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(71) Applicant: RUSH-PRESBYTERIAN-ST. LUKE'S MEDICAL CENTER [US/US]; 1653 West Congress Parkway, Chicago, IL 60612 (US).

(72) Inventors: RAWLINS, Richard, Graham; 520 Forest Avenue, Oak Park, IL 60302 (US). DMOWSKI, Wojciech, Paul; 300 North Maple, Apartment 9, Oak Park, IL 60302 (US).

(74) Agents: WATT, Phillip, H. et al.; Fitch, Even, Tabin & Flannery, Room 900, 135 South LaSalle Street, Chicago, IL 60603 (US).

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(54) Title: SPERMATOZOA SEPARATION METHOD

(57) Abstract

The present invention provides a process for preparing, from a first sample of spermatozoa of a mammalian species, a second sample wherein the fraction of male spermatozoa is different from said fraction in the first sample.
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SPERMATOZOA SEPARATION METHOD

TECHNICAL FIELD

The present invention relates to separation of spermatozoa of a mammalian species into fractions which differ significantly in the fractions of male and female sperm which they contain.

BACKGROUND OF THE INVENTION

It has long been recognized that methods to predictably increase the probability that a mammal of a particular sex would be conceived, during sexual intercourse, by artificial insemination or by in vitro fertilization, would be desirable. The advantages that such methods would provide in agriculture and to human couples that desire to have a child of a particular sex are apparent.

Perhaps the most widely employed methods now in use to increase such probabilities are those described by Ericsson in numerous publications and in U. S. Patent Nos. 4,009,260 and 4,339,434, both of which are incorporated herein by reference.

Ericsson's methods rely on the use of gradients of density and/or viscosity in an aqueous solution which is physiologically acceptable to sperm to separate a fraction of sperm, with a high fraction of male sperm, from a sperm sample. Ericsson's method of sperm separation is not capable of providing a fraction with a significant increase in the fraction of female sperm. Ericsson's methods depend on high concentrations of substances such as serum albumins to provide the density and/or viscosity gradients required for the methods.

Attempts have been made to establish alternatives to the albumin-gradient sperm separation methods of Ericsson. Thus, for example, Shishito et al., Intl. J. Fertil. 20, 13 (1975) have reported the separation of a fraction of sperm enriched in male sperm by electrophoresis and Steeno et al., Andrologia 7, 95 (1975) have reported the separation of a fraction of
sperm enriched in female sperm by gel filtration (e.g., Sephadex™ gel filtration).

Ericsson's methods, as well as the alternatives thereto, have a number of drawbacks. First, the methods require complex media or apparatus to accomplish the separation of a desired fraction from a sperm sample. Second, the methods are quite time consuming and labor intensive. Third, the desired fractions obtained by the various methods have concentrations of sperm that are low compared to concentrations in the ejaculates subjected to the methods and, consequently, conception rates achieved with such fractions are low. Finally, the methods permit the separation, from a single sample, of either a fraction enriched in male sperm or a fraction enriched in female sperm but not both.

SUMMARY OF THE INVENTION

We have now discovered a simple method for separating a mammalian sperm sample simultaneously into a fraction enriched in male-chromosome-bearing (i.e., male) sperm and a fraction enriched in female-chromosome-bearing (i.e., female) sperm. The method entails simply swim-up, against gravity, of sperm from a sample, such as a "pellet" provided by low speed centrifugation from a suspension of sperm in an aqueous solution that is physiologically acceptable to the sperm, into an aqueous solution that overlays the sample and also is physiologically acceptable to the sperm. The method of the invention is significantly improved over prior art methods in that it is significantly less time-consuming than such methods and does not employ the viscosity or density gradients of the type required by the Ericsson methods or the complex media or apparatus required by other prior art methods.
Surprisingly and unexpectedly, in view of the teachings of Ericsson and others that male sperm are more motile than female, we have discovered that the upper layer of solution, into which the sperm swim against gravity in the method of the invention (e.g., typically, the 40% to 70% of the volume of that solution that is further in the vertical direction from the pellet, or sample in other form, being separated, than the remainder of the volume of said solution (the lower layer)) is enriched in female sperm while the lower layer of said solution (which is in interfacial contact with the pellet (or sample in other form), which, in turn, rests on the bottom of the tube or other vessel in which the method is carried out) is enriched in male sperm.

Surprisingly and unexpectedly, the simple method of the invention advantageously permits the simultaneous separation from a sperm sample of a fraction enriched in male sperm and a fraction enriched in female sperm and both fractions have a yield of sperm significantly improved over the yields in the fractions that can be obtained by the Ericsson methods and other prior art methods.

DETAILED DESCRIPTION OF THE INVENTION

The method of our present invention depends on the different rate of movement of motile male sperm and motile female sperm of a mammal (including a human) against gravity through an aqueous solution starting with a sperm pellet or other sperm sample at the bottom of the solution in a tube or other suitable vertically elongated vessel.

The aqueous solution, up into which the sperm swim in the method of the invention, is any of various solutions, known to the skilled in the art, which are physiologically acceptable to mammalian sperm (i.e., in
which such sperm remain viable and motile). and which, immediately prior to application to the pellet (or sperm sample in other form) are homogeneous. Unlike in the methods of Ericsson, gradients of density and/or viscosity are not established in the solutions employed in the methods of the present invention prior to application of a sperm sample to a solution for separation in accordance with the invention. Any such gradients that might arise in a solution employed in carrying out the invention would be only slight gradients that might arise as a consequence of the fractionation of the sperm or as a consequence of the slight settling of solution over the short duration (typically about 1 hr. at 37 °C) of the sperm-separation process. Typical among the aqueous solutions that can be employed in carrying out the methods of the invention are Tyrode’s Solution, Ham’s F-10 Medium, and Ham’s F-10 Medium supplemented with 5 % - 15 % human serum (e.g., 10 % heat inactivated maternal serum).

Although the teachings of Ericsson and others suggest that male sperm of a mammal are more motile than female sperm of the mammal, we have found that male sperm move upward against gravity through an aqueous solution employed in the present invention more slowly than female sperm.

More particularly, the invention is a process for preparing from a first sample of sperm of a mammal, said first sample held in a vessel, a second sample, wherein the fraction of sperm with the male sex chromosome is different from said fraction in said first sample, which process comprises: (a) placing in interfacial contact with said first sample in said vessel an aliquot of an homogeneous aqueous solution that is physiologically acceptable to the sperm, said aliquot having a volume consisting of a lower layer and an upper
layer, said lower layer being in contact with and extending vertically upward from said first sample and, together with said first sample, filling the entire volume of said vessel below said upper layer; (b) maintaining said first sample and said aliquot of aqueous solution at substantially the same temperature, within a range of temperatures at which the sperm of the species remain motile, and maintaining said vessel substantially stationary, until a portion of the sperm of said first sample migrates into said upper layer to provide in said upper layer a fraction of sperm with the male sex chromosome which is less than said fraction in said first sample; and (c) removing said upper layer from said vessel.

In the vessel used in carrying out the method of the invention, the sample of sperm, from which a fraction enriched in male sperm and a fraction enriched in female sperm are made in accordance with the invention, is located at the bottom (i.e., at the position of lowest gravitational potential in the vessel).

This sample of sperm will typically be a "pellet" of sperm prepared by methods well known in the art by low speed centrifugation of a suspension of sperm (from an aliquot of liquefied semen) in an aqueous solution that is physiologically acceptable to the sperm followed by aspiration of solution and then, preferably, resuspension of the pellet from the first centrifugation into another aliquot of physiologically acceptable solution followed, in turn, by low speed centrifugation and aspiration of solution. As understood in the art, the volume of such a pellet will depend on the volume and sperm count of the liquefied semen from which the pellet is prepared. With human sperm, a typical pellet volume will be 100 µl to 200 µl per 250 µl of liquefied semen. Methods of preparation of liquefied mammalian semen are well known in the art.
Of the solution, into which the sperm swim (migrate) from the sample of sperm in accordance with the invention, the lower layer will completely cover said sample and will extend vertically upward (i.e., to higher potential in the gravitational field) from the uppermost point of said sample (i.e., the point furthest in the vertical direction from the bottom of the vessel). The upper layer of said solution, which is the entire solution volume that is not in the lower layer, extends vertically upward from the uppermost surface of the lower layer.

The boundary between the upper layer and lower layer of the solution is defined operationally by the volume of said solution (together with sperm that has swum up from the first sample) that is removed in step (c) of the method described above. To minimize mixing of the entire solution (and the reduction, that would accompany such mixing, in the separation of female sperm from male sperm that had occurred during the swim up), to obtain an upper layer of solution with a maximum increase in fraction of female sperm, and to obtain a lower layer of solution with a maximum increase in fraction of male sperm, the layer of solution removed in said step (c) of the method is a top layer (i.e., a layer the top of which is the solution surface that is in contact with the air (or other gas) above the solution in the vessel in which the method is carried out and that is most distant vertically from the bottom of the vessel). That is, the upper layer is a top layer, as just described, and not a layer with a top (an upper surface) below the solution surface that is in contact with the air (or other gas) above the solution in the vessel in which the method is carried out and that is most distant vertically from the bottom of the vessel.
This volume of solution removed in step (c) of the method as described above is the volume of the upper layer. A number of factors are significant in determining what these volumes are. The volumes will depend to some extent on the shape and dimensions of the part of the vessel in which the first sperm sample and solution rest, as it is important to remove upper layer without mixing or otherwise disrupting the solution and sperm sample in a way that will reduce significantly the separation of sperm that was achieved during the swim up and the shape and dimensions of the pertinent part of the vessel will influence the volume of upper layer that can be so removed. Further, this volume will be selected to achieve, on the one hand, one or both of an acceptably high fraction of female sperm in the upper layer and an acceptably high fraction of male sperm in the lower layer (with acceptability depending on whether it is intended to use only one or both of the fractions for fertilization by artificial insemination or in vitro fertilization and on what is desired for the probability that fertilization with a fraction will result in a zygote of one of the sexes) and, on the other hand, a sufficiently high number and concentration of healthy, motile sperm in the fraction or fractions of interest to make the probability that fertilization can be accomplished with the fraction (or fractions) acceptably high. Further, we have discovered unexpectedly that, with some human sperm samples, which are usually those with at least average concentrations of forwardly motile sperm, when the solution in which the separation method is carried out is as described in the Example below, a marked difference in optical density arises spontaneously between a lower layer, which is in contact with and extends upward from the sperm pellet and is milky in appearance, and an upper layer, which extends vertically
upward from the top surface of said lower layer to the top surface of the solution and is translucent. The boundary between such lower layer and such upper layer is clearly visible (after a swim up for 1 hour at 37 °C as described in the Example) from the rapid change in optical density (as a function of vertical distance upward from the bottom of the vessel) at said boundary. Typically, the volume of the upper layer removed in accordance with step (c) of the method of the invention described above will between about 40 % and about 70 % (preferably about 50 % to 60 %) of the volume of the solution placed in contact with the first sperm sample. When a boundary, defined by optical density differences, occurs between an upper layer and a lower layer, as just described, substantially all of the translucent layer is preferably taken as the upper layer; typically the volume of such an upper layer is about 50 % to about 60 % of the total volume of solution.

In accordance with the invention, the lower layer of solution is usually removed from the vessel (and from the sperm pellet or sperm sample in other form) after the upper layer is removed as described above. (If the lower layer is removed, it is removed after the upper layer.)

Removal of the layers is achieved by any of many methods known in the art, typically with a sterile, plastic squeeze pipette or micropipette or like apparatus. As indicated above, the removal of the layers is carried out carefully to minimize mixing of solution or of solution with first sperm sample.

If desired, sperm from a sample of liquefied semen can be divided into a plurality of samples of convenient size and each of these samples can be processed to yield a first sperm sample for separation of fractions increased in male sperm and in female sperm in
accordance with the invention. We have found, for example, that the preparation of eight "first semen samples" for simultaneous processing, each with 500 µl of solution for sperm swim up, by the method of the invention is suitable with samples of human semen.

Once an upper layer, or a lower layer, or both are removed from the vessel, in which the swim up for sperm separation occurred, the layer or layers are treated appropriately, by methods known in the artificial insemination and in vitro fertilization arts, so that the sperm they contain can be used in artificial insemination or in vitro fertilization. Both the upper layer and the lower layer are "second samples," within the meaning of that term in the above-definition of the invention, as both layers have a fraction of male sperm that differs from said fraction in the first sample (e.g., sperm pellet) from which swim-up occurred. If sperm of a semen sample has been separated into fractions, each of which is processed in accordance with the method of the invention, the upper layers from all or part of the processed fractions can be combined and the lower layers from all or part of the processed fractions can be combined for an artificial insemination or in vitro fertilization. As the skilled will understand, providing a sperm count sufficiently high for an acceptable probability that artificial insemination or invitro fertilization will be achieved may require that the layers from more than one fraction are combined. In our work with human sperm, where, as indicated above, we typically process a semen sample in eight fractions, we combine the upper layers from all eight fractions and the lower layers from all eight fractions for use in artificial insemination or in vitro fertilization.
In the method of the invention, the vessel employed will typically be a test tube or centrifuge tube made of a material, typically a plastic, that does not adversely affect the viability or motility of the sperm and to which the sperm do not readily adhere. The tube will typically be cylindrical with a rounded or tapered bottom portion and have a length much greater than the diameter of the cylindrical portion. The volume of the tube will be selected for convenience in adding and removing the volumes of solutions that are to be added and removed (without undesirable mixing or other disruption) in carrying out the method of the invention. Such vessels, suitable for carrying out the invention, will be familiar to the skilled and are readily available commercially.

In carrying out the method of the invention, the solution and first sample are maintained at a substantially constant temperature, at which the sperm remain motile (about 20 °C to about 40 °C, preferably about 37 °C), and the vessel, in which the method is carried out is maintained substantially stationary. As indicated above, mixing or convection of the solution during the swim-up process, whereby a fraction of sperm increased in female sperm and a fraction of sperm increased in male sperm arise from the first sample, disrupts and interferes with the process of separating female from male sperm. Thus, reference to "substantially constant" temperature means temperature that is sufficiently constant everywhere in the solution and first sample and during the swim-up process to avoid convection that would prevent the separation of female from male sperm from being achieved. The skilled will understand how to hold the temperature sufficiently constant to avoid such convention. A temperature that varies no more than plus or minus one or two degrees
Celsius during the swim-up/separation process and no more
than about one degree Celsius between any two points in
the solution and first sample will be sufficiently
constant to carry out the invention. Similarly,
5 maintaining the vessel "substantially stationary" means
not subjecting the vessel to shaking, jostling or other
movements that will cause mixing of the solution that
would prevent the separation of female from male sperm to
be achieved in the swim-up process. Maintaining the
vessel sufficiently stationary is easily within the skill
of the ordinarily skilled in the art.

The swim-up process (i.e., the process whereby
separation of female from male sperm occurs during step
(b) of the method of the invention) will be allowed to
continue for a period of time that can be readily
ascertained by the skilled to achieve an acceptable
fraction of female sperm in the upper layer, or an
acceptable fraction of male sperm in the lower layer, or
both. This time period will depend on temperature (the
sperm having greater motility at higher temperature),
other factors (such as solution composition) that may
affect the motility of the sperm, the vertical distance
from the pellet (or first sperm sample in other form) to
the middle of the upper layer, and the ratio of the
25 volume of the upper layer to the lower. If too short a
time is allowed for swim-up, less than the maximum
attainable fraction of female sperm will be achieved in
the upper layer and less than the maximum attainable
fraction of male sperm will be achieved in the lower
layer. If too long a time is allowed for swim-up, the
male sperm will eventually catch-up with the female sperm
in the upper layer and the fraction of female sperm in
the upper layer and the fraction of male sperm in the
lower layer will be reduced from the maxima. In a
30 typical system, as illustrated in the Example, at the
preferred temperature of 37 °C, a swim-up period of
between about 30 minutes and 90 minutes, preferably about
1 hour, is suitable.

The invention is now illustrated in the
following Example, which concerns human spermatozoa.

EXAMPLE

A semen sample was collected by masturbation
into a sterile plastic container and was held for 30
minutes at room temperature to permit liquefaction. Then
the specimen was aspirated into a 10.0 ml syringe through
a 20 ga needle to ensure complete liquefaction and then
expelled back into the original container.

The liquefied semen was then pipetted into eight
15.0 ml plastic centrifuge tubes (Corning Co., Corning,
New York, USA) in 250 μl aliquots and mixed with 500 μl
of IM ("insemination medium") per each of the eight
tubes. IM is Ham’s F-10 medium supplemented with 10 %
heat-inactivated and filtered maternal serum (serum taken
from a female at day 21 of her menstrual cycle, said
female to be the recipient of an in vitro fertilized
embryo prepared with sperm separated by the method of the
invention). The IM was equilibrated with 5 % CO₂ in
air to a pH of 7.4 and held at 37 °C in an incubator
prior to use.

Each of the eight aliquots was then centrifuged
at 200 x g for 10 minutes at room temperature, and the
centrifuge was slowed to a stop without use of the centrifuge break. The supernatant was aspirated from
each tube using a 1.0 ml sterile plastic squeeze pipette
(222-1S, Samco Inc., San Fernando, California, USA) and
discarded. Each pellet was then resuspended in 500 μl of
IM and centrifuged again for 10 minutes at 200 x g at
room temperature, with the centrifuge again being stopped
without use of the brake. The supernatant from each
tube was also drawn off and discarded, but this time the pellet was left undisturbed and carefully overlain with another 500 μl of IM. To avoid disrupting the pellet, each tube was tilted 45 degrees from the vertical and the IM slowly added drop by drop down the side, using a new sterile 1.0 ml plastic squeeze pipette. Once the pellets were overlain with the IM, the tubes were placed vertically in a rack and transferred to an incubator for a swim-up of 60 minutes at 37 °C in 5 % CO₂ and humidified air.

After the one hour for swim-up, the tubes were removed from the incubator and the upper 250 - 300 μl of IM was carefully aspirated from each with a sterile 1.0 ml plastic squeeze pipette and pooled into a 3.0 ml plastic test tube (Falcon 2003, Lincoln Park, New Jersey, USA). These pooled upper layers were labeled the "A" fraction. The lower 200 - 250 μl from each tube was similarly collected and pooled, and the pooled lower layers were labeled the "B" fraction.

During the incubation for swim-up, jostling or shaking of the tubes was avoided, as was mixing during the withdrawal of the upper and lower fractions.

In many of the 49 semen samples treated, the upper layer in each tube was visually distinct from the lower layer, with a marked difference in the opacity of the medium at a boundary that could be observed between the two layers at the time of aspiration (after the 1 hour swim-up). The lower layers were milky in appearance, while the upper layers were translucent.

With samples in which this difference in opacity was observed, the translucent layer was taken as the upper layer and the milky layer taken as the lower layer. With the samples in which this difference in opacity was not observed, the top 300 μl were taken as the upper layer and the bottom 200 μl as the lower layer.
Analysis of the A and B samples for male sperm was carried out with fluorochrome quinacrine mustard. The mean percentage of male sperm in the upper fractions (A samples) was significantly lower (36.1 %, standard deviation +/- 10.3 %) and in the lower fractions significantly higher (60.4 %, standard deviation +/- 12.4 %) than in the initial specimens (47.1 %, standard deviation +/- 4.0 %).

Sperm from B fractions were used to inseminate in a series of in vitro fertilization cases. Of 23 conceptions, 17 males and 6 females were conceived. Of these, 15 males and 5 females were delivered (including 8 males and 2 females in 5 sets of fraternal twins).

Sperm from A fractions were used to inseminate in a series of in vitro fertilization cases. Of three conceptions, two females and one male have been delivered.

Although the invention has been described herein with some specificity, those of ordinary skill in the art will recognize modifications and variations that are within the spirit of the invention. It is intended that such modifications and variations be encompassed within the scope of the invention as described and claimed.

Various features of the invention are also apparent from the following claims.
WHAT IS CLAIMED IS:

1. A process for preparing from a first sample of sperm of a mammalian species, said first sample held in a vessel, a second sample, wherein the fraction of sperm with the male sex chromosome is different from said fraction in said first sample, which process comprises: (a) placing in interfacial contact with said first sample in said vessel an aliquot of an homogeneous aqueous solution that is physiologically acceptable to the sperm, said aliquot having a volume consisting of a lower layer and an upper layer, said lower layer being in contact with and extending vertically upward from said first sample and, together with said first sample, filling the entire volume of said vessel below said upper layer; (b) maintaining said first sample and said aliquot of aqueous solution at substantially the same temperature, within a range of temperatures at which the sperm of the species remain motile, and maintaining said vessel substantially stationary, until a portion of the sperm of said first sample migrates into said upper layer to provide in said upper layer a fraction of sperm with the male sex chromosome which is less than said fraction in said first sample; (c) removing said upper layer from said vessel.

2. A process according to Claim 1 wherein the volume of said upper layer is between 0.2 and 0.8 times the volume of said aliquot of physiologically acceptable solution and wherein the mammalian species is selected from the group consisting of bovine, canine, caprine, equine, human and ovine.

3. A process according to Claim 2 wherein, after removal of said upper layer from said vessel, at least a portion of said lower layer is removed and is said second sample.

4. A process according to Claim 3 wherein the species is human.
5. A process according to Claim 3 wherein the first sample is a pellet of sperm prepared by a process which comprises centrifuging a suspension of sperm, said suspension prepared by diluting liquefied semen with an aliquot of an aqueous solution that is physiologically acceptable to the sperm.

6. A process according to Claim 4 wherein the first sample is a pellet of sperm prepared by a process which comprises centrifuging a suspension of sperm, said suspension prepared by diluting liquefied semen with an aliquot of an aqueous solution that is physiologically acceptable to the sperm.

7. A process according to Claim 5 wherein the physiologically acceptable aqueous solution is selected from the group consisting of Tyrode’s Solution, Ham’s F-10 Medium, and Ham’s F-10 Medium supplemented with 10% serum of the species of the sperm being fractionated.

8. A process according to Claim 6 wherein the physiologically acceptable aqueous solution is selected from the group consisting of Tyrode’s Solution, Ham’s F-10 Medium, and Ham’s F-10 Medium supplemented with 10% human serum.

9. A process according to Claim 7 wherein, in the process of preparing the first sample, the pellet obtained from the first centrifugation is suspended in a second aliquot of an aqueous solution of the same composition as the aqueous solution employed in diluting the liquefied semen, the resulting suspension is centrifuged, and the supernatant from the second centrifugation removed and wherein the aqueous solution, placed in interfacial contact with the first sample in the process of preparing the second sample with a fraction of male sperm different from that of first sample, has the same composition as the aqueous solution employed in diluting the liquefied semen; and wherein the
volume of said upper layer of the volume of said aqueous solution in which the first sample is immersed is 0.5 to 2 times the volume of said lower layer.

10. A process according to Claim 8 wherein, in the process of preparing the first sample, the pellet obtained from the first centrifugation is suspended in a second aliquot of an aqueous solution of the same composition as the aqueous solution employed in diluting the liquefied semen, the resulting suspension is centrifuged, and the supernatant from the second centrifugation removed; wherein the aqueous solution, placed in interfacial contact with the first sample in the process of preparing the second sample with a fraction of male sperm different from that of first sample, has the same composition as the aqueous solution employed in diluting the liquefied semen; and wherein the volume of said upper layer of the volume of said aqueous solution in which the first sample is immersed is 0.5 to 2 times the volume of said lower layer.

11. A process according to Claim 9 wherein the first sample and the aqueous solution in which the first sample is immersed are maintained at a temperature between 20 °C - 40 °C until the fraction of male sperm in the upper layer is less than 0.45.

12. A process according to Claim 9 wherein the first sample and the aqueous solution in which the first sample is immersed are maintained at a temperature between 20 °C - 40 °C until the fraction of male sperm in the upper layer is less than 0.45.

13. A process according to Claim 11 wherein, in addition, the second sample of sperm is used to impregnate a female of the species by artificial insemination or used to prepare a viable embryo of the species by in vitro fertilization.
14. A process according to Claim 12 wherein the first sample and the aqueous solution in which it is immersed during preparation of the second sample are maintained between 35 °C and 39 °C and wherein the upper layer of said aqueous solution is not removed until the fraction of male sperm in said upper layer has decreased to less than 0.40.

15. A process according to Claim 14 wherein, in addition, the second sample of sperm is used to impregnate a human female by artificial insemination or used to prepare a viable human embryo by in vitro fertilization.

16. A process according to Claim 14 wherein the physiologically acceptable aqueous solution employed to prepare the pellet, which is the first sample, and to immerse said pellet in preparation of the second sample, is Ham's F-10 Medium supplemented with 10% maternal serum of said female artificially inseminated with sperm of said second sample or of the female in which the in vitro-fertilized embryo is to be implanted.

17. A process according to Claim 13 wherein the upper layer of the aqueous solution in which the first sample is immersed during preparation of the second sample is not removed until the fraction of male sperm in said upper layer has decreased to less than 0.40.
INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 89/00800

I. CLASSIFICATION OR SUBJECT MATTER (If several classification symbols apply, list all).
According to International Patent Classification (IPC) or to both National Classification and IPC.
IPC (4): C12N; 5/02
U.S. Cl: 435/2; 424/105

II. FIELDS SEARCHED
Minimum Documentation Searched

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<th>Classification System</th>
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<td>424/105</td>
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Documentation Searched other than Minimum Documentation to the extent that such documents are included in the fields searched.

III. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of Document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to Claim No.</th>
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<tbody>
<tr>
<td>Y</td>
<td>US A, 4,009,260 (Ericsson) 22 February 1977 (See entire document).</td>
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<td>Y</td>
<td>US A, 4,067,965 (Bhattacharya) 10 January 1978 (See entire document).</td>
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<td>Y</td>
<td>US A, 4,225,405 (Lawson) 30 September 1980 (See col. 15 and 16).</td>
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<td>Y</td>
<td>US A, 4,326,026 (Sarkar) 20 April 1982 (See entire document).</td>
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<td>Y</td>
<td>US A, 4,327,177 (Shrimpton) 27 April 1982 (See entire document).</td>
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  "E" earlier document but published on or after the international filing date.

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  "P" document published prior to the international filing date but later than the priority date claimed.

  "Q" later document published after the international filing date and not in conflict with the application but cited to understand the principle or theory underlying the invention.

  "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step.

  "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.

  "A" document member of the same patent family.

IV. CERTIFICATION

Date of the Actual Completion of the International Search
28 April 1989

Date of Mailing of this International Search Report
05 JUL 1989

International Searching Authority
ISA/US

Signature of Authorized Officer
Sam Rosen

Form PCT/ISA/210 (second sheet) (October 1991)