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(54) Device for the separation of motile cells from less motile cells in a mixed cell sample

(57) The device comprising a tubular member which includes a sample loading area (P), a motile cell collecting area (4) and an elongate separation area (1) connecting the sample loading area and the cell collecting area, in which the separation area is substantially upright in use, whereby motile cells are able to move from the sample loading area in the separation area through medium preferably alkaline, placed in the device, into the collecting area, the non motile, or less motile, cells being left in the sample loading area and separation area.

The sample loading area may include an area 11 of convex glass protrusions H which can protect the fluid from flowing back out of the tube and reflects light to the upper level (4).

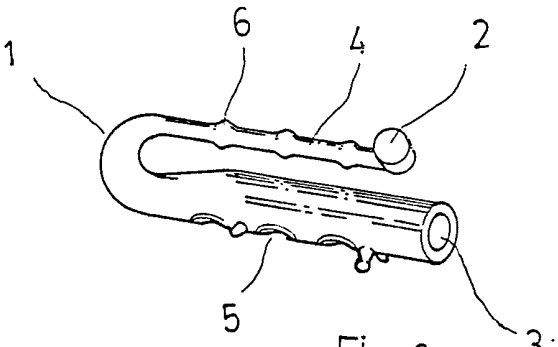

Fig. 6

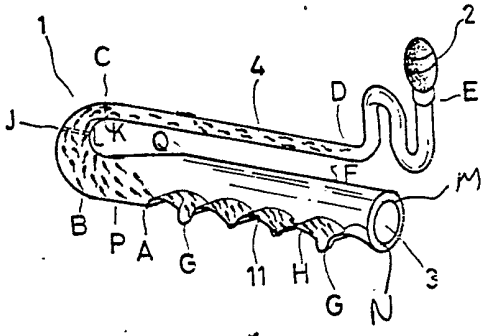

Fig.1

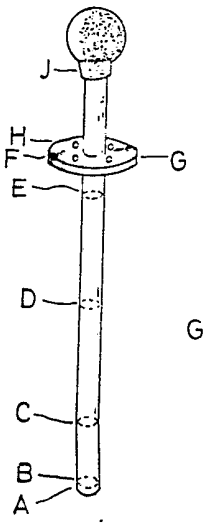

Fig.5

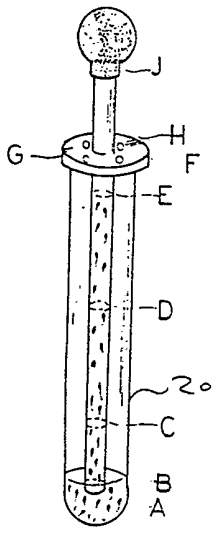

Fig. 4

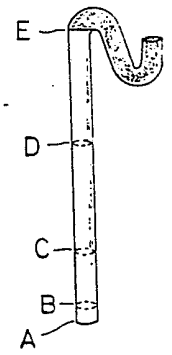

Fig.2

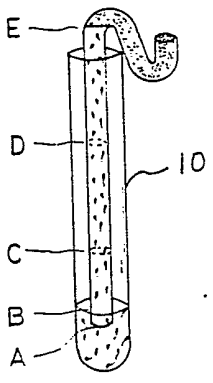

Fig. 3

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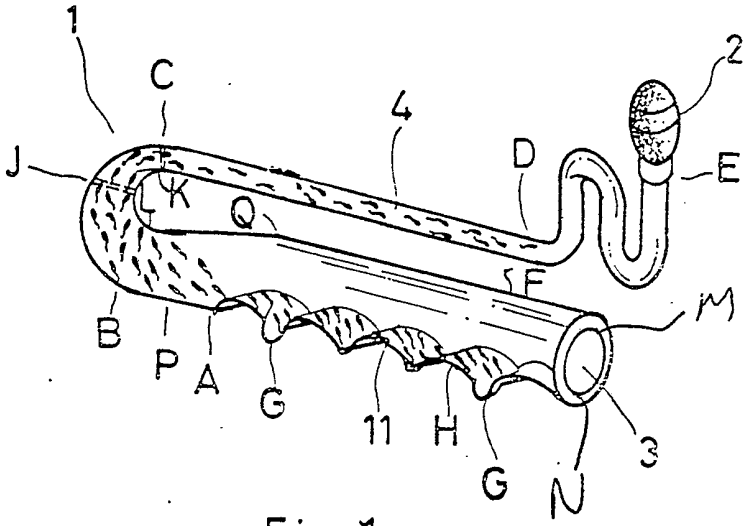

Fig.1

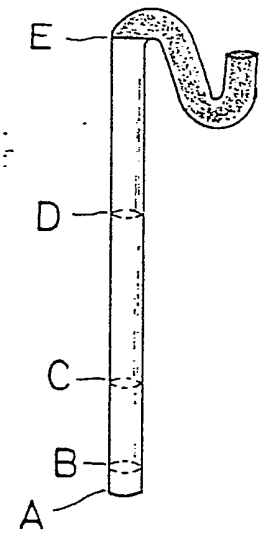

Fig.2

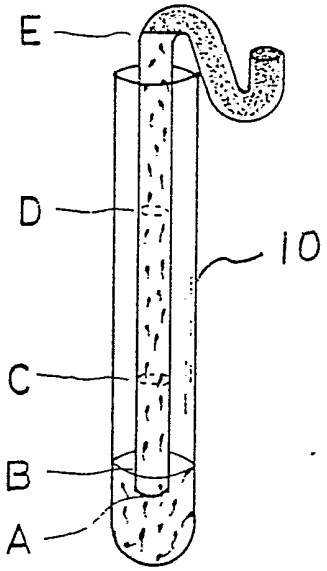

Fig.3

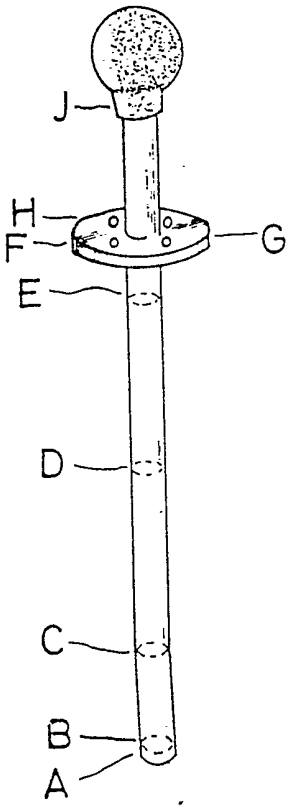

Fig.5

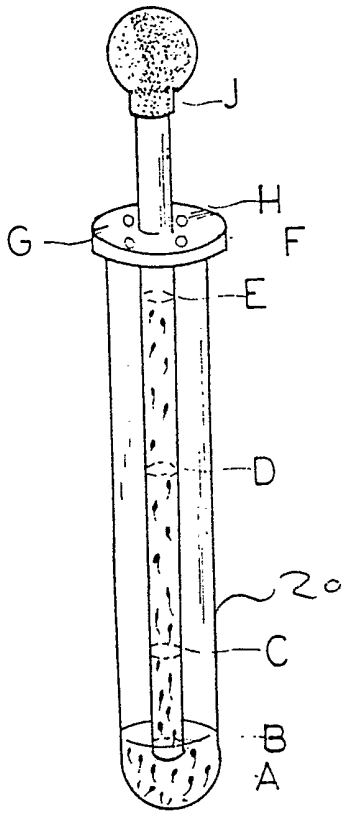

Fig. 4

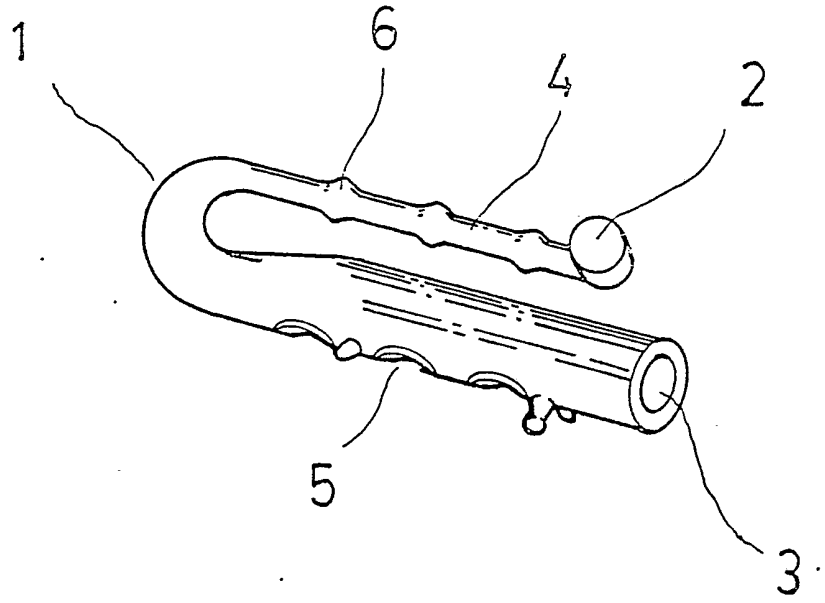

Fig. 6

A METHOD AND/OR DEVICE FOR SEPARATING MOTILE CELLS FROM NON-MOTILE, OR LESS MOTILE, CELLS IN A MIXED CELL SAMPLE

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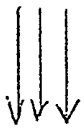
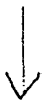
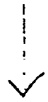
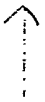
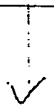
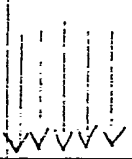
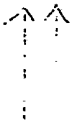
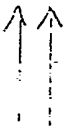
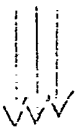
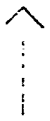
This invention relates to a method and/or device for separating motile cells from non-motile, or less motile, cells in a mixed cell sample.

Conventional advanced technology methods for treatment of patients of sterility and genesiology include in-utero artificial insemination, external fertilization (in vitro baby), embryo transplantation, in-oviduct sperm-ovum transplantation. During these methods, the processed sperm specimens may contain a variety of bacteria or other microbes, because the raw semen collected is contaminated and regular process techniques cannot separate bacteria and other microbes from sperm specimens. Conventional techniques for collecting sperm specimens includes following methods:

1. Simple swim-up method (technique) described by Makler et al in 1983.
2. Conventional or Regular Swim-up method (technique)
3. Fall-down method.
4. Discontinuous Percoll's gradient.
5. Centrifigation method for use in in utero artificial insemination.

The following is a comparison chart between above-mentioned methods and the present sperm/microbe separation system:

Item / Method	Conc. of Sperm	Rate of Activity %	Morpho-logy % normal	Conc. of microbes (CFU's/ml)
Raw Semen	Within normal range	Within normal range	Within normal range	1×10^5 below (or more)
Method 1 Simple swim-up method (technique)				
Method 2 Conventional Swim-up method				
Method 3 Fall-down method				
Method 4 Discontinuous Percoll's Gradient				
Method 5 Centrifigation method			no variation	
Method 6 The present method				Sterile

Remarks: * : indicates increasing in direct proportion.
& : indicates decreasing in direct proportion.
CFU's : Colony Forming Units.

Each c.c. of normal raw semen of 90% contains approximately 1×10^5 bacteria, of which Staphy lococous epidermidis is most frequent and comprises about 77%, Corynebacterium sp, comprises about 66%. Viridans streptococci comprises about 50%, and furthermore, there are some aerobic bacteria such as alpha-hemolytic Streptococcus, Neisseria sp. (not Neisseria gonorrheoae), Pseudomonas aeruginosa, Diphytheriods, and some anaerobic bacteria such as Peptococcus prevotic, Pepto-streptococcus etc.

Result / Specimen	Method 1	Method 2	Method 3	Method 4	Method 5	Method 6
Raw Semen	Bacteria existed but amount reduced	Bacteria existed but amount reduced	Bacteria existed but amount reduced	Bacteria existed amount apparently reduced	Bacteria existed amount apparently reduced	Bacteria not existed
Processed semen		Bacteria existed amount reduced	Bacteria existed amount reduced	Bacteria existed amount reduced		Bacteria not existed
Raw Semen innoculated with E. coli	E. coli still existed	E. coli still existed	E. coli still existed	E. coli still existed	E. coli still existed	E. coli not existed
Raw Semen innoculated with Staphlycocous aureus	S. aureus still existed	S. aureus still existed	S. aureus still existed	S. aureus still existed	S. aureus still existed	S. aureus not existed
Raw Semen innoculated with Neisseria gonorrhoeae	Neisseria gonorrh. still existed	Neisseria gonorrh. still existed	Neisseria gonorrh. still existed	Neisseria gonorrh. still existed	Neisseria gonorrh. still existed	Neisseria gonorrh. not existed
Processed semen innoculated with E. coli		E. coli still existed	E. coli still existed	E. coli still existed		E. coli not existed

Result Specimen	Method 1	Method 2	Method 3	Method 4	Method 5	Method 6
Processed semen innoculated with Stapphylcocous aureus		S. aureus still existed	S. aureus still existed	S. aureus still existed		S. aureus not existed
Processed semen innoculated with Neisseria gonorrhoeae		Neisseria gonorrh. still existed	Neisseria gonorrh. still existed	Neisseria gonorrh. still existed		Neisseria gonorrh. not existed

From the above chart we can understand that it is rather difficult to collect sperm specimens without containing microbes. Thus, physicians add an antibiotic to sperm specimens while making in utero insemination or gamete intra-fallopian transfer (GIFI). The microbes or bacteria may be killed by the antibiotic, but the killed microbes or the terminated microbes may lyse and release endotoxins which are harmful to sperm, ova, embryos, and cause failure of the operation or increase the risk of infection to those treated women for sterility to cause clinical or subclinical complications. Therefore, the present method and device have been created to solve the problems. The present separating systems are based on the following principles:

1. By means of the basic principle of biology that sperm cannot transfer microbes in low-viscosity of culture media, so as to collect germ-free sperm specimens without adding antibiotics.

2. By matching with the physiological properties of the sperm to develop new swim-up technique and conduction, letting the sperm swim-up from the right to the left and from the bottom to the upper part and then horizontally from the left to the right, to run through two challenge angles to delete residues and any poisonous chemical substances.

3. According to the biological principle of the feature of the activity of sperm that horizontal conduction can collect more sperm than vertical orientation, a gradually narrowed tube containing weak alkaline culture media of low viscosity is created to achieve the process so that the sperm can be well seen under microscope, the sperm can be well separated and a sufficient amount of fluid containing enough germ-free sperm can be collected. Moreover, the longer the separating tube used and the longer the incubation time applied the

better the separating effect.

The present method and device has been proved to provide following advantages and effect:

1. Avoid contamination of microbes or inflammatory reaction of infections.

2. Increase the successful rate after artificial insemination.

3. Reduce patient's mental pressure and financial loss.

4. Help patients to reduce the requirement of operation and thereby.

5. Help patients to control the sex of offspring and reduce the risk of sex-linked diseases.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a plan view of a duck-shaped tube embodying the present invention.

Fig. 2 is a plan view of a U-shaped separating tube embodying the present invention.

Fig. 3 illusrtates a U-shaped separation tube of the present invention in operation.

Fig. 4 is a plan view of a Four-holes type separation embodying the present invention.

Fig. 5 illustrates a Four-holes type separation tube of the present invention.

Fig. 6 illustrates a fourth embodiment according to the present invention.

The separation system of the present invention is mainly composed of a transparent tube and a rubber or silicon rubber ball. For full understanding, are four different types of preferred embodiments hereinafter described:

As shown in Fig. 1, the duck-shaped separation tube (1) is composed of a glass tube and a rubber bulb (silicon rubber bulb). The part "H" is a hemispherical protrusion (11) protruding beyond inner tube wall. The number of such hemispherical protrusion (11) can be one or more. At the top end of the separation tube (1) there is a rubber bulb (2) which can be replaced by a silicon rubber bulb. The rest of the separation tube (1) is a hollow tube (3) containing culture medium fluid for sperm. The culture medium fluid may be B.W.W. medium or Ham's F-10 medium with 10% human serum collected before ovulation or with 10% serum collected from the umbilical cord. The medium is to added up to point A and raw semen is to be placed point P. The point H of the convex glass of protrusion (11) can protect the fluid from flowing back out of the tube and reflects light to make upper level (4) of the duck-shaped tube more clear while examining sperm under microscope.

In the F part, the inner diameter of the tube is 6mm, i.e. point M to point N is 6mm. The air collecting space point Q to point A is also 6mm. The tube smoothly inclines from point Q to become narrower and then to curve upward to form an air collection area. The rear part of the tube is horizontally in a U-shape extending up to point D. The inner diameter at point D is 1mm. The tube is pre-engraved circularly by a glass cutter at point C, point D and point J for easy breaking while in clinical process to collect selected sperm.

The capacity from point P to point C is about 0.15ml and from point C to point D is about 0.12ml, from point D to point E is about 0.06ml, from point A to point P is about 0.05ml and the capacity of the rubber ball is 0.5ml. The length of each segment from point N to point B is 3cm, point M to point L is 3cm, point A to point B is 1cm,

point Q to point L is 1cm, point B to the outer surface of point C is 2cm, the distance from point K to point L is 1cm (range 0.5-1.0cm), point C to point D is 6cm, point D to point E is 4cm, and point A to point D is 9cm. The point G is to support the four fulcrums of the tube to allow the tube be able to stably stand up.

The U-shaped tube of point D to point E is for protection against mis-operation or air contamination. The sterile sperm can be collected for further processing after 30 minutes to one hour incubation. The prepared tube is to be incubated at a 37°C incubator containing high humidity (96% up) and 5% carbon dicoxide. The concentration of sperm at C-D segment is to be 20-30 x 10^6 or more. The rubber bulb can protect culture medium from back-flowing and can also squeeze out sperm specimens contained in medium fluid. When raw semen is placed at specimen position at the tube, sometimes air bubbles may present. Therefore, the air collecting area of point Q can prevent the air bubbles from swimming up to carry bacteria to C-D segment. Point P is semen specimen loading area. Because semen specimen has higher gravity than culture medium, the placed semen will stably located at the bottom between point A and point B.

Please refer to Fig. 2 and Fig. 3, wherein the U-shaped tube is made of glass, the inner diameter is 3.5mm, the thickness of tube wall is below 0.5mm; the length from point A to point C is 2mm, point B to point C is 15mm, point C to point D is 25mm; the capcity from point A to point B is about 0.02ml, from point A to point C is about 0.15ml, from point C to point D is about 0.25ml, from point D to point E is about 0.25ml; from point E to the end of the separating tube is a solid tube. The total capacity of the U-shaped tube is about 0.65ml. The tube is engraved circularly by glass cutter at point C and point D

for easy breaking of the tube to collect C-D segment and D-E segment that contain culture medium and selected sperm specimens. According to the method, culture medium is added to the U-shaped tube from the opening (point A). Place proper semen specimen into a Boroosilicate tube 10 of 75mm in length and 22mm in inner diameter. Then, hang up the U-shaped tube at the top opening of the Borosilicate tube, letting the front segment of point A to point B be dipped into central part of the semen specimen. The incubator is controlled at 37°C, in high humidity and with 5% carbon dioxide. The prepared tube is to be incubated for about one hour. After well incubation, the tube can be broken off at point C for the collection of segment C-D which is to be labelled #1. and then for the collection of segment D-E which is to be labelled #2. The concentration of segment #1 is about 6-12 x 10^6/ml and its total capacity is 0.25ml, and therefore, segment #1 contains about 20 x 50^5 sperm. The segment #2 contains about 75 x 10^5. Both segment #1 and #2 contain no bacteria. The specimen of segment #1 can be available for fertilization with 10-40 ova, the average is about 25 ova. The specimen of segment #2 can be available for fertilization with 3-12 ova, and the average is about 7 ova.

Please refer to the four-holes type separation tube as shown in Fig. 4 and Fig. 5, wherein the tube is composed of a glass tube and a hollow rubber ball, the inner diameter of the tube is about 3.5mm; the thickness of the tube wall is below 0.5mm; the length of each segment is 2mm for point A to point B, 15mm for point A to point C, 25mm for point C to point D, 25mm for point D to point E, and 7mm for point E to point F; the thickness of part G is 3mm and its diameter is 25mm; the diameter of each holes at part G is 2mm; the length from point H to point J is 15mm, the length from point A to point F is 72mm, and

the full length of the tube is 90mm; the capacity of each segment is about 0.02ml for point A to point B, 0.15ml for point A to point C, 0.25ml for point C to point D, 0.25ml for point D to point E, 0.07ml for point E to point F, 0.18ml; for point F to point J, and 0.72ml for point A to point F; the capacity of the rubber ball is about 0.65ml. Since the capacity of point A to point E is 0.65ml.

The four holes of the part C of the tube for the admission of carbon dioxide into the tube of semen specimen area during incubation. The sperm should be supplied with dextrose, aminoacids and carbon dioxide to give energy. The tube wall at point C should be pre-engraved circularly by glass cutter for easy breaking of the tube while collecting the segment A-G. The rubber ball is for squeezing out semen specimen medium fluid from segment C-D and segment D-E.

Because the capacity of the rubber ball is 0.65ml, equal to that of segment of point A to point E, when sucking up semen culture medium from the opening of the separation tube at point A the medium will be sucked up to point E. In use, a raw semen specimen is to be placed at the bottom in Borosilicate tube of 75mm in length and 22mm in inner diameter. Then, place the four-holes type separation tube into said Borosilicate tube, letting the part G cover the opening of said Borosilicate tube and letting the front end of the separation tube from point A to point B be dipped in the central part of the placed semen specimen. Then the attached separation tube and Borosilicate tube are placed in an incubator where contains high humidity and 5% dioxide carbon and incubated therein for 1 hour so as to allow sperm swim-up the separation tube. After proper incubation, break off the tube at point C and squeeze out the medium fluid in the segment C-D and segment D-E that are respectively labelled #3 and #4. The segment

#3 is same as the segment #1 in previously described U-shaped tube, and the segment #4 is same as the segment #2, wherein all the segments contain no microbes or semen residue and no antibiotic is to be required. The sperm obtained are pure and excellent for fertilization.

What is described above is regarding the process of separating sperm from microbes. By means of the glass tube as shown in Fig. 1, it can also be applicable for the separation of X/Y sperm.

Because the Y chromosome of sperm is about 1/6 the size of X sperm, Y chromosome is rather smaller and lighter than that of X chromosome. The head of Y sperm is small and can resist alkaline solution better than X sperm. Y sperm has a higher survival rate in a relative high pH weak alkaline medium and within a fixed period of time, Y sperm normally can swim up for longer distance than X sperm. For said reason, the longer the separation tube is the better the separation of X sperm and Y sperm can achieve. This explains when at the rear end of the separation tube we can collect more Y sperm.

When in application to separate X sperm and Y sperm, please refer to Fig. 1. Firstly add weak alkaline medium of low viscosity in the hollow tube up to point A. The medium adopted can be B.W.W. medium or Ham's F-10 medium with 10% human serum collected before ovulation or with 10% serum collected from umbilical cord, and the medium should be adjusted for pH value 8.0 up before use. When the prepared medium is added to the separation tube, the raw semen that originally contains microbes is to be placed at the position of semen specimen area as indicated at point P. The "H part" is to protect the fluid medium from back-flowing or overflowing. The inner diameter of the tube is 6mm and the shape and size as well as its capacity are all same as previously described for Fig. 1. The sperm in the semen specimen need about one

to one and half hour to swim up to the culture medium. After this process is completed, the ratio of Y sperm and X sperm collected from the rear part of C-D segment is about 4-5:1.

For better understanding, we can refer to the following comparison list:

Method / Item	germ-free X/Y sperm separation system	conventional familiarized separation method
1. Culture medium	low viscosity culture medium	Albumin medium
2. Antibiotic	No requirement	Needed or should be added
3. Background of principle	(1) Apply sperm/germ separating system (2) Multi-direction swim-up technique (3) Long distance swim-up technique (4) Special separation tube is required (5) Apply hydrodynamics & Bernoulli's equation	(1) Lack of concept oof germ-free sperm processing technique (2) One way swim-up technique (3) Short distance swim-up technique (4) No special separation tube is applied (5) No hydrodynamics theory is required
4. Swim-up	Beginning from the right to the left, then from the bottom to the upper surface, and then from the left to the right to swim up horizontally. This multi-direction swim-up technique needs to pass through two challenge angles.	Downward or one way swim-up technique, no challenge angle available
5. Separation	Custom-made to match with the present technique	Regular centrifugal tube or test tube is applicable, no specific separation tube is applied
6. Length of separation tube	Long	Short
7. Calibre of separation tube	Calibre is thin and gradually reducing	Calibre is thick and constantly in same size
8. Position of separation tube	Stand lying horizontally, no supporting frame is required	Stand vertically, supporting frame is required

9. Breaking of tube for the collection of preferred specimen	Yes, and easy	No
10. Applicability of microscope examination before specimen collection	Yes, perfect	No, not appropriate
11. Swim-up distance of sperm during separation	9 cm or more	About 3cm only
12. Length of separation tube	selectable, to match with raw semen specimen in accordance with physiological science	Fixed type
13. Process of collection	Applicable for direct in-utero insemination	Suspension process is required, not applicable for direct in-utero insemination
14. Contamination of pre-existing bacteria during process	No. because the tube needed to be broken off segmentally and the medium fluid is in low viscosity, sperm pellet is to be placed at the bottom and will not carry microbes	Yes, convection current makes contamination of bacteria that existed in raw semen, the amount of bacteria is directional to the amount that originally existed in raw semen which can not be eliminated after suspension process.
15. Existance of microbes or bacteria in preferred sperm specimens after separation	Non existance	Existance. Antibiotic can not guarantee complete elimination of microbes and bacteria.
16. Existance of toxic substance in selected sperm specimens	Non existance	The bacteria that killed by antibiotic will release toxic substances
17. Separation time required	Short period of time is required	Longer period of time is required

18. Effect	Excellent	Good
19. Patient condition after operation	(1) Few cases of bellyache are encountered (2) No complication of pelviperitonitis	(1) Bellyache is often encountered (2) The complication of pelviperitonitis is quite oftenly seen, rigor and pyrexia may usually accompanied
20. Weak	No	Shortage of amount of sperm collected is oftenly happened

Referring to Figure 6, the bottom (3) of the separation tube (1) according to the present invention comprises a plurality of concave wells (5) which are presented as convex lenses when watched from the top. The separation tube (1) is a hollow tube with wider bottom and is getting narrower to turn deflected upward with concomitant smaller caliber. The separation tube (1) also comprises a bulbous portion (6) at the top that is preferably accomodated in vertical to the lower convex lenses that formed by the concave wells (5) such that light can be foused on the fluid medium through the effect of the convex lenses while observing through a microscope. The upper portion (4) of the separation tube (1) has a horizontal level and a rubber bulb (2) is accomodated therein at the turning corner, and ova are preaccomadated in the bulbous portion (6), when sperm are swimming up through the curved tube they can be fertilized with the ova in the sphere (6) to complete the in-vitro artificial insemination process. Any inseminated ovum will start cell division process inside the sphere (6) and the physician can use a glass cutter to pick up embryo of the inseminated ovum for transplantation into the uterus of a female for further conceiving process. The in-vitro insemination process applied in the present preferred embodiment is managed in same manner as previously described and has to be passed the process of incubation at 37°C in an incubator containing high humidity and 5% of carbon dioxide.

CLAIMS:

1. A device for separating motile cells from less motile, or immotile, cells in a mixed cell sample, the device comprising a tubular member which includes a sample loading area, a motile cell collecting area, and an elongate separation area connecting the sample loading area and the cell collecting area, whereby when the device is arranged with the collecting area at a higher level than the sample loading area, motile cells are able to move from the sample loading area into the separation area through a medium contained within the device, into the collecting area, the non motile, or less motile, cells being left in the sample loading area and/or separation area.

2. A device according to Claim 1 in which the loading area is transverse to the separation area.

3. A device according to Claim 1 or 2 in which the collecting area is transverse to the separation area.

4. A device according to Claim 1,2 or 3 in which the loading area and collecting area are substantially parallel.

5. A device according to any preceding claim in which the tubular member is weakened in the region of the collecting area to facilitate collection of purified motile cells by breaking open the tubular member at the weakened point.

6. A device according to Claim 5 in which the member is weakened by scoring its surface.

7. A device according to any preceding claim which includes means for expelling the purified motile cells from the motile cell collecting area.

8. A device according to Claim 7 in which the said means comprises a flexible bulb connected to an end of the tubular member whereby air can be expelled from the bulb to expell motile cells from the motile cell collecting area.

9. A device according to any preceding claim in which the bore of the motile cell collecting area is less than that of the sample loading area.

10. A device according to any preceding claim in which the wall of the tube is provided with protrusions which reflect light upwardly in use.

11. A device according to Claim 10 in which comprises lens means in the wall of the tube.

12. A device according to any preceding claim in which sample loading area, separation and collecting areas are provided by a substantially straight length of tube.

13. A device according to Claim 12 in which the device includes at least one solid section.

14. A method of separating motile cells from non motile, or less motile, cells in a mixed cell sample, comprising loading the sample into the sample loading area of a device according to any preceding claim which contains suitable medium, ensuring that the separating area of the device is substantially upright, incubating the loaded device for a period of time and collecting pure motile cells from the cell collecting area after the period of time.

15. A method according to Claim 14 in which the motile cells are sperm cells.

16. A method according to Claim 15 in which the motile cells are Y sperm cells and the less motile cells, and/or non motile cells are X sperm and/or microbial contaminants.

17. A method according to any one of Claims 14 to 16 in which the medium is alkaline.

18. A method and/or device substantially as described herein with reference to the accompanying drawings.

19. Sperm/germ and germ-free Y, X sperm separating systems wherein the sperm/microbes separation system being completed in way to priorily add culture medium fluid to a separation tube and then to place into the separation tube (like duck-shaped separation tube) at point P to let sperm absorb the nutrition of the medium fluid so that the sperm can automatically swim up to get rid of the attached microbes or bacteria; after a proper period of time, germ free sperm being obtained from the C-D segment of the duck-shaped separation tube or from the C-D segment and the D-E segment of the Four-holes type separation tube.

20. Sperm/germ and germ-free Y/X sperm separation systems as claimed in Claim 19, wherein the X/Y sperm separation system being completed in a way that a prepared weak alkaline culture medium fluid being fully filled in a test tube, said duly filled test tube being attached with a rubber bulb and properly incubated; raw semen specimen being added with culture medium fluid in a volume 1:3, the mixed semen specimen and medium fluid thus obtained being well stirred and centrifuged for separation of supernatant and sperm pottet, the sperm pellet thus obtained being placed in the bottom of said test tube that contains weak alkaline medium fluid to let sperm contact the alkaline medium fluid for one hour or more after one hour contact of the sperm with the alkaline medium fluid, examining the upper segment of the test tube by microscope to see that number of sperm that swam up; at the time that the sperm in the upper segment being in proper volume, cutting off the rear part of the upper volume, cutting off the rear part of the upper segment of the test tube by a glass cutter with the help of the rubber bulb to squeeze out the contained medium fluid so as to obtain germ-free Y sperm.

21. Sperm/germ and germ-free Y/X sperm separating systems as claimed in Claim 19 or 20, wherein the duck-shaped tube comprising an upper segment and a lower segment, said lower segment having a transparent support at the opening and one or more half-moon shaped convexes protruding beyond the inner wall of the tube at the front end, said lower segment being turning upward in U shape with inner diameter gradually reducing; said upper segment being in parallel with the lower segment with its roar end in U-shape for the attachment of a hollow and elastic rubber ball; said duck-shaped separation tube being

circularly engraved by glass cutter at front end as well as rear end for the convenience of breaking off the tube to collect a preferred segment.

22. Sperm/germ and germ-free Y/X sperm separating systems as claimed in Claim 19 or 20, wherein the U-shaped tube used being in hollow condition from the bottom up to 65mm height with the other part in solid condition; said U-shaped tube being circularly engraved by glass cutter at point C and point D for easy collection of sperm.

23. Sperm/germ and grem-free Y/X sperm separating system as claimd in Claim 19, wherein the separation tube used being in a Four-holes type made of a hollow glass tube with a rubber ball attached at the top end, said four-holes type separation tube comprising a tube cover at a position 72mm above the bottom of the tube, said tube cover comprising four holes as air outlets, the outer tube wall of the tube being circularly engraved by glass cutter atpoint C and point D for the convenience of breaking off the tube to collect prefered sperm.

24. Sperm/germ and germ-free Y/X sperm separation systems as claimed in Claim 19, 20 or 21, wherein the duck-shaped separation tube used having an air collecting space between point Q and point A to prevent air bubbles from floating upward, said air collecting space being formed in a manner that the upper tube wall being inclining downwad from point Q to reduce the inner diameter and then turning upward in curved shape.

25. Sperm/germ and germ-free Y/X sperm separation systems as claimed in Claim 19, wherein the separation tube comprising a plurality of spheres accommadated symmetrically, in vertical to the lower convex lenses formed by the concave wells, a microscope being focused upon the swim-up sperm or the embryo of the division of inseminated ovum for observation.

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