

## **TITOLO**

Successo della Iniezione Intra-Citoplasmatica degli Spermatozoi crioconservati di un paziente affetto da seminoma ed azoospermia post chemioterapia. Descrizione di un caso.

## **TITLE**

Successfully Intra-Cytoplasmic Sperm Injection with cryopreserved-thawed spermatozoa in a male with seminoma and azoospermia post chemotherapy. A case report.

## **AUTHORS**

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## SUMMARY

Male fertility is affected by the treatment of testicular cancer. The time recovery can be achieved in 84% of cases within ten years. In most cases the cryopreservation of spermatozoa, in order to use them for assisted reproductive techniques, can solve the secretory infertility due to the treatment of the cancer.

In this study we report a case of a man 35 years old with azoospermia after treatment of a testicular seminoma. He underwent chemio and radiotherapy. Before the treatment his spermatozoa were cryopreserved. After a five years period from the end of the treatment, he demanded for paternity. We used his cryopreserved-thawed spermatozoa to perform an IntraCytoplasmic Sperm Injection. The recovery of motile spermatozoa after thawing was very low. All the five Metaphase II oocytes recovered from his partner, after a stimulated cycle according to the long protocol, were injected with immotile spermatozoa. Regular fertilization was obtained only in three out of the five injected oocytes. One zygote did not cleaved and we could transfer two embryos of quite good quality. A singleton pregnancy was obtained and a healthy girl was born.

Therefore, cryopreservation of spermatozoa is useful to solve the problem of infertility in men that underwent the oncology therapy. It has to be part of the therapeutic program of these patients. After failing the preventive cryopreservation, the testicular sperm extraction can be performed for the IntraCytoplasmic Sperm Injection procedure.

## INTRODUCTION

The incidence of malignant tumours in infertile men is 0.4-1.1%. Testicular cancer and Hodgkin's disease represent two of the most frequent cancers in young adult males (Meirow & Schenker, 1995; Padron *et al.*, 1997).

50-70% of testicular cancer patients have poor seminal quality before oncology treatment. In particular, the parameters of semen in non-seminoma testicular tumours appear statistically significantly lower than in seminoma (Botchan *et al.*, 1997; Gandini *et al.*, 2003).

This impairment might relate to the direct effect of the tumour, to the production of  $\beta$ -hCG by some cancer histotypes, and in Hodgkin's disease to the common symptoms such as fever, weight loss and general stress (Vigersky *et al.*, 1982; Petersen *et al.*, 1998).

Seminoma is a tumour of the undeveloped germ cell. The spermatozoon used for the intracytoplasmic sperm injection (ICSI) is a matured cell, so it is not a malignant cell, but it can carry chromosomal or gene defects. Even, it is postulated that the chromosomal instability may be a factor in the development of malignancy for testicular tumours (Gundy *et al.*, 1992).

Nevertheless, some authors do not report any statistically significant difference in the frequencies of chromosomal aberrations between controls and testicular cancer patients (Alvarez *et al.*, 1998; Chan *et al.*, 2001).

Chemotherapy and radiotherapy damage germ cells, while surgical treatment impairs the capability to ejaculate; 80% of cases develop retrograde ejaculation. These side effects may be avoided in part by selective lymphadenectomy with preservation of thoracolumbar ganglia and by shielding the contra lateral testis from radiation (Reš *et al.*, 1994).

The improvement in prognosis over the past years, the young age of the patients and information from the existing literature regarding poor semen quality, still before anticancer therapy, make semen cryopreservation prior to the oncology treatment mandatory, as such a treatment may induce minor or major alterations in spermatogenesis, including possible

transitory or irreversible azoospermia (Bahadur *et al.*, 2000; Richards *et al.*, 2000 Laguna *et al.*, 2001).

The following case demonstrates the significance of the deep freezing semen previous to anti cancer therapy, for a young man with a testicular seminoma, in order to successfully plan his fatherhood.

## **MATERIALS AND METODS**

### **Case report**

The patient was a 35 years old man. He was diagnosed a seminoma of the right testicle. Pathohistology confirmed the above diagnosis. The spermiculture was positive for staphilococcus aureus. Hormonal and genetic examinations were normal and no Y-microdeletion was found. The right testicle was 25 ml volume, the left one was 20 ml volume. The deference vases were palpable.

Staging tests such as the ultrasound scan of the abdomen, lung X-rays and computed tomography (CT) scan of the retroperitoneal space were performed. The CT scan of the abdomen showed several pathologically enlarged lymph nodes, up to 3 cm in diameter, between the aorta and the inferior vena cava. No metastases were observed in the parenchymal organs. The above tests established a clinical radiological stage of II A. The tumour markers  $\alpha$ -fetoprotein (AFP) and  $\beta$ -human chorionic gonadotrophin ( $\beta$ HCG) were normal.

Before oncology treatment, patient was counselled to cryopreserve his semen. Then, he underwent orchidectomy and a combined chemotherapy composed of etoposide plus cisplatin and bleomycin. The above therapy was administered on three cycles.

Finally, complete remission was achieved and the patient stopped the treatment.

Five years later the couple demanded for parenthood. Azoospermia was ascertained in the ejaculate by three repeated monthly controls.

Therefore, the cryopreserved semen was used for the assisted reproduction. The seminal parameters before freezing were as follows: volume up to 2,5 ml, sperm concentration equal to 4,1 millions per ml and there was 4% of progressive motile spermatozoa.

### **Freezing protocol**

Ejaculated semen was allowed to liquefy for about 30 minutes. A 10 µl aliquot was put in Makler chamber to assess the concentration and motility of spermatozoa (WHO 1999 criteria). Sperm morphology was assessed based on the Kruger strict criteria 1993.

Freezing was performed in sperm freezing medium (Life Global<sup>®</sup>). According to the recommendations for use, to avoid cold shock at first the medium was warmed to room temperature. To each 1 ml of sperm 0,7 ml of medium was added in drops while gently swirling. The mixture was allowed to equilibrate at room temperature for 10 minutes and then drawn into 0,3 ml CBS<sup>™</sup> high security straws with hydrophobic plug, leaving approximately 1.5 cm of air at the end of the straw. The straws were sealed by the CBS<sup>™</sup> SYMS unit.

The programmable freezer CryoLogic CL-8800 (Freeze Control<sup>®</sup>), operating with a computer IBM compatible, and the CryoGenesis<sup>™</sup> software were used to freeze. The fast protocol was applied. The temperature started at 32°C and cooled until 4.0°C at 4.00°C/min. It was stable for 5 minutes and then went up to -8.0°C at 4.00°C/min. After one minute, manual seeding was performed and the temperature reached -20.0°C by 10.00°C/min. Finally, it went up to -50.00°C at 8.00°C/min and the straws were directly plunged into liquid nitrogen.

### **Thawing protocol and semen preparation before ICSI**

The straws were kept at room temperature for 5 minutes and then immersed in 37° C water bath for 10 minutes. Then, the content was processed through the swim up method as follows. At first, it was washed at 300 g for 10 minutes in 2 ml HTF plus with hepes (Life Global®) and then the pellet was overlaid by 1 ml aliquot of life global medium (Life Global®). It was incubated at 37° C and 5% atmosphere for up to one hour. Thereafter, microscopic examination was performed to assess the number and the motility of spermatozoa harvested. Hypoosmotic swelling test (HOST) was performed (Hypo®, IVF Science) for vitality examination.

### **Induction of multiple follicular growth**

The partner was 36 years old. She had normal physical, hormonal and genetic examinations. She underwent controlled ovarian stimulation according to the long protocol. The gonadotrophin-releasing hormone agonist (Decapeptyl depot®, IPSEN) was administered on the 21<sup>st</sup> day of the previous menstrual cycle to obtain pituitary down regulation. The gonadotrophins (Fostimon®, AMSA) administration started on the 2<sup>nd</sup> day of the current menstrual cycle at a dosage of 150 IU for up to 18 days. Monitoring by the trans vaginal ultrasound and the 17 beta oestradiol plasmatic assays were carried out on the 5<sup>th</sup>, 7<sup>th</sup> and 12<sup>th</sup> day of the stimulated cycle.

### **Ovum pick up and ICSI procedure**

On day 0, oocytes were collected by the transvaginal follicular aspiration ultrasound guided 36h after 10.000 IU human Chorionic Gonadotrophin injection (Gonasi HP®, AMSA) and cultured for up two hours in medium without hepes (Global®, LifeGlobal).

Thereafter, they were denuding in hyaluronidase (hyaluronidase, LifeGlobal) and checked for the evaluation of the grade of maturation. The metaphase II oocytes underwent ICSI procedure.

ICSI was performed in droplets of HTF plus with hepes (RTF plus, LifeGlobal) under oil (LiteOil<sup>®</sup>, LifeGlobal) with the use of polyvinylpyrrolidone (PVP, LifeGlobal) in the droplet containing the prepared sperm. After ICSI, the injected oocytes were washed and then placed in droplets of culture medium (Global<sup>®</sup>, LifeGlobal) under oil (LiteOil<sup>®</sup>, LifeGlobal).

### **Assessment of fertilization and embryo development**

On day +1, fertilization was checked 16-18 h after injection taking into account the following parameters: the pronuclear morphology, the nucleolar morphology and the polar body alignment with respect to the longitudinal axis of pronuclei.

The fertilized oocytes were transferred to fresh culture medium (Life Global<sup>®</sup>) until the control of the cleavage and morphological assessment that were performed on the day after (day +2).

The cleaved oocytes were transferred again to fresh culture medium.

### **Embryo transfer**

On day +3, immediately before the embryo transfer procedure, the embryos were checked for the evaluation of the grading. It was carried out on the base of the assessment of the following parameters: the number of blastomers, their symmetry, the percentage of fragmentation and the quality of cytoplasm. Four grades were thus distinguished: Grade A, even blastomeres, no fragmentation; Grade B, even or uneven blastomeres, fragmentation < 20%; Grade C, even or uneven blastomeres, fragmentation > 20% and < 50%; Grade D fragmentation > 50%.

### **Luteal phase support**

The luteal phase was supported by daily intramuscularly injections of 50 mg progesterone (Prontogest<sup>®</sup>, AMSA) starting on the day 0 for up to 15 days after the embryo transfer. Then, the plasmatic evaluation of  $\beta$ -HCG was performed.

## RESULTS

A total of five oocytes were obtained. They were all metaphase II grade.

After thawing, the spermatozoa appeared immotile and morphologically abnormal above all for the high frequency of atypical heads and vacuols. Only spermatozoa with functionally intact membrane and less morphologically abnormal were used for ICSI.

Three out of the five oocytes injected were normally fertilized. Two of them showed the pronuclei centralized and juxtaposed (pattern A), the third one had the pronuclei centralized but not juxtaposed (pattern C). The nucleoli were large size aligned in the first one (pattern 1), large size scattered in the second one (pattern 2) and small size scattered in the third one (pattern 4). The polar bodies alignment, with respect to the longitudinal axis of pronuclei, was longitudinal in the first two oocytes (pattern  $\alpha$ ). The third oocyte displayed different alignment for each of its polar bodies (pattern  $\gamma$ ).

At cleavage control on day +2, the first two oocytes cleaved (pattern A1 $\alpha$  and A2 $\alpha$  respectively), the third one did not (pattern C4 $\gamma$ ). Both cleaved zygotes had four blastomeres. The first one showed four even cells, clear cytoplasm, one visible nuclei in each blastomer and no fragmentation. The second one had slightly uneven cells, with about a 10% of fragmentation, clear cytoplasm and one cell with two nuclei.

On day +3, immediately before transfer, the first embryo had eight even blastomeres with initial compacting and the second one had eight uneven blastomeres still with about 10% of fragmentation.

After two weeks the pregnancy test, assessed by the dosage of the plasmatic  $\beta$  HCG, was positive.

One month later, the trans vaginal ultrasound examination evidenced one gestational sac with one embryo in the uterus displaying the fetal heartbeat.

At the first trimester, bleeding with pelvic pain took place. They spontaneously stopped with bed rest.

The ultra screen at the twelfth week of gestation was normal.

The couple refused to undergo amniocentesis.

At the 38<sup>o</sup> gestational week the women underwent elective caesarean operation and a 2,950 kilos weight female was born. She was in good health.

## DISCUSSION

The importance of cryopreservation of semen before treatment for cancer derives from the evidence that most patients undergoing chemotherapy become azoospermic about 7-8 weeks after starting treatment (Pont *et al.*, 1997).

Approximately two thirds of these men have retrievable testis sperm, which may be used with ICSI to have healthy offspring (Kohn *et al.*, 2001; Damani *et al.*, 2002; Meseguer *et al.*, 2003).

The retrieved testicular spermatozoa can be frozen but they may reduce the pregnancy rate (Meseguer *et al.*, 2003; Thompson-Cree *et al.*, 2003).

It could be assumed that the fertilization rate depends on sperm parameters (Olsen *et al.*, 2001), still, according to the data from existing literature, there is no simple and universal model for predicting the sperm fertilizing potential in vitro (Jedrzejczak *et al.*, 2005).

In particular, as with the general population with non-obstructive azoospermia, clinical and histologic parameters were unable to predict with certainty the testicular sperm extraction (TESE) outcome in an individual patient (Mulhall *et al.*, 1997; Tournaye *et al.*, 1997). The stage of spermatogenesis is also very important, as the fertilization rate is 64% with spermatozoa and 40% with round spermatids (Gianaroli *et al.*, 1999).

We know that cytotoxic anti-neoplastic therapy acts mainly on proliferating type B spermatogonia. However, recovery of spermatogenesis depends on the survival of type A spermatogonia and the severity and duration of spermatogenic impairment after chemotherapy correlates with the numbers of type A spermatogonia that are destroyed (Meistrich *et al.*, 1982).

At this regard, some studies suggest that the highest risks for sterility in young cancer survivors are radiotherapy, particularly doses > 40 Gy delivered to para-aortic and pelvic lymph nodes, and alkylating chemotherapy (Damenwood *et al.*, 1986).

It is not possible to know with certainty why some patients remain persistently azoospermic after chemotherapy (Naysmith *et al.*, 1998). Individual susceptibility to gonadotoxic effects of chemotherapy may be implicated in some patients; in others, a pre-existing spermatogenic impairment, caused by the testicular tumour itself, may contribute to a more severe degree of spermatogenesis damage than usual (Meseguer *et al.*, 2003).

Finally, one important matter of concern is the potential genetic risk associated with the use of assisted reproductive techniques in men with non-obstructive azoospermia after treatment for cancer.

Actually, it is postulated the possibility of an increased incidence of tumours in the offspring and the appearance of the consequences of the mutagenic effects of chemotherapy on germ cells (Arnon *et al.*, 2001).

Even if an increased incidence of sperm chromosomal abnormalities during and immediately after receiving chemotherapy is considered to be a transient effect of cytotoxic drugs, it is demonstrated that significant increase in the frequency of sperm aneuploidy may persist until least 18months after the initiation of chemotherapy (De Mas *et al.*, 2001).

The clinical impact of such a rise in sperm chromosomal abnormalities remains still to be clearly defined, but, theoretically, it could lead to spontaneous abortions, stillbirths or birth defects in chromosomally abnormal children (Hassold *et al.*, 1996).

## **CONCLUSIONS**

The reported case demonstrates that patients who are azoospermic, because of treatment by surgery, chemotherapy and radiotherapy for a testicular cancer, can plan parenthood thanks to new methods in assisted reproductive technologies.

The collaboration of all specialists participating in the treatment of these patients is of great importance.

Because of the potential genetic risks of the TESE-ICSI programme, we emphasize the cryopreservation of the ejaculate, even in cases of very poor semen quality, and the freezing a biopsy of healthy testicular tissue in cases of azoospermia before starting gonadotoxic therapy.

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