presence of DNA damaged [15,29]. In our case report, no association was observed between fertilization rate and level of sperm DNA damage since the fertilization rates between the second and third cycles did not differ significantly.

Regarding the association of embryo development and sperm DNA integrity, results are still debatable. Several authors suggested that early embryo cleavage is a strong indicator of embryo quality [10,16,18,28] and that embryonic genome activation may occur progressively through pre-implantation development (4–8 cell stage) with sperm DNA damage having a negative effect on continued development to the blastocyst stage [24,25,30]. In a systematic review on sperm DNA damage and embryo quality, half of the 28 studies cited, reported a positive correlation between sperm DNA damage and embryo development/quality [35]. In the present case, we observed that the quality of embryos obtained on the third cycle compared to the previous cycles, showed a better

early cleavage development that in turn lead to a successful twin pregnancy.

Recently, we reported that the level of enrichment in different categories of sperm samples varies from 20 % to 96 % [7]. In our case, although the percentage of enrichment as measured by TUNEL was reduced from 72.5 % to 58.8 %, the post MACS sperm fragmentation rate was still considered high [27]. Therefore, we cannot eliminate the possibility that the positive outcome is a result of regression to the mean. Further, since all embryo transfers were carried out at day 2/3, we do not have data on the potential of the embryos to form blastocysts. Thus, it is impossible to eliminate that the success of the third ICSI cycle was due to chance alone. Nonetheless, judging from the fact that, with the use of MACS, embryo quality was improved as well as that both embryos implanted resulted in live birth, alternative explanation should be explored.

First, it is possible that the extent of reduction of the DNA fragmentation rate was enough to increase the reproductive success as observed. However, further studies are required to evaluate the thresholds of sperm DNA quality enrichment that can contribute to improvement in assisted reproductive outcomes. Second, though we were not able to repeat SCSA® evaluation of the sperm after MACS due to their limited quantity available, it may be possible that the reduction in sperm DNA fragmentation as measured by SCSA® reflects a larger extent of improvement than TUNEL assay. It is known that both assays evaluate different aspects of sperm DNA chromatin quality and one does not necessarily predict the other [27]. Further studies are required to evaluate the correlations of sperm DNA quality improvement post MACS processed by the different assays along with the reproductive outcomes.

In summary, this case highlights the use of annexin V-MACS on cryopreserved spermatozoa of a cancer survivor patient. Although the underlying mechanisms involved on sperm DNA fragmentation and embryo development are unclear, the detection of such anomalies before ART are essential to achieve healthy reproductive outcomes. Further studies are needed to evaluate the efficacy and potential risks of integrating annexin V-MACS in the ART laboratory.

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