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(54) Title: METHODS OF PRESERVING THE FUNCTIONALITY OF AN ORGAN.

(57) Abstract: A method of preserving functionality of an organ. The method includes removing a whole organ and associated vasculature, cryo-preserving the whole organ, allowing a period of time to elapse, thawing the whole organ including vasculature and introducing the whole organ into a recipient so that the organ is supplied with blood by vasculature belonging to the recipient. Further disclosed is a method of preserving fertility of a patient undergoing a treatment expected to cause sterility. The method includes removing a whole gonadal organ from the patient, cryo-preserving the whole gonadal organ, conducting the treatment and waiting for an effect thereof to subside, thawing the whole gonadal organ and introducing the whole gonadal organ where it is supplied with blood by the vasculature system of the patient. Further disclosed is a method of assuring a supply of viable gametes for future use, the method includes removing a whole gonadal organ from a patient, cryo-preserving the whole gonadal organ, waiting until viable gametes are desired by the patient, thawing the organ and introducing the gonadal organ into the patient so that it is supplied with blood by a vasculature system belonging to the patient.



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Method of preserving the functionality of an organ

5 FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to methods of preserving functionality of an organ, preserving fertility of a patient undergoing a treatment expected to cause sterility and assuring a supply of viable gametes for future use and, more particularly, to a methods which rely upon removal of an organ, 10 cryopreservation of the removed organ and reintroduction of the cryopreserved organ into a recipient in such a way that the vasculature of the recipient supplies blood to the introduced organ or a portion thereof.

Undoubtedly, successful cryopreservation of solid organs of clinical interest would have a significant impact on the field of organ transplantation. However, despite several 15 decades of research freezing and thawing of solid organs remains largely impractical (Dietzman et al., 1973; Guttman et al., 1977; Pegg et al., 1987; Karow, 1981; Jacobsen et al., 1982).

Attempts to understand the mechanism by which cold-blooded animals survive freezing in nature (Storey and Storey, 1988; Storey, 1990) have led to successful, short-term 20 cryopreservation of hearts and livers by freezing at high subzero temperatures (-3.4°C to -4°C), (Wang et al., 1992; Banker et al., 1991, Rubinsky et al., 1994, Soltys et al., 2001).

Cryopreservation at lower temperatures, which is necessary for long-term storage, was attempted in dog intestines, but vascular injury was observed after thawing (Karlsson et al., 1996). Livers have regained partial function after freezing to -60°C (Mazur, 1977), dog 25 spleens (Fuller, 1987) and ureters (Pegg, 1987) have survived deep freezing and transplantation, and kidneys have survived freezing to -80°C .

(Fahy et al., 1984) proposed vitrification (ice free cryopreservation) as an alternate strategy for for organ cryopreservation (Kheirabadi et al., 2000) . Vitrification produced a high survival rate in cryopreserved small organisms such as *Drosophila* embryos at -196°C 30 (Steponkus et al., 1990; Mazur et al., 1992).

Thus, successful cryopreservation of organs apparently requires a specific optimal cooling rate, because damage may occur if organs are frozen either too rapidly or too slowly (Karlsson and Toner., 1996; Mazur, 1977). Cooling too slowly ($<1^{\circ}\text{C}/\text{min}$) causes extracellular crystallization which may physically disrupt the vital tissue architecture

(Fuller, 1987; Pegg, 1987), whereas at higher cooling rates intracellular crystallization will cause irreversible damage.

Further, cryopreservation of large-volume samples, such as tissues or organs, introduces heat transfer problems. In macroscopic samples there is a large thermal gradient from the surface of the sample to the interior. For example, it was shown that cells survived equally when frozen as isolated cells or in a monolayer, only when the applied cooling rate was less than $0.5^{\circ}\text{C}/\text{min}$. Moreover, the survival of cells in monolayer was higher than isolated cells using the determined optimal cooling rate of $0.2^{\circ}\text{C}/\text{min}$. This indicates that the attached cells were more tolerant of slow cooling injury (Armitage et al., 1996). The need for a slow cooling rate is further illustrated by the cooling rate at which a Wood frog survives freezing (less than $0.1^{\circ}\text{C}/\text{minute}$; Schmid, 1982).

Typically, prior art freezing techniques produce temperature gradients within the freezing chamber which make it difficult to achieve an optimal cooling rate (Koebe et al., 1993). In addition, cooling rates slower than $0.1^{\circ}\text{C}/\text{minutes}$ are hard to control in a programmable freezing apparatus since the accuracy of the temperature measurement is within that range (OMEGA ENGINEERING, INC). United States Patent 5,873,254 to Arav teaches a device capable of producing a uniform cooling rate of $0.1^{\circ}\text{C}/\text{min}$ throughout a biological sample. However, the earlier teachings of Arav do not disclose methods for cryopreservation of whole organs and subsequent introduction of those whole organs into a recipient subject. Specifically, the earlier teachings of Arad do not include methods for thawing a whole organ without impairing functionality thereof and surgical techniques for anastomic transplantation.

It is well established that treatment of a malignant disease by radiotherapy or chemotherapy can have dramatic and irreversible effect, on fertility, especxially in female patients (Byrne et al., 1987; Ataya, 1989; Familiari, 1993).

Thus, there is a recognized need, but no established method, for cryopreservation of a gonadal organ for subsequent reimplantation for patients at risk for premature sterility as a result of planned cancer treatment. While oocyte cryopreservation theoretically offers a means of preserving fertility for these patients, severe practical problems for oocyte cryopreservation remain unsolved (Arav et al., 1996; Zeron et al., 1999; Zenzes et al., 2001). Similarly, while cryopreservation of semen for male patients is available, it is not an optimum method of assuring male sterility. Cryopreservation of oocytes and/or semen typically require laboratory intervention in the form of IVF procedures. This prospect is daunting to many patients for a

variety of reasons including emotional and religious reasons. Thus, storage and later use of a gonadal organ for conception by conventional methods offers significant advantages over previously available alternatives.

A high proportion of viable follicles have been found to survive in human ovarian tissue after freeze-thawing (Hovatta et al., 1996; Newton et al., 1996; Oktay et al., 1997), and this has aroused interest in the procedure as a potential means of preserving the fecundity of patients at risk of premature ovarian failure (Donnez et al., 1998; Newton et al., 1998). However, freezing and thawing of the whole ovary was not reported.

Gosden and colleagues (1994) have achieved limited success in sheep using ovarian cortex freezing leading to speculation that this technique may be applicable to humans. The sheep ovary is similar to the human ovary in that it has a dense fibrous stroma and relatively high density of primordial follicles in the ovarian cortex.

Autotransplantation of frozen-banked and fresh ovarian cortex cryopreservation ovarian cortex, have resulted in two pregnancies (Gosden et al., 1994). Baird et al. (1999) performed frozen tissue autotransplantation in eight sheep, which were monitored for up to 22 months. All the animals resumed cyclicity and showed hormone production, however, it was established that there was a drastic reduction in the total follicular number, and the resumption of cyclical ovarian function was temporary. Thus, despite recent advances in this area, transplantation of a functional portion of an ovary for purposes of restoring full ovarian function remains unreliable. Therefore, freezing and grafting of human ovarian tissue is not considered clinical option (Kim et al., 2001).

Clinical acceptability of ovarian tissue transplantation will require higher numbers of follicles survive and retain the capability to develop and ovulate. Recently it has been demonstrated that during freezing of cortical ovarian slices granulosa cells are more damaged than oocytes (Siebzehnruhl et al., 2000).

Further, currently accepted experimental practice dictates use of ovarian slices resulting in ischemic damage as a result of non-vascular transplantation. Still further, the need for an IVF procedure in addition to preparing tissue for cryopreservation is time consuming and increases costs of the procedure. Finally, in those cases where ovarian function has been restored, long post-implantation delays are observed (Radford et al., 2001).

Transplantation of whole ovary including vascular has been recognized as a theoretical method of solving the problems described above. While Ovary transplantation has been known for decades, (Nobel Lecture, December 11, 1912: "Suture of blood-vessels and

transplantation of organs”), autotransplantation has been considered impractical because of the absence of long term organ cryopreservation.

There is thus a widely recognized need for, and it would be highly advantageous to have, methods of preserving functionality of an organ, methods of preserving fertility of a patient undergoing a treatment expected to cause sterility and methods of assuring a supply of viable gametes for future use devoid of the above limitation.

SUMMARY OF THE INVENTION

According to one aspect of the present invention there is provided a method of preserving functionality of an organ. The method includes: (a) removing at least a whole organ including vasculature from a donor; (b) cryo-preserving the whole organ including vasculature; (c) allowing a period of time to elapse; (d) thawing the whole organ including vasculature so that at least a portion of cells contained therein resume biological activity; and (e) introducing the whole organ including vasculature into a recipient so that a blood vessel present in the vasculature of the whole organ including vasculature is supplied with blood by a vasculature system belonging to the recipient. For purposes of this specification and the accompanying claims, the phrase "whole organ including vasculature" refers to those blood vessels responsible for local supply of blood to the removed organ.

According to another aspect of the present invention there is provided a method of preserving fertility of a patient undergoing a treatment expected to cause sterility, the method includes; (a) removing at least one whole gonadal organ including vasculature from the patient; (b) cryo-preserving the at least one whole gonadal organ including vasculature; (c) conducting the treatment and waiting for an effect thereof to subside; (d) thawing the at least one whole gonadal organ including vasculature so that at least a portion of cells contained therein resume biological activity; and (e) introducing at least one whole gonadal organ including vasculature into the patient so that a blood vessel present in the gonadal organ is supplied with blood by a vasculature system belonging to the patient.

According to yet another aspect of the present invention there is provided a method of assuring a supply of viable gametes for future use. The method includes; (a) removing at least one whole gonadal organ including vasculature from a patient; (b) cryo-preserving the at least one whole gonadal organ including vasculature; (c) waiting until viable gametes are desired by the patient; (d) thawing the gonadal organ so that at least a portion of cells

contained therein resume biological activity; and (e) introducing the at least one whole gonadal organ including vasculature the patient so that the at least one whole gonadal organ including vasculature is supplied with blood by a vasculature system belonging to the recipient.

5 According to further features in preferred embodiments of the invention described below, a single individual serves as both the donor and the recipient.

 According to still further features in the described preferred embodiments the whole organ including vasculature has a capacity to fulfill at least one function selected from the group consisting of oogenesis, spermatogenesis, supply of a hormone, supply of a growth
10 factor, metabolism of a precursor into an end-product, elimination of waste and regulation of a physiologic process.

 According to still further features in the described preferred embodiments the method further includes facilitating angiogenesis of the whole organ including vasculature.

 According to still further features in the described preferred embodiments the organ is
15 a gonadal organ selected from the group consisting of an ovary and a testicle.

 According to still further features in the described preferred embodiments the period of time is selected from the group consisting of: (i) sufficient time for resolution of an acute medical condition of the donor; (ii) sufficient time for a change in a physiologic state of the recipient; (iii) sufficient time for identifying a recipient unlikely to reject tissue from the
20 donor.

 According to still further features in the described preferred embodiments the single individual is a cancer patient attempting to avoid sterility.

 According to still further features in the described preferred embodiments the single individual is a woman wishing to alleviate menopausal symptoms by implantation of a
25 previously removed ovary including vasculature after onset of menopause.

 According to still further features in the described preferred embodiments the single individual is a woman wishing to achieve altered fertility status by implantation of a previously removed whole ovary including vasculature.

 According to still further features in the described preferred the at least one whole
30 gonadal organ including vasculature is selected from the group consisting of an ovary and a testicle.

 According to still further features in the described preferred embodiments the method of further includes installing at least one prosthetic testicle in a scrotum of the patient.

According to still further features in the described preferred embodiments the method of further includes administering sex hormones to the patient.

The present invention successfully addresses the shortcomings of the presently known configurations by providing methods of preserving functionality of an organ, preserving fertility of a patient undergoing a treatment expected to cause sterility and assuring a supply of viable gametes for future use.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only, and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention, the description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

In the drawings:

FIG. 1 is a cartoon of a device used for freezing of a whole organ suitable for use in the context of the present invention ;

FIG. 2 is a flow diagram illustrating essential steps in methods according to the present invention;

FIGs. 3a-d are photomicrographs of oocytes (panels a and b) and embryos (panels c and d) from experimental (panels a and c) and control (panels b and d) ovaries; and

FIG. 4 is a graph illustrating estradiol and progesterone levels as a function of time post transplantation.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of methods of preserving functionality of an organ, preserving fertility of a patient undergoing a treatment expected to cause sterility and assuring a supply of viable gametes for future use which can be used to remove an organ, cryopreserve the removed organ and reintroduce the cryopreserved organ into a recipient.

Specifically, the present invention assures that the vasculature of the recipient supplies blood to the introduced organ or a portion thereof.

For purposes of better understanding the present invention, as illustrated in Figures 2 - 4 of the drawings, reference is first made to the construction and operation of a conventional (i.e., prior art) thermal gradient device as illustrated in Figure 1.

A freezing device **20** (Multi-Gradient-Directional (MGD) IMT technologies; Israel) according to earlier teachings of Arav (United States Patent 5,873,254) was employed to freeze removed whole organs. In brief, device **20** includes a plurality of temperature domains (e.g. **22**, **24**, **26** and **28**) within 270 mm copper blocks **36**. A tube containing a whole organ **30** is pushed at a constant velocity (V ; **32**) through the predetermined temperature gradient (**34**; $G = \Delta T/d$ where ΔT is temperature differences and d is the distance between temperatures) resulting in a cooling rate (B) according to the equation $B = G * V$. The cooling rate may be set to, for example, $0.03^{\circ}\text{C}/\text{min}$ by varying velocity **32** at which tube **30** containing the organ passes through the gradient **34**. Seeding is typically performed at the tip of the tube **30** and ice interphase is propagated according to the freezing point of the solution.

The principles and operation of methods according to the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

Referring now to the drawings, Figure 2 illustrates a method **40** of preserving functionality of an organ. Method **40** includes removing **42** at least a whole organ including vasculature from a donor. Method **40** further includes cryo-preserving **44** the whole organ including vasculature and allowing **46** a period of time to elapse. Method **40** further includes thawing **48** the whole organ including vasculature so that at least a portion of cells contained therein resume biological activity. Method **40** further includes introducing **50** the whole organ including vasculature into a recipient so that a blood vessel present in the vasculature of the whole organ including vasculature is supplied with blood by a vasculature system belonging to the recipient. Preferably, a single individual serves as both the donor and the recipient. The

single individual may be, for example, a cancer patient attempting to avoid sterility. Alternately, the single individual may be a woman wishing to alleviate menopausal symptoms by implantation of a previously removed ovary including vasculature after onset of menopause. Alternately, but also preferably, the single individual is a woman wishing to achieve altered fertility status by implantation of at least one previously removed whole ovary including vasculature. In this case, a woman may elect to have one or both ovaries remove when she is relatively young, and then have one or both ovaries re-implanted at a later time when she wishes to conceive.

According to various preferred embodiments of method 40, the whole organ including vasculature may be introduced to fulfill at least one function including, but not limited to, oogenesis, spermatogenesis, supply of a hormone, supply of a growth factor, metabolism of a precursor into an end-product, elimination of waste or regulation of a physiologic process. Most preferably, the organ is a gonadal organ i.e. an ovary or a testicle.

Preferably, method 40 further includes facilitating angiogenesis of the whole organ including vasculature.

Allowing a period of time to elapse may be for a variety of reasons, such as, for example, allowing sufficient time for resolution 52 of an acute medical condition of the donor, or allowing sufficient time for a change 54 in a physiologic state of the recipient, or allowing sufficient time for identifying 56 a recipient unlikely to reject tissue from the donor.

The invention is further embodied by method 40 of preserving fertility of a patient undergoing a treatment expected to cause sterility. Method 40 includes removing 42 at least one whole gonadal organ including vasculature from the patient. Method 40 further includes cryo-preserving 44 the at least one whole gonadal organ including vasculature. Method 40 further includes conducting 45 the treatment and waiting for an effect thereof to subside. Method 40 further includes thawing 48 the at least one whole gonadal organ including vasculature so that at least a portion of cells contained therein resume biological activity; and introducing 50 the at least one whole gonadal organ including vasculature into the patient so that a blood vessel present in the gonadal organ is supplied with blood by a vasculature system belonging to the patient. The at least one whole gonadal organ including vasculature is either an ovary or a testicle depending upon the sex of the patient.

In cases where the gonadal organ is a testicle, method 40 preferably further includes installing 58 at least one prosthetic testicle in a scrotum of the patient. This step is performed to prevent damage to the patient's self image.

Whether the patient is male or female, method 40 further includes administering 60 sex hormones to the patient if both gonadal organs are removed so that secondary sex characteristics are not affected.

The present invention is further embodied by method 40 of assuring a supply of viable
5 gametes for future use. Method 40 includes removing 42 at least one whole gonadal organ including vasculature from a patient. Cryo-preserving 44 the at least one whole gonadal organ including vasculature and waiting until viable gametes are desired 47 by the patient. Method 40 further includes thawing 48 the gonadal organ so that at least a portion of cells contained therein resume biological activity and introducing 50 the at least one whole gonadal
10 organ including vasculature into the patient so that the at least one whole gonadal organ including vasculature is supplied with blood by a vasculature system belonging to the recipient.

Freezing of whole organs in conjunction with methods 40 may be accomplished, for example, using devices taught by Arav (United States Patent 5,873,254) for example by
15 placing the whole organ in a volume of freezing solution; pushing the solid tissue at a velocity (V) through a predetermined temperature gradient (G) thus determining a cooling rate (B) according to the equation $B=G*V$; initiating crystallization at a predetermined position within the temperature gradient (G); and allowing the solid tissue to continue to move through the temperature gradient (G) at the velocity. According to Arav $G = \Delta T/d$ where ΔT is the
20 temperature difference and d is the distance over which it is maintained. Thawing of the cryo=preserved organ may be accomplished, for example, by placing the cryopreserved organ into a 66°C environment for a first period of time and placing the cryopreserved solid tissue into a 38°C water bath for a second period of time as described hereinabove.

Additional objects, advantages, and novel features of the present invention will
25 become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

Additional objects, advantages, and novel features of the present invention will
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EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, and cell biology techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Culture of Animal Cells - A Manual of Basic Technique" by Freshney, Wiley-Liss, N. Y. (1994), Third Edition; "Animal Cell Culture" Freshney, R. I., ed. (1986); "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990);); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

Materials and Methods

Reference is first made to the following materials and methods which are generally employed in performing experiments described in examples set forth hereinbelow.

Cryopreservation of organs: Organs (e.g. ovaries) were removed from donor animals and perfused with Belzer UW solution(ViaSpan®, Do Pont Pharma, USA) +10% dimethyl sulfoxide(DMSO; Sigma Chemicals, St. Louis, USA) by insertion of a catheter into a blood vessel of the organ.

Organs, and their vasculature network, were then transferred to 16x100mm glass tubes (Manara, Israel) and frozen using a commercially available (IMT, Israel) multi-gradient directional freezing apparatus (described in United States Patent 5,873,254). Different freezing temperatures were assayed as described hereinbelow.

Cryo-preservation apparatus: A Cryopreservation apparatus as described by Arav (United States Patent 5,873,254; Figure 1) was employed for freezing the ovaries as described hereinabove. Briefly, the apparatus includes 4 temperature domains within 270 mm copper blocks. A test tube containing the sample is pushed at a constant velocity (V) through the predetermined temperature gradient ($G = \Delta T/d$ where ΔT is temperature differences and d is the distance between temperatures) resulting in a cooling rate (B) according to the equation $B = G * V$. The cooling rate was set to $0.1^{\circ}\text{C}/\text{min}$ by varying the speed the tube passes through the temperature gradient. Seeding was performed at the tip of the test tube and ice interphase was propagated according to the freezing point of the solution.

In-vitro follicular assays:

Follicles were aspirated using a 22 gauge needle connected to a 5ml syringe and thin ovarian cortex slices were cut. The granulosa cells and follicular viability was assessed using FDA (fluorescein diacetate)/DAPI (4,6-diamidino-2-phenylindole) fluorescent live/dead staining (Molecular Probes, Leiden, the Netherlands and Merk, Germany).

Thawing of cryopreserved organs:

Organs were first immersed in a high temperature ($66-68^{\circ}\text{C}$) water bath for 10 to 20 seconds followed by immersion in a $38-40^{\circ}\text{C}$ water bath for 10 to 120 seconds. Ovaries were then perfused with 0.5M sucrose and 150iu heparin to remove cryoprotectants and prevent blood clotting.

Introduction of organs into recipients:

Transplantation was performed by re-anastomizing arteries and veins of the transplanted organ with 10 zero sutures (Ethilon; Johnson and Johnson, U.S.A.). Success of the procedure was evaluated by observing arterial blood pulse and venous return flow in the newly introduced organ. A hyaluronic acid gel was employed to prevent adhesions (Intergel; Johnson and Johnson, U.S.A.). For ovary transplantation was autologous.

Fertility evaluation of transplanted ovaries:

Two months after transplantation, 600iu of PMSG were administered to the recipient to cause superovulation (Intervet International BV; Boxmeer, Netherlands). Three days later follicles were aspirated from the transplanted organ using a syringe and 20 gauge needle. Oocytes were transported in a mobile incubator (Minitube, Germany) at 38°C in buffer medium to an IVF laboratory. Aspirated oocytes were then put into TCM-199 maturation medium supplemented with 10 % (V/V) heat-inactivated fetal calf serum

(FCS) (Bio-lab, Jerusalem, Israel), 0.2mmol Na pyruvate l^{-1} , 5 μ g gentamicin l^{-1} , 10 μ g ovine LH ml^{-1} (NIADDK-NIH-26, AFP5551B, Bethesda, MD), 1 μ g ovine FSH ml^{-1} (NIADDK-NIH-20, AFP7028D, Bethesda, MD) and 1 μ g estradiol ml^{-1} and incubated in a 38.5°C in a humidified atmosphere of 5 % CO₂ in air. After maturation, oocytes were
5 denuded from cumulus cells in the maturation wells and placed for 5 min. in the ionomycin medium: 10 ml TCM-199, supplemented with 10 % (V/V) heat-inactivated FCS, 0.2mmol Na pyruvate l^{-1} , 5 μ g gentamicin l^{-1} , and 5 μ mol of ionomycin l^{-1} . Oocytes were transferred to 6-dimethylaminopurine (6-DMAP) medium (10 ml of TCM 199 supplemented with 2mmol of 6-DMAP l^{-1}) for 4.5h. The oocytes were then washed three
10 times in a cleavage medium (Sydney IVF Cleavage Medium, Cook, Australia) and transferred into 50 μ l cleavage drops under mineral oil for another three days. Cleaved embryos were counted on day 4 (activation day = 0) and transferred to 50 μ l drops under mineral oil of blastocysts medium (Sydney IVF Blastocyst Medium, Cook, Australia). Blastocysts were counted after 8-10 days.

15 Hormonal status: Progesterone and estradiol levels were analyzed in serum samples bi-weekly. Progesterone was measured using COAT-A-COUNT Progesterone DPC®, USA. Estradiol levels were measured by ESTER-US-CT, ORIS Group, France.

Example 1:

In vitro assay of viability of sheep ovaries after thawing

20 In order to determine the effect of freezing temperature on subsequent viability, fifteen Assaf sheep ovaries were collected 10 min after slaughter. The ovaries were perfused with a Belzer UW solution (ViaSpan®, Do Pont Pharma, USA) + 10% DMSO (Sigma, St. Lois, USA) for 3 min, in order to obtain maximal permeability of the organ to DMSO. After
25 perfusion, the ovaries were loaded into 16X100mm glass test tubes (Manara, Israel) in UW+10% DMSO and transferred to the freezing apparatus (IMT, Israel) described hereinabove. Control ovaries were perfused as above but were not cryopreserved. Ovaries were frozen to different temperatures as detailed in table 1.

Table 1: Freezing parameters of whole ovine ovaries

EXP.	T 1	T 2	T 3	T 4	T5	V	CR
1	0°C	-6°C	-10°C	-14°C	-	0.03 mm/s	0.1°C/min
2	0°C	-6°C	-18°C	-30°C	-	0.01 mm/s	0.1°C/min
3	0°C	-6°C	-18°C	-30°C	-196°C	0.01mm/s	0.1°C/min

Briefly, the cooling rate was set to 0.1°C/min by varying the speed of the tube through the temperature gradient. Seeding was performed at the tip of the test tube and ice interface was propagated according to the freezing point of the solution. 15 minutes after reaching the final temperature the test tube was thawed. When -196°C was used as the final temperature, ovaries were stored in LN for 2 days to one week before being thawed.

Frozen ovaries were thawed as described hereinabove. The frozen-thawed whole ovaries were then washed in buffer medium at 38°C. Follicles were aspirated with a 22G needle connected to a 5ml syringe in order to obtain granulosa cells. Thin slices were cut from the ovarian cortex. The granulosa cells and primordial and small antral follicles were evaluated for viability using fluorescent microscopy as described by Oktay et al. (Fertil. and Steril. 1997(67):481-486) FDA (fluorescein diacetate)/DAPI (4,6-diamidino-2-phenylindole) fluorescent live/dead staining (Molecular Probes, Leiden, the Netherlands and Merk, Germany) was performed.

Granulosa cells that were evaluated after freezing and thawing showed no statistically significant differences from controls (Table 2). The survival of small follicles (small antral and primordial follicles) showed no statistically significant differences from controls (Table 2). Means were calculated using the General Linear Model procedure of JMP (SAS Institute, 1994) and differences between treatments examined by analysis of variance. Significance was $P < 0.05$.

These results clearly demonstrate that small follicles and granulosa cells are not damaged by freezing and thawing using conditions described herein.

Table2: In vitro study survival of ovaries after freezing to different temperatures

Exp.	Number of ovaries	Storage temp.	Viability of granulosa cells($\pm$ SE)	Live follicles /dead follicles
Cont.	3	Room temp.	50% $\pm$ 7	101/105
1	3	-14°C	38% $\pm$ 29	69/69
2	3	-30°C	40% $\pm$ 16	18/37
3	5	-196°C	58 $\pm$ 16	68/73

Example2:

In vivo assay of viability sheep ovaries after thawing

In order to verify the results of Example 1 in an *in vivo* system, eleven-month-old Assaf sheep were subjected to oophorectomy under general anaesthesia via longitudinal laparotomy. The uterus and ovaries were exposed and a dissection of the right ovarian artery and vein was performed. The ovary was perfused *in vivo* with UW + 10% DMSO for 3 min. It was then excised and inserted into a freezing tube containing UW + 10% DMSO. Slow freezing was performed by cooling from 0 to -6°C over 2 min, seeding was done before entering -6°C and cooling to -14°C, -30°C and thawing after 15 minutes or to -30°C and then direct plunging into LN and then thawing. The cooling rate of the three different procedures was 0.1 °C/min. After 15 min at the final temperature thawing was performed by plunging the test tube into a 66°C water bath for 20 s and then into a 40°C water bath for 2 min. Ovarian vascular transplantation was performed by re-anastomosing the ovarian artery and vein with 10 zero sutures (Ethilon, Johnson and Johnson). After the arterial anastomosis was completed, blood flow was verified by observing blood pulse in the ovary and venous return through the ovarian vein.

Two months after autotransplantation, 600 iu PMSG (intervet international BV, Boxmeer-Holand, France: intervet SA 49100-Angers, Pais-Bas) was administered. Oocytes were aspirated three days later, matured, and parthanogenetically activated *in vitro* as described hereinabove. Parthenogenetic activation resulted in normal development of 3/5 retrieved oocytes., pictures of oocytes (Figures 3a and b)and embryos (Figures 3c and)

indicate no apparent difference between experimental (Figures 3a and c) and control (Figures 3b and d) oocytes or embryos. Following oophorectomy of the unfrozen contralateral ovary in the recipient, blood was taken bi-weekly for two months and progesterone and estradiol levels were evaluated using a commercially available assay (Coat-A-Count Progesterone, DPC, LA, USA and ESTR-US-CT, ORIS Group, France) Results are summarized in Figure 4.

Briefly, estrogen and progesterone levels dropped to negligible levels immediately post transplantation. Estrogen levels surged four weeks following transplantation of an ovary frozen in liquid nitrogen. Cyclical estrogen and progesterone thereafter were measured for over thirty weeks. These results clearly demonstrate that the implanted ovary restored normal hormonal balance to the recipient. Further, normal oocyte division and development (Figure 3) indicate that the primordial and small antral follicles in the transplanted ovary recover from freezing without ill effect. In summary, these results indicate that it is now feasible to remove and freeze a solid organ for subsequent transplantation into a recipient and to achieve full physiologic activity from the thawed transplanted organ. This capacity is expected to find utility in a variety of clinical contexts as detailed hereinabove.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims. All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention.

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WHAT IS CLAIMED IS:

1. A method of preserving functionality of an organ, the method comprising:

(a) removing at least a whole organ including vasculature from a donor;

(b) cryo-preserving said whole organ including vasculature;

(c) allowing a period of time to elapse;

5 (d) thawing said whole organ including vasculature so that at least a portion of cells contained therein resume biological activity; and

(e) introducing said whole organ including vasculature into a recipient so that a blood vessel present in said vasculature of said whole organ including vasculature is supplied with blood by a vasculature system belonging to said recipient.

10

2. The method of claim 1, wherein a single individual serves as both said donor and said recipient.

3. The method of claim 1, wherein said whole organ including vasculature

15 has a capacity to fulfill at least one function selected from the group consisting of oogenesis, spermatogenesis, supply of a hormone, supply of a growth factor, metabolism of a precursor into an end-product, elimination of waste and regulation of a physiologic process.

20 4. The method of claim 1, further including facilitating angiogenesis of said whole organ including vasculature.

5. The method of claim 1, wherein the organ is a gonadal organ selected from the group consisting of an ovary and a testicle.

6. The method of claim 1, wherein said period of time is selected from the group
5 consisting of:

- (i) sufficient time for resolution of an acute medical condition of said donor;
- (ii) sufficient time for a change in a physiologic state of said recipient;
- (iii) sufficient time for identifying a recipient unlikely to reject tissue from said donor.

10 7. The method of claim 2, wherein said single individual is a cancer patient attempting to avoid sterility.

8. The method of claim 2, wherein said single individual is a woman wishing to
15 alleviate menopausal symptoms by implantation of a previously removed ovary including vasculature after onset of menopause.

9. The method of claim 2, wherein said single individual is a woman wishing to achieve altered fertility status by implantation of a previously removed whole ovary
20 including vasculature.

10. A method of preserving fertility of a patient undergoing a treatment expected to cause sterility, the method comprising;

- (a) removing at least one whole gonadal organ including vasculature from the
25 patient;

- (b) cryo-preserving said at least one whole gonadal organ including vasculature;
- (c) conducting the treatment and waiting for an effect thereof to subside;
- (d) thawing said at least one whole gonadal organ including vasculature so that at least a portion of cells contained therein resume biological activity; and

5 (e) introducing at least one whole gonadal organ including vasculature into the patient so that a blood vessel present in said at least one gonadal organ is supplied with blood by a vasculature system belonging to the patient.

11. The method of claim 10 wherein said at least one whole gonadal organ including vasculature is selected from the group consisting of an ovary and a testicle.

10

12. The method of claim 10, further comprising installing at least one prosthetic testicle in a scrotum of the patient.

13. The method of claim 10 further comprising administering sex hormones to the
15 patient.

14. A method of assuring a supply of viable gametes for future use, the method comprising;

(a) removing at least one whole gonadal organ including vasculature from a patient;

20 (b) cryo-preserving said at least one whole gonadal organ including vasculature;

(c) waiting until viable gametes are desired by the patient;

(d) thawing said gonadal organ so that at least a portion of cells contained therein resume biological activity; and

(e) introducing said at least one whole gonadal organ including vasculature the patient so that said at least one whole gonadal organ including vasculature is supplied with blood by a vasculature system belonging to the patient.

5 15. The method of claim 14 wherein said gonadal organ is selected from the group consisting of an ovary and a testicle.

 16. The method of claim 14, further comprising installing at least one prosthetic testicle in a scrotum of said patient.

10

 17. The method of claim 14 further comprising administering sex hormones to said patient.

FIGURE 1 20

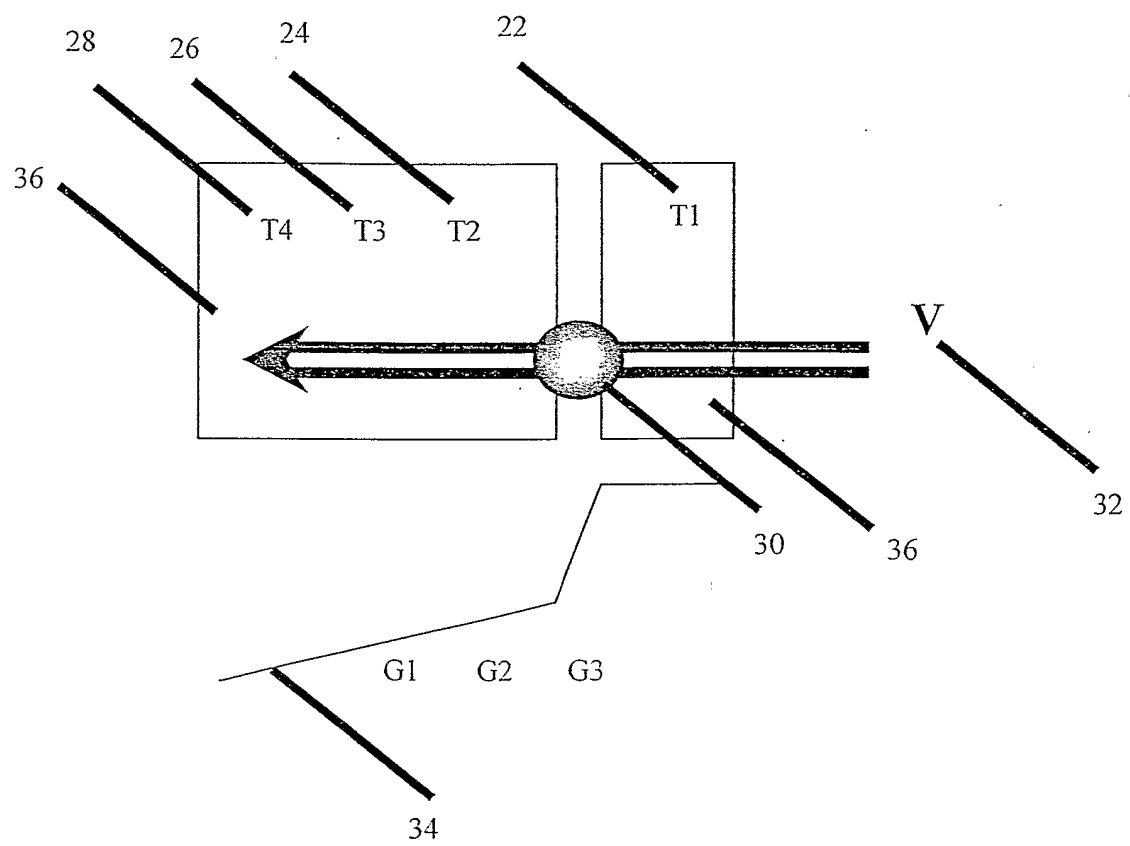


FIGURE 2 40

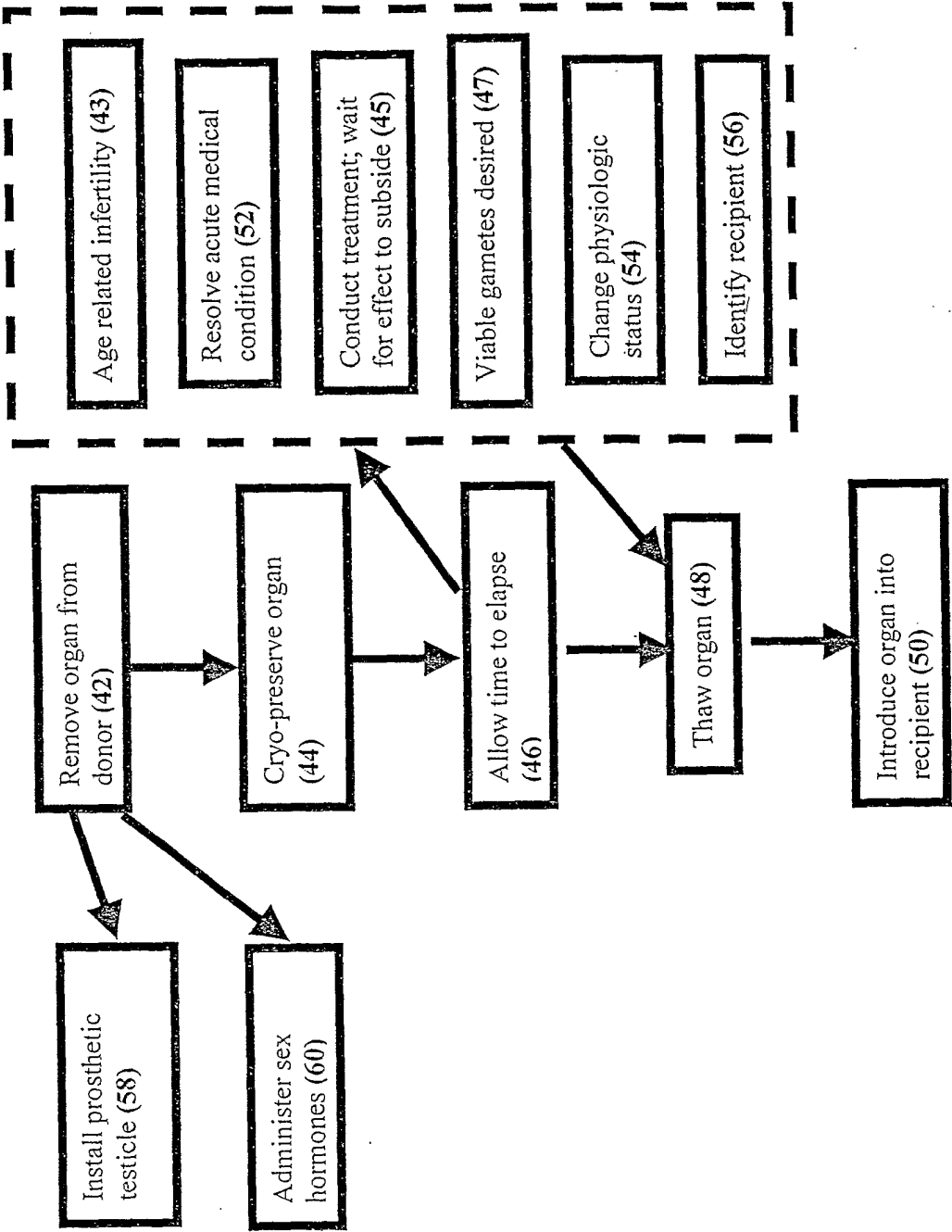


FIGURE 3

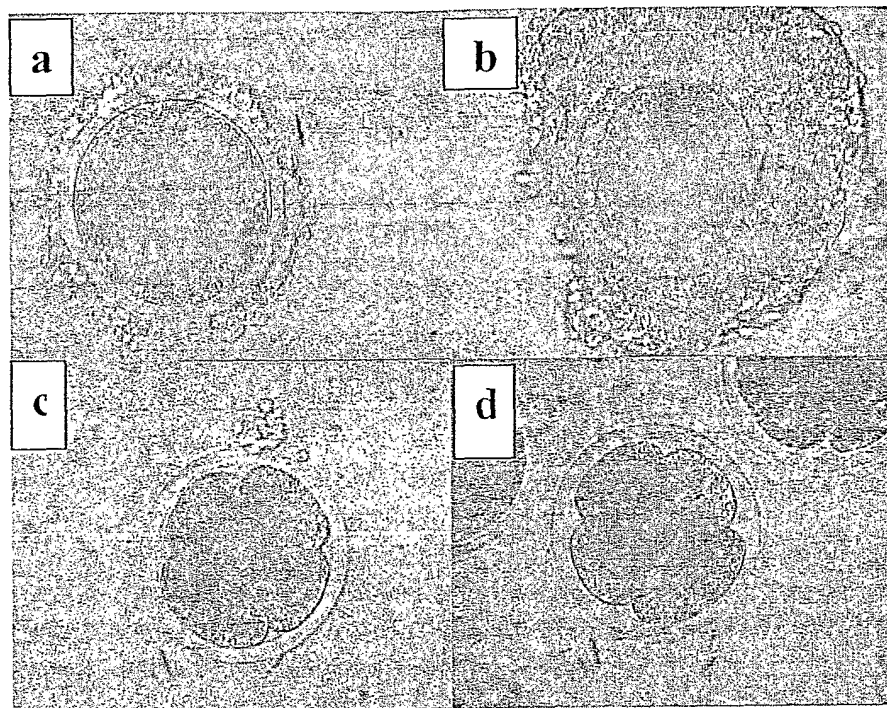
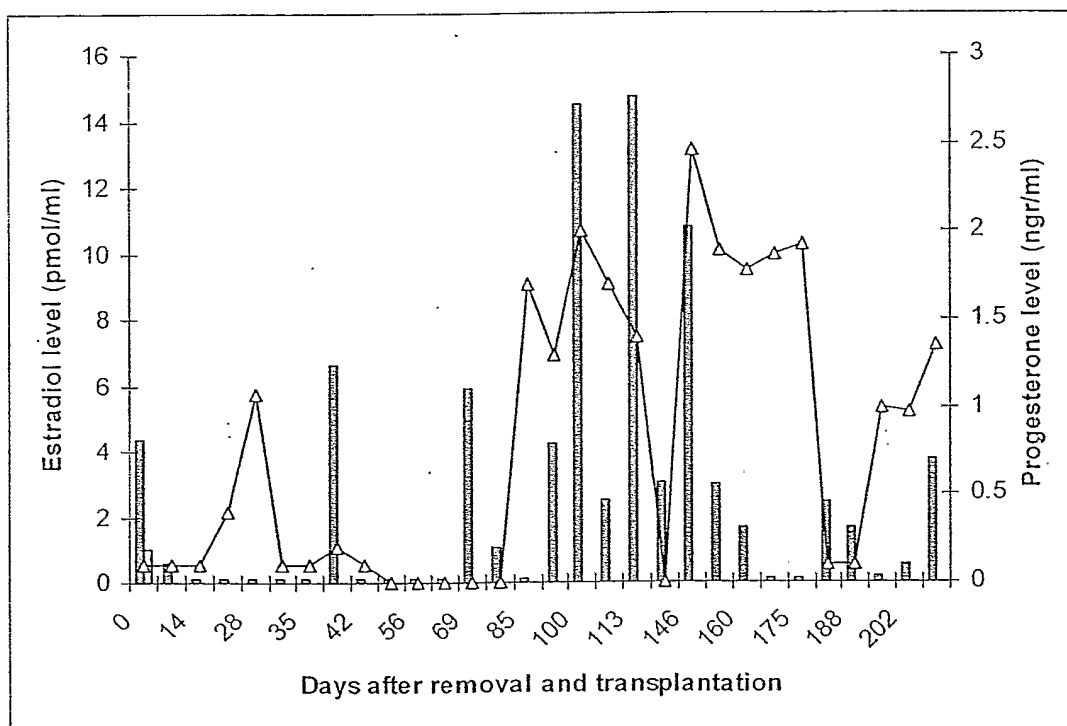


FIGURE 4



INTERNATIONAL SEARCH REPORT

International application No.

PCT/IL02/00709

A. CLASSIFICATION OF SUBJECT MATTER														
IPC(7) : A01N 1/00 US CL : 435/1.3														
According to International Patent Classification (IPC) or to both national classification and IPC														
B. FIELDS SEARCHED														
Minimum documentation searched (classification system followed by classification symbols) U.S. : 435/1.3														
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched														
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) Medline, WPIDS, Biosis, USPATFUL														
C. DOCUMENTS CONSIDERED TO BE RELEVANT														
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.												
X	SALLE et al., Restoration of Ovarian Steroid Secretion and Histologic Assessment after Freezing, Thawing and Autograft of a Hemi-Ovary in sheep. Fertility and Sterility. August 1999, Vol. 72, No. 2, pages 366-370, especially abstract.	1-5, 14, 15 ----- 6-11, 13, 17												

X	SALLE et al., Normal Pregnancies and Live Births after Autograft of Frozen-Thawed Hemi-Ovaries into Ewes. Fertility and Sterility. February 2002, Vol. 77, No. 2, pages 403-408, abstract.	1-7, 9-11, 14, 15 ----- 8, 13, 17												

Y	AUBARD et al., Greffes et Transplantations Ovariennes Chez la Femme: le Point. Revue Francaise de Gynecologie et d'Obstetrique. December 1993, Vol. 88, No. 12, pages 583-590, abstract.	8												
Y	HILLIER, The Parks Lecture: Controlled Ovarian Stimulation in Women. Journal of Reproduction and Fertility. November 2000, Vol. 120, No. 2, pages 201-210, abstract.	13, 17												
☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.														
<table border="0"> <tr> <td>* Special categories of cited documents:</td> <td>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td> </tr> <tr> <td>"A" document defining the general state of the art which is not considered to be of particular relevance</td> <td>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td> </tr> <tr> <td>"B" earlier application or patent published on or after the international filing date</td> <td>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td> </tr> <tr> <td>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td> <td>"&" document member of the same patent family</td> </tr> <tr> <td>"O" document referring to an oral disclosure, use, exhibition or other means</td> <td></td> </tr> <tr> <td>"P" document published prior to the international filing date but later than the priority date claimed</td> <td></td> </tr> </table>			* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	"B" earlier application or patent published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family	"O" document referring to an oral disclosure, use, exhibition or other means		"P" document published prior to the international filing date but later than the priority date claimed	
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"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone													
"B" earlier application or patent published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art													
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family													
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Date of the actual completion of the international search 15 May 2003 (15.05.2003)		Date of mailing of the international search report 23 JUL 2003												
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (703)305-3230		Authorized officer <i>Felicia D. Roberto for</i> Sandra Saucier Telephone No. (703) 308-0196												

INTERNATIONAL SEARCH REPORT

PCT/IL02/00709

Continuation of Item 4 of the first sheet:

The title is too long under PCT Rule 4.3. It has been shortened to:

Method of preserving the functionality of an organ