

ISSUES IN ASSISTED REPRODUCTION

Preimplantation Genetic Diagnosis (PGD)

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The role of this new technology, developed as part of an advancement in IVF (In Vitro Fertilization) treatment for infertile couples has had a profound effect, on the one hand, in reducing the incidence of transmission of genetic disease to the next generations and on the other hand on the treatment of life threatening diseases by now being able to produce suitable for transplantation HLA (Human Leucocyte Antigen) matched, cord blood, stem cells.

Infertility, in the community is an unexpected and dramatic event for couples who think that childbearing is a normal process and rightful in its own sense...

....but statistics show otherwise:

- 15-18% of all couples cannot conceive after 12 months of free intercourse
- There are an estimated 250,000-300,000 infertile couples in Greece
- Over 15,000 IVF cycles are performed annually and
- Over 5,000 IVF children are born here in Greece annually (approximately 5% of all births).

In Slide 5 we see all stages of fertilization and embryo development.

Preimplantation genetic diagnosis

PGD as a new modality is a well accepted reproductive choice for couples at genetic risk and it involves the removal of cells from embryos after in vitro fertilization, the diagnosis and detection of **genetic disorders**, either chromosomal or gene defects, and finally the transfer of unaffected healthy IVF embryos.

PGD is indicated when **both parents** are carriers of a recessive autosomal gene disorder and one such disorder is **b-thalassemia** common in mediterranean countries – it is estimated that 10% of the Greek population has the recessive trait, and the second most common in 2% of the Greek population is cystic fibrosis. You yourselves can appreciate the importance of eradicating the transmission of these

diseases as I have been told that the operating surplus of this conference is funding further research into the disease of thalassaemia.

Other indication is when **one** parent has a **dominant autosomal or sex-linked disorder**.

Further indications for the application of PGD in order to detect chromosomal abnormalities, include

- Couples with a history of offspring or fetus with **chromosomal abnormality**
- Women of **advanced age** (>36 years)
- Couples with history of **recurrent pregnancy loss** (>2)
- Couples with history of **multiple IVF failures**

One of the major drawbacks for transplants, in general, is graft rejection if there is no proper match to the recipient.

Appropriately related or unrelated donors are restricted to those matched for major histocompatibility antigens. As such Human Leucocyte Antigen (HLA) matching is required to prevent devastating immune complications of graft rejection. Thus, PGD can be used to appropriately select and transfer to the mother HLA typed embryos and thence collect cord blood stem cells when this baby is born for transplantation.

Hence, the European Fertility Society - ESHRE Consortium in 2005, dictates that PGD is also applied for HLA typing in couples who already have a child suffering from a malignant or genetic disorder, and the child is likely to be cured or its life expectancy will be substantially prolonged by cord blood stem cell transplantation from an HLA-matched identical sibling.

There are three potential sources of embryonic genetic material for preimplantation genetic analysis :

1. **polar bodies**....This is genetic material excreted during miosis of the oocyte just before and during fertilization. We can biopsy the first polar body from oocytes before sperm insemination or both polar bodies after sperm insemination.
2. **blastomeres** ...These are cells from cleavage stage embryos; and,
3. **trophectoderm cells**, ie. from embryos at the blastocyst stage.

Still the most common approach to attaining embryonic genetic material for PGD analysis involves the aspiration of **one or two blastomeres**, ie cells, from the cleavage stage embryo. Precompaction eight-cell embryos are usually biopsied early on day 3, once they have completed their 3rd mitotic division. Following genetic diagnosis, the embryo transfer may be performed on the same day or delayed to day 4 or left to reach the blastocyst stage.

It seems that the biopsied embryos have the ability to adapt to the cell loss as their further developmental rate is not compromised and there is no evidence to suggest of increased embryonic abnormalities due to the procedure.

Since the application of extended culture using more complex media or new generation sequential media, high rates of development to the blastocyst stage have been reported. Pregnancies following blastocyst biopsy and PGD have now emerged and I will share with you further on my talk our experience on this manner of PGD.

The PGD at the blastocyst stage involves **zona dissection** with laser, hatching of the trophectoderm cells through the hole, and finally, excision of the trophectoderm cells for diagnostic purposes. The advantages of this method is

- its ability to remove 8-10 cells,
- embryonic cells are not affected
- less likely that the result will be affected by mosaicism and finally of utmost importance
- to transfer fewer embryos and such to reduce the likelihood of multiple pregnancies

Looking at the three methods of PGD just described, the main disadvantage of PGD based on analysis of polar body or early cleavage biopsy procedures is the limited amount of material available for genetic analysis.

When diagnosing monogenic disorders in single cells using PCR-based protocols, there is a high risk of PCR failure - no result – and as such an incomplete result may be obtained, potentially resulting in a reduced number of unaffected embryos available for transfer.

Increasing the amount of starting DNA template should in principle increase the sensitivity and reliability of genetic diagnosis. As such biopsy at the blastocyst stage provides the advantage that more cells can be removed for genetic analysis and this in turn will improve PGD outcome.

There has been extensive animal and research studies on the application of blastocyst trophectoderm biopsy. The development of **noncontact lasers** has greatly facilitated trophectoderm biopsy leading now to various reports of pregnancies, following this method, in the literature today.

Some years ago our IVF unit together with Monash IVF designed and developed a **new method of trophectoderm cell biopsy from blastocysts**. The original purpose of this design protocol was to study genetic parameters which are expressed in human blastocysts and which in turn determine the success of pregnancy in IVF.

The technique we developed had **the sole purpose of removal up to 10 trophectoderm cells and in the same time avoiding the inner cell mass or the embryo body**. The removal of so many cells increased the accuracy of diagnosis while providing material for multiple genetic analysis.

To this affect of major contribution was the donation to our center of a ZILOS-tk laser optical system by **Hammilton Thorne Biosciences**.

All embryos were cultured to the blastocyst stage, and at day 5 after fertilization they were assessed for blastocyst formation. On the morning of day 5 an opening was made at the **zona pelucida directly opposite the inner cell mass of each blastocysts**, using the lowest setting of the ZILOS-tk non contact laser.

Blastocysts were incubated for a further four hours to allow blastocoele **expansion and herniation** of the trophectoderm cells from the opening of the zona.

Applying gentle suction with the biopsy pipette trophectoderm cells were encouraged to herniate from the zona at which time dissection of the trophectoderm cells was performed with 3-4 laser pulses.

The biopsied cells were immediately specifically treated and sent directly for genetic analysis. On the morning of the sixth day, morphology and growth of the biopsied blastocysts were assessed.

It seems that the **morphology and viability** of the blastocysts were not affected by this biopsy technique and as is seen from the powerpoint slides the **healing** process was complete even before the embryotransfer.

Applying the same biopsy technique for PGD of b-thalassaemia in coordination with the Genetics laboratory of the University of Athens, we succeeded in having the first birth of a child in November 2004.

And this was published in Human Reproduction mutually with Professor Trounson's Australian team and Professor Kanavakis, of the Greek Genetics laboratory, as **the**

first report on live birth following trophectoderm biopsy at the blastocyst stage of development for the PGD of β -thalassaemia.

This case report describes a 39 year old lady who she and her partner were β -thalassaemia carriers. They had 2 previous cleavage stage PGD cycles which were unsuccessful and on her 3rd attempt on a frozen thaw cycle, 10 cryopreserved zygotes were thawed, 8 survived and on day 5, 3 early blastocysts had developed. 4-6 trophectoderm were dissected from each of the blastocysts, and the results were:

-  **one affected** blastocyst: re-expanded and totally hatched from the zona
-  **one carrier** blastocyst: re-expanded and 50% of the blastocyst had herniated through the biopsy hole
-  **one normal** blastocyst: reformed its blastocoel cavity but with no significant expansion from the preceding day

Both the carrier and the normal blastocyst were transferred to the uterus, and a **singleton** pregnancy was confirmed and at 12 weeks of gestation. CVS confirmed the unaffected status of the embryo (N/N) and a normal healthy female baby was delivered at 38 weeks of gestation by Caesarean section.

And of course this was reported at the 6th World Conference on PGD which took place in London in 2005.

This year we will further report comparative results of blastocysts biopsy versus day 3 cleavage stage biopsy, for PGD of β -thalassaemia, where it seems that the implantation rate is almost double in the former, 47.6% per embryo in blastocyst biopsy to 26.7 % per embryo in day 3 biopsy.

The first case of PGD and simultaneous human leucocyte antigen (HLA) typing performed for fanconi anaemia complementation group C(FA-C), resulted in the delivery of an HLA matched offspring, the collection of stem cells from this offspring, their transplantation and consequent successful haematopoietic reconstitution of an affected sibling as described by Verlinsky et al, in 2001. To improve access to HLA identical bone marrow transplantation in sporadic bone marrow failures this approach was then applied with the purpose of ensuring the birth of HLA identical offspring from preimplantation HLA typing, which resulted in the radical treatment of a sibling with a disease known as sporadic Blackfan-Diamond anaemia (BDA), by stem cell transplantation.

Stem Cells

Just a few words on stem cells. Stem cells are defined by their ability to self-renew and to form one or more differentiated cell types with the potential for **organ regeneration**. What distinguishes different populations of stem cells are the types of specialised cells that they generate. One clear division of the stem cell family is between those isolated from the embryo, known as embryonic stem cells, and those in adult somatic tissue, known as adult stem cells.

Another source of stem cells, with more practical application, is from blood of the umbilical cord of newborn children. These are usually collected from the placenta after the delivery of the new born. This provides an easy source of stem cells and as such has prompted the creation of cord blood banks around the world.

In the future the treatment of diseases will never be the same, once we realize the immense therapeutic potential of stem cells. As to how different the future will be, can be seen through the personal medical history of young Sentric Selton, published 2 years ago in National Geographic who **suffered from leukaemia, now cured, and his bone marrow reconstituted after transplant of cord blood stem cells taken from the umbilical cord of a newborn girl at Duke University in the USA**.

Sentric Selton belongs to a new generation of people having cord blood stem cell transplants. In his case however the transplanted stem cells, turned into bone marrow, produce till today blood cells with two X chromosomes, since they originally were derived from the umbilical cord of a newborn girl. Even though his sexual characteristics will not be affected, this practically means, as his own mother laughingly points out, that this minor detail may, at some point, save him, if he ever commits a crime and leaves behind stains of blood. The resulting DNA tests of this blood will leave no doubt as to the sex of the person committed the crime as everybody will be searching for a woman!!

In September of 2005 in our clinic we applied PGD and HLA typing to a couple with a 2 year old child suffering from chronic granulomatosis disease. This life threatening monogenic form of immunodeficiency allows children a life span of 10-15 years before dying from common infections. The couple requested PGD in order to have a healthy next child and at the same time to do HLA typing, collect stem cells and perform bone marrow transplantation in order to save the affected sibling.

After egg collection and subsequent embryo culture, 11 blastocysts developed and were biopsied on day 5, 3 blastocysts were determined to be genetically healthy, free of the disease and HLA identical to the sick sibling. 2 unaffected embryos were transferred resulting in a singleton pregnancy. A healthy female HLA matched baby was born in June 2006, cord blood was collected at birth and stem cells have now been frozen.

Till the end of the year, hematopoietic stem cells will be transfused to the sick brother and hopefully will contribute to its cure on the one hand and to science in general on the other hand as this process can now be applied to a variety of other diseases.

This case has been sent and accepted for publication and represents the first reported case for HLA typing in chronic granulomatous disease. A second healthy HLA matched child has also been born this February in a similar case of a couple having a child again with granulomatous disease.

Further from the previous said, a unique opportunity seems to now stand out, and this is no other than applying PGD technique for the determination of embryo viability.

In other words, for couples going through an IVF program we could do PGD not only **to avoid genetic diseases** but also **to determine the gene expression profiling of viable IVF blastocysts**.

As such, trophectoderm biopsy can provide ample cell material for the study of genes involved in implantation.

As such, multiple genetic analysis could be performed for:

- DNA typing,
- Genetic screening,
- the creation of DNA libraries.

With this in mind, in 48 cycles in our center, with the informed consent of the patients, we performed trophectoderm biopsy in blastocysts just before ET, and the cells were sent to Monash University for genetic analysis and fingerprinting.

From the 48 ET's we had 27 pregnancies, and 27 deliveries of 37 babies. At birth, we collected cord blood from each baby born and as such we were able to trace each baby to the blastocyst we had originally transferred. Using the microarray technique an analysis has been performed to determine the genes activated in the embryos proceeding to pregnancy. This will be very useful to form a common profile for embryos able to implant and the results will soon be published.

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