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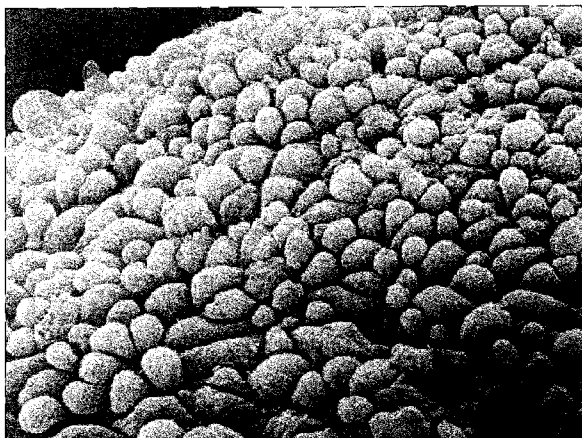
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(54) Title: USE OF N-ACETYL-5-METHOXYTRYPTAMINE OR ANALOGUES THEREOF, FOR PROMOTING THE MECHANISM OF IMPLANTATION OF THE EMBRYO AND RELATED COMPOSITIONS AND CULTURE MEDIA

FIG.3



(57) Abstract: The present invention refers to the use of N-acetyl-5-methoxytryptamine (melatonin) and/or an analogue thereof, for use in the medical or veterinary field in the assisted reproduction for promoting the mechanism of implantation of the embryo, and in particular for the prevention of implantation failure into the uterus, by topical administration of an effective amount in a mammalian subject female in need of such treatment, and related compositions, culture media and medical devices.

"Use of N-acetyl-5-methoxytryptamine or analogues thereof, for promoting the mechanism of implantation of the embryo and related compositions and culture media"

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The present invention relates to the use of N-acetyl-5-methoxytryptamine (melatonin) and/or an analogue thereof, for use in the medical or veterinary field in the assisted reproduction for promoting the mechanism of implantation of the embryo, and in particular for the prevention of implantation failure into the uterus, by topical administration of an effective amount in a mammalian subject female in need of such treatment, and related compositions, culture media and medical devices.

The implantation of the human embryo into the uterus is a complex mechanism, which involves both the embryo, and the endometrial epithelium. The phases of apposition, adhesion and invasion involves a multiplicity of molecules, which play an unique role in the process, the molecular dialogue between the conceived and the endometrium implies interactions among cells, and between cells and biochemical factors.

These mechanisms, if suitably expressed or inhibited, are of help to determine the receptivity or non-receptivity state of the endometrium versus the embryo.

The state of endometrial receptivity or non-

receptivity, although it is a largely shared concept, is clinically hard to be defined: the histological normality of the endometrium does not necessarily imply a functional normality; on the other side, the  
5 time and space expression of particular endometrial structures, named pinopodes, is strongly indicative of the receptivity state itself.

The implantation is a complex sequence of signals which are crucial for setting up pregnancy; it is  
10 supposed that a great number of molecular mediators under the influence of ovarian signals are involved in the embryo-endometrial interaction. These mediators comprise a wide range of molecules like hormones, cytokines, growth factors, lipids, adhesion  
15 molecules and else (1).

Failure in the implantation in the technologies of medically assisted reproduction (MAR) still remains an unsettled problem and it is considered one of the main reasons of infertility in healthy women.  
20 Considering then that the percentage of implantation of the techniques of MAR is of about 25%, the inadequate uterine receptivity is deemed as being responsible for about two thirds of all failures (for one third the embryo is considered as being  
25 responsible) (2).

The endometrium is receptive of the invasion of blastocyst for a range of a limited time and defined as "window of implantation". The "dialogue" for the synchronization between "ovulation-first phases of

embryonic growth - cell modifications of endometrial epithelium for the implantation" is hold by a series of hormonal and biochemical messengers.

During the "implantation window", the endometrial  
5 epithelium expresses, through hormonal and biochemical control, some structures named pinopodes, capable to allow adhesion and invasion of blastocyst. The implantation window would be characterized, according to studies of electron microscopy (3) by  
10 the maximum expressivity of pinopodes.

The formation of pinopodes is considered as a specific marker of the endometrial receptivity, they appear about one week after ovulation, but their complete expression changes from patient to patient  
15 and seems to be maintained for only one day (4).

The expression of pinopodes is directly proportional to the increase of plasmatic levels of progesterone (5).

The concept of endometrial receptivity or  
20 "implantation window", connected to the expression of pinopodes, unanimously accepted by the scientific community, is for some decades subject-matter of study for the ones who face the problems related to assisted reproduction.

25 In summary: progesterone, the pro-gestation hormone, is the main mediator and around it rotate a plurality of other mediators which may promote the implantation occurrence.

From the aforesaid it comes out how the problem of

obtaining pregnancy through techniques of assisted reproduction is mostly ascribable to the short knowledge of the implantation phenomenon.

It appears thus clear, also in view of the numerous  
5 legislative restrictions in the field of assisted reproduction, the necessity to optimize the phase of implantation.

The authors of the present invention have now found that one of the mediators capable to have a key role  
10 along with progesterone in the expression of pinopodes, with much positive effects for the embryonic nesting is N-acetyl-5-methoxy tryptamine (melatonin).

Melatonin is produced by the pineal, neuroendocrine  
15 gland controlled by light; darkness stimulates its synthesis and secretion.

The use of melatonin has no side effects or overdose problems, as well as no contraindication (6) is reported in literature.

20 Many are the advantages already known and associated with the use of melatonin also in different processes, which may positively interfere with the implantation. In this respect, it exists moreover an inversely proportional correlation with the risk of  
25 carcinoma of the endometrium and the serum levels of melatonin with ascertained protective action.

It has been proved by some authors (7) that melatonin inhibits proliferation of atypical endometrial cells, defined as Ishikawa, responsible for carcinoma of the

endometrium. Through experiments with radioligands and antagonists of melatonin such as luzindole, the results of the group of researchers have proved that the inhibition effect is produced by melatonin  
5 through the MT2 receptors present in the membranes of the proliferative cells of Ishikawa.

Another determinant aspect to consider is the menopause period, during which melatonin diminishes its production and, indeed, during such a period the  
10 risk of endometrial carcinoma is higher.

For example, *in vitro* and *in vivo* (6, 8) studies have shown a strong antioxidant action of melatonin, said action being higher than that of mannitol, of glutathione and vitamin E in the protection from  
15 oxidative damage due to hydroxyl toxic radicals and to other catabolic cell products deriving from variations in the degenerative or proliferative metabolism and from/for lipid oxidation.

Melatonin (9-10) has moreover important anti  
20 estrogenic properties and stimulates production of progesterone which is in antithesis with the action of estrogens themselves (high levels of estrogens associate with a worst result in the techniques of assisted reproduction) (11).

25 In this regard, it is pointed out that super-ovulation through gonadotropins induced in MAR cycles, determines a hyperestrogenization with increase of endometrial hyperplasia, a significant risk factor for endometrial carcinoma as above

mentioned.

Some authors (12) affirm that it exists a synergism between human chorionic gonadotropins (hCG) and melatonin: indeed, melatonin increases, by increasing  
5 inter alia the production of progesterone (both *in vivo* and *in vitro*), 6-7 days after the ovulatory peak of hCG.

In this regard it is highlighted how the post-ovulatory peak of melatonin perfectly coincides with  
10 the endometrial implantation window and with the formation of blastocyst (the step of embryonic growth in which the nesting into the uterus occurs). The positive correlation between melatonin and progesterone and the negative correlation between  
15 melatonin and estradiol would be enhanced by hCG: indeed, in the absence of the latter, melatonin has a very weak effect on production of progesterone.

An object of the present invention is N-acetyl-5-methoxy tryptamine or an analogue thereof, for use in  
20 the medical or veterinary field in assisted reproduction for the prevention of implantation failure into the uterus by topical administration of an effective amount in a mammalian subject female in need of such treatment.

25 Preferably, said analogue of N-acetyl-5-methoxy tryptamine is selected from agomelatine, 6-hydroxymelatonin, serotonin, 5 hydroxytryptophan or their derivatives.

In a preferred embodiment of the invention the

mammalian subject female is a woman suffering from infertility or polyabortion.

According to a further preferred embodiment, the topical administration of N-acetyl-5-methoxy  
5 tryptamine or an analogue thereof, preferably of N-acetyl-5-methoxy tryptamine, takes place through endometrial irrigation or uterine washing or endometrial washing.

Still according to a further preferred embodiment of  
10 the invention, said topical administration occurs in a single administration at the time of oocyte retrieval.

Preferably, said active principle is present in a concentration ranging from  $4 \times 10^{-9}$  g/ml to  $25 \times 10^{-9}$   
15 g/ml, still more preferably greater than or equal to  $10 \times 10^{-9}$  g/ml.

In an alternative embodiment, it is possible the concurrent use with a systemic therapy based on N-acetyl-5-methoxy tryptamine, hCG or progesterone, or  
20 a combination thereof, from the day of oocyte retrieval.

In alternative preferred embodiments of the present invention, the techniques of assisted reproduction are selected from the group consisting in basic or of  
25 I level techniques, simple and not much invasive as targeted intercourse or intrauterine artificial insemination and II or III level techniques, which are more complex and invasive selected from in vitro fertilization (IVF), in vitro fertilization and



embryo transfer (FIVET), intracytoplasmic sperm injection (ICSI), intracytoplasmic morphologically selected sperm injection (IMSI) and Tese-Tesa techniques (Testicular Sperm Aspiration-Extraction).

5 Artificial fertilization stands mainly for the technique of intrauterine insemination (IUI), which is a technique of medically assisted reproduction, wherein the seminal fluid is introduced inside the uterine cavity. It may be suggested in all those

10 cases of incompatibility between cervical mucus and seminal fluid, in that it allows to go beyond the cervical length and insert spermatozoa directly into the uterus. It is moreover used in case of unexplained sterility, of male infertility of light

15 or moderate degree, in cases of reiterated failures in the induction of pregnancy with stimulation of ovulation and targeted intercourse and in cases of sexual disorders which do not allow a complete sexual intercourse.

20 In vitro fertilization and embryo transfer (FIVET) is a MAR technique wherein human gametes are withdrawn, placed under culture and, after fertilization and production of one or more embryos, these are transferred into the uterus. This technique is

25 suggested in cases of:

- Acquired or congenital tubal pathology;
- male infertility of moderate degree;
- endometriosis of III or IV level;
- unexplained infertility;

- cryo-preserved semen in relation to the seminal quality, subsequent to thawing;
- failing of the therapeutic procedure of I level techniques.

5 After ovarian stimulation, oocytes retrieval (PICK-UP) is carried out via transvaginal operation, under echographic control, in local anaesthesia and/or deep sedation, a preparation of the sperm is effected and the oocytes to be fertilized are selected. Next, the  
10 extracorporeal culture of gametes is prepared and after verifying the occurred fertilization, the transfer into the uterus of a definite number of embryos is performed.

The intracytoplasmic sperm micro-injection (ICSI)  
15 uses various steps of the FIVET. Also in this case the fertilization is extracorporeal, but it takes place with the injection of a single spermatozoon inside the cytoplasm of the oocyte. Then, after the occurred fertilization, the embryos are transferred  
20 into the uterus.

This technique is suggested in cases of:

- male infertility of severe degree;
- occlusive and secretive azoospermia (testicular spermatozoa or epididymis);
- 25 - failed or reduced fertilization in previous cycles of in vitro fertilization (IVF);
- thawed oocytes;
- reduced number of oocytes;
- cryo-preserved semen in relation to the seminal

quality subsequent to thawing.

Also this technique can be carried out under spontaneous cycle or through induction of multiple follicular growth and after having stimulated the  
5 ovary to produce more follicles and so having obtained more oocytes, oocyte retrieval (PICK-UP) is carried out via transvaginal operation. Contemporaneously to PICK UP, preparation of the sperm is effected. In case of azoospermia, the  
10 techniques used for withdrawal of spermatozoa are: Testicular Sperm Percutaneous Aspiration (TESA), Testicular Sperm Extraction (TESE), Microchirurgical Epididymal Sperm Aspiration (MESA), Percutaneous Epididymal Sperm Aspiration (PESA).

15 Subsequently, the preparation of the oocyte and the insemination of oocytes through intracytoplasmic micro-injection technique of a single spermatozoon takes place. After verifying the occurred fertilization of each oocyte, the embryos are  
20 transferred into the uterus.

It is a further object of the present invention a composition suitable for topical administration comprising N-acetyl-5-methoxytryptamine or an analogue thereof, or their combination as active  
25 ingredient, in an effective amount in a mammalian subject female in need of such treatment, along with one or more physiologically acceptable excipients or adjuvants, for use in the medical or veterinary field in the assisted reproduction for the prevention of

implantation failure into the uterus.

Preferably, said active ingredient is present in the composition in a concentration ranging from  $4 \times 10^{-9}$  g/ml to  $25 \times 10^{-9}$  g/ml, preferably greater than or  
5 equal to  $10 \times 10^{-9}$  g/ml.

According to a preferred embodiment, the active ingredient in the above mentioned composition is formulated as endometrial/uterus irrigation/washing in a cell culture medium in a final concentration  
10 ranging from  $4 \times 10^{-9}$  g/ml to  $25 \times 10^{-9}$  g/ml, preferably greater than or equal to  $10 \times 10^{-9}$  g/ml.

Such a culture medium for blastocyst may preferably comprise the following components:

- source of D-glucose;
- 15 - Antibiotic, preferably gentamicin;
- Human serum albumin;
- Essential and non-essential amino acids, preferably L-aurine;
- buffer salts preferably selected between:
- 20 - Calcium salts: calcium lactate, calcium pantothenate;
- Sodium salts: sodium chloride, sodium bicarbonate and sodium pyruvate;
- Potassium salts: potassium chloride, potassium  
25 phosphate;
- Magnesium salts: magnesium chloride, magnesium sulphate  
and mixtures thereof;
- Water;

and has a pH between 7.5 and 7.8.

According to a preferred embodiment said culture medium may be Sydney IVF ®Blastocyst medium.

According to an alternative embodiment of the present  
5 invention, the active ingredient of the above mentioned composition is formulated as endometrial irrigation, endometrial washing or uterine washing in physiological solution with a final concentration ranging from  $4 \times 10^{-9}$  g/ml to  $25 \times 10^{-9}$  g/ml,  
10 preferably greater than or equal to  $10 \times 10^{-9}$  g/ml.

In females, the procedure of washing of the uterine cavity has been commonly used with the main objective to set non-invasive prenatal diagnosis, innovative with respect to invasive traditional methods of  
15 amniocentesis and chorionic villus sampling [(13)(14)]. In recent years, such technique has been applied to evaluate the conditions of the uterine cavity before the transfer of the embryo in the cycles of assisted reproduction. The application of  
20 this procedure consists in the insertion into the uterus of a buffer solution with the aim at collecting uterine secretes and evaluate their organic and inorganic biological components. It has the purpose to identify possible markers, predictive  
25 of implantation success [(15)(16)(17)(18)].

Still preferably, said composition suitable for topical administration through uterine washing further comprises essential and non-essential amino acids, and buffer salts. Preferably said buffer salts

are salts of calcium, sodium, potassium and magnesium, and mixtures thereof. The use of buffer salts and amino acids is provided for producing a formulation with a pH comprised between 7 and 8  
5 (preferably between 7.5-7.8), to replicate the micro-environment suitable for the embryo (and similar to that of culture media in which the embryo grows before the transfer into the uterus).

According to an alternative embodiment, the  
10 compositions of the invention may be formulated as gel suitable for uterine administration, more precisely for in situ administration in the uterine cavity, preferably with a medical device being an intrauterine "T shaped" device. It is also possible  
15 to foresee controlled release gel formulations for mucosal administration.

Considering that the uterine washing technique as above described revealed to be a non-invasive practice and that allows restoration of the uterine  
20 physiological conditions through mechanical removal of secretes, which may alter the implantation conditions, the invention has as a further object a medical device for uterine washing comprising a sterile container pre-filled or to be filled with a  
25 composition as above defined.

More precisely, the device according to the present invention can be filled at the moment of use with a sterile solution suitable for the uterine or endometrial washing supplemented with the active

principle melatonine or analogue thereof according to the present invention already formed or it can be filled with such sterile solution supplemented just at the moment of use with the active principle.

5 According to a preferred embodiment the sterile container is disposable. More preferably the sterile container (preferably a disposable sterile container) is pre-filled with physiological solution supplemented with melatonin (in a concentration  
10 ranging from  $4 \times 10^{-9}$  g/ml to  $25 \times 10^{-9}$  g/ml, preferably greater than or equal to  $10 \times 10^{-9}$  g/ml), buffer salts and amino acids such as to produce a formulation with pH comprised between 7-8, preferably between 7.5 and 7.8.

15 The uterine washing shall be performed with the aid of a catheter after pick-up with echographic guide. The next embryos transfer or implantation shall take place averagely three days after the washing (2-5 days).

20 Therefore, according to preferred embodiments of the invention the medical device according to the invention may further comprise a sterile flexible catheter, preferably with a single terminal hole.

The sterility of the medical device components is  
25 achieved preferably through sterile filling or final sterilization, such as heating treatment or through gamma rays treatment.

In addition, the medical device can be accompanied by instructions for use in the various MAR techniques.

The purpose of the medical device is that of removing oxidant substances and possible secreted products in response to the stimulation preceding the MAR techniques and thus of restoring the physiological composition of the endometrial exudate. The objective is that of avoiding that such oxidizing substances and secreted products reduce the percentage of implantation in patients subjected to MAR. As above anticipated, the use of buffer salts and amino acids is provided for setting a pH comprised between 7-8, preferably between 7.5 and 7.8, for replicating the micro-environment suitable for the embryo. The addition of melatonin, additional to the effects shown within the present invention, exerts a very strong antioxidant effect and a safety action versus endometrial cells, of the oxidative damage due to toxic radicals and other catabolic products.

Preferably, said disposable sterile container is selected from syringe, dispenser, cartridge for self-injection /pen.

According to particularly preferred embodiments of the invention the syringe is of the luer-lock type 3 ml made of polycarbonate or borosilicate glass. When a syringe of the luer-lock type is used, the intrauterine catheter possibly associated with the medical device according to the invention is provided with a luer lock female joint for coupling with the syringe.

Alternatively, if a self-injection system or a pen is



used (insulin type) it can be used a glass cartridge  
3 ml of borosilicate glass.

Still preferably said disposable sterile container is  
pre-filled with volume of 1.5 ml of washing solution

5 containing:

- $10 \times 10^{-9}$  g/ml (10 ng/ml) of melatonin
- essential and non-essential amino acids
- calcium salts
- potassium salts
- 10 - magnesium salts

a pH comprised between 7,5-7,8 and osmolarity  
comprised between 280-290 mOsm/kg.

The invention has also as an object the medical  
device as above illustrated for use in a single  
15 administration as endometrial washing at the moment  
of the oocyte retrieval in protocols of medical  
assisted reproduction.

The invention also refers to a cell culture medium *in*  
*vitro* or *in vivo*, comprising the following  
20 components:

- source of D-glucose;
- Antibiotic, preferably gentamicin;
- Human serum albumin;
- Essential and non-essential amino acids,  
25 preferably L-aurine,
- Calcium salts: calcium lactate, calcium  
pantothenate;
- Sodium salts: sodium chloride, sodium bicarbonate  
and sodium pyruvate;

- Potassium salts: potassium chloride, potassium phosphate;
- Salts of magnesium: magnesium chloride, magnesium sulfate;
- 5 - Water;

characterized in that it further comprises N-acetyl-5-methoxy tryptamine or an analogue thereof, or their combination, wherein said active ingredient is present in a concentration ranging from  $4 \times 10^{-9}$  g/ml  
10 to  $25 \times 10^{-9}$  g/ml, preferably greater than or equal to  $10 \times 10^{-9}$  g/ml.

It stands for a further object of the present invention a cell culture medium for the expansion of pinopodes and the achievement of an *in vitro* model  
15 study.

Such a cell culture medium can be used also for embryonic cultures, by achieving for example a culture medium which is used for transferring the embryo into the uterus, said medium being thus  
20 inserted into the uterus.

Particularly advantageous aspects of use according to the present invention are connected to the proposed route of topical administration, which does not compromise the MAR treatment cycle, being such a  
25 route much less invasive than endometrial biopsy, already applied to women subjected to a cycle of MAR (13).

At last, thanks to the properties of synthesis inhibition by side of prostaglandins (14), which are

typical of melatonin, this washing reduces uterine contractions and further promotes implantation.

The present invention will be now described in an illustrative manner, but not limitative, according to  
5 two preferred embodiments, with particular reference to the annexed figures, wherein:

- Figure 1 illustrates the endometrium before treatment through the composition according to the present invention, therefore in the absence of  
10 pinopodes;

- Figure 2 illustrates the endometrium 1/2 days after treatment through the composition according to the present invention: the formation of pinopodes starts to come into sight;

15 - Figure 3 illustrates the endometrium 3/4 days after treatment through the composition according to the present invention: maximum growth of pinopodes;

- Figure 4 illustrates the endometrium 5/6 days after treatment through the composition according to the  
20 present invention: it starts cleavage of pinopodes, which appear to be irregular (cell apoptosis).

- Figure 5 shows the results of the FACS analysis (fluorescence intensity measurement) of the antioxidant activity of melatonin, agomelatine and 6-  
25 hydroxymelatonin (170 nM corresponding to  $40 \times 10^{-9}$  g/ml) carried out at  $H_2O_2$  concentrations of 20  $\mu M$  and 200  $\mu M$ ;

- Figure 6 shows the results of apoptosis assay (% cell death) carried out at different concentrations

(80 nM, 170 nM, 340 nM; corresponding respectively to  $18 \times 10^{-9}$  g/ml,  $40 \times 10^{-9}$  g/ml,  $80 \times 10^{-9}$  g/ml) of melatonin, agomelatine and 6-hydroxymelatonin.

Merely for example, but not limitative of the present invention, hereinafter are reported the comparative studies carried out (*in vitro* and *in vivo*) by the authors of the present invention to evaluate the percentage increase of human embryonic implantation in MAR techniques through the use of melatonin in different solutions.

EXAMPLE 1: *In vivo* studies

MATERIALS AND METHODS

*Solutions of melatonin used for endometrial washings*

The melatonin (or N-acetyl-5-methoxy tryptamine; CAS Number 73-31-4) used has been obtained at Farmalabor as product in form of powder with title  $\geq 99\%$ .

Two clinical trials have been carried out providing the administration of melatonin (concentration  $10 \times 10^{-9}$  g/ml) through endometrial washing in:

- 1) culture medium Sydney IVF® Blastocyst medium (Clinical trial 1) supplied by Cook Ireland Ltd (Catalogue Number G20722 and G20929). Said culture medium is normally used to improve cleavage, differentiation and expansion *in vitro* of blastocyst. The medium contains D-glucose, all the 20 essential L-amino acids, L-taurine, Gentamicin, human serum albumin, calcium lactate, calcium pantothenate; sodium chloride, sodium bicarbonate and sodium

pyruvate; potassium chloride, potassium phosphate; magnesium chloride, magnesium sulfate; purified water. Further characteristics of the culture medium:

- 5       - pH: 7,5 - 7,8 (use of bicarbonate buffer)  
      - Osmolarity: 280-290 mOsm/kg  
      - Endotoxins : < 0.4 EU/ml  
      - MEA:  $\geq$  80%

2) physiological solution (Clinical Trial 2)

10   *Statistical Analysis:*

It has been carried out a statistical analysis (IMPRUN.TXT) of linear regression with more variables.

*Statistical Methods:*

- 15   The role of the number of implanted embryos, of their quality and of the washing process with the solution containing melatonin in the increasing of the chance of pregnancy has been computed by means of non-conditional logistic regression, by adjustment  
20   according to the mother's age.

The ratio between the chance of pregnancy associated with each procedure (number of embryos, their quality and application of the washing process with saline solution or Blastocyst medium containing melatonin)  
25   and the chance of pregnancy with reference respectively to the lower category of number of embryos (one), to the quality less than optimal of implanted embryos, or to the absence of the washing process, has been defined as Odds Ratio (OR), and it

has been computed through non-conditional logistic regression, by adjustment according to the mother's age.

In the logistic regression, OR corresponds to the  
5 antilogarithm on a natural basis of the regression coefficient  $\beta$  associated with each covariate in the regression model.

The value thus calculated expresses therefore the advantage obtained through each procedure,  
10 irrespective of the other conditions.

The two-tailed confidence intervals at 95% (IF95%) of the OR have been calculated through the formula of Wald ( $e^{\beta \pm (z_{\alpha/2} * se_{\beta})}$ ).

*Sterility Controls (culture medium)*

15 To carry out the sterility controls of the culture medium supplemented with melatonin it has been used the BACT/ALERT 3D-60 device (BIOMERIEUX). The bottles of Bact/ALERT culture are applied to systems of microbial detection in qualitative procedures for  
20 recovering and detecting optional anaerobic and aerobic (bacteria) in the blood and other fluids normally sterile as the Blastocyst medium used in the present experimentation.

The Bact/ALERT system of microbial detection is used  
25 to determine whether microorganisms are present in the samples of blood or of other fluids normally sterile as the blastocyst medium used in our invention, with suspected bacteremia. The Bact/ALERT system and the culture bottles offer a system of

microbial detection and a culture medium with environmental and nutritional conditions suitable for microorganisms commonly present in blood infections and other fluids normally sterile.

5 The inoculated bottles (from a minimum of 5 ml to a maximum of 10 ml of sample at issue) are incubated in the device, where they are subjected to continuous monitoring in order to detect a possible growth of microorganisms in the Bact/ALERT bottles.

10 The Bact/ALERT system of microbial detection uses a colorimetric sensor and reflected light for monitoring the presence and the production of carbon dioxide (CO<sub>2</sub>) dissolved in the culture medium.

The microorganisms possibly present in the sample  
15 metabolize the substrates in the culture medium producing carbon dioxide. The production of CO<sub>2</sub> determined by the growth of microorganisms induces the gas permeable green-blue sensor present on the bottom of each culture bottle to take a yellow  
20 colour.

The lighter colour indicates an increase of the units of monitoring reflectance of the system.

The reflectance of the bottle is monitored and traced by the device every 10 minutes.

25 The culture bottles are established as being positive or negative by the managing software of Bact/ALERT systems of microbial detection after 6 days of incubation.

No intervention is necessary up to the moment in

which the Bact/ALERT device alerts that a culture bottle is positive or negative.

Before carrying out any endometrial irrigation culture tests have been carried out for testing  
5 sterility in 2-4-6 days and once the negative culture was found it was used into the uterus.

*Criteria of inclusion*

Restricted criteria of inclusion have been used for selecting the patient population involved in the  
10 studies:

- 3) age  $\geq 20$  and  $< 44$  years
- 4) body mass index (BMI)  $\geq 20$  and  $\leq 28$  kg/m<sup>2</sup>
- 5) Basal FSH  $\leq 19$  UI/l
- 6) Male and female factors

15 *Patients*

The patients involved in all the two clinical studies, after collecting informed consent, have been subjected to assisted reproduction.

In this first two-arms randomized multi-centre  
20 clinical study (control and study group) involved about 430 patients/arm.

The patients undergoing assisted fertilization have been randomized the day of oocyte retrieval in three groups:

25 **Group A:** 430 patients to be subjected to endometrial irrigation with 1.5 ml of physiological solution (controls).

**Group B:** 436 patients to be subjected to endometrial irrigation with a solution of 1.5 ml of



culture medium supplemented with final concentration of melatonin ( $10 \times 10^{-9}$  g/ml).

**Group C:** patients not subjected to endometrial irrigation.

5 The study has been carried out involving patients in several private centers specialized in MAR, such as the Center of BRA, the Promea Center of Torino and Cagliari, the Genera Center of Perugia and the polyclinic Public Center of Bari. To these centres it  
10 has been sent the culture medium supplemented with melatonin after having been subjected to sterility tests to carry out the following protocol of treatment.

After *in vivo* enrolling for assisted fertilization by  
15 endometrial irrigation with known concentration (final concentration of  $10 \times 10^{-9}$  g/ml) of melatonin at the time of oocyte retrieval, patients were subjected to related ultrasound-guided visualization of the diameter of liquid stratum created on the bottom of  
20 the uterus.

The second clinical study with a single prospective centre involved 64 patients in the group of control and 92 patients in the group of study.

In said study melatonin has been administered in  
25 sterile physiological solution, through endometrial washing inside the uterine cavity of patients, following to oocyte retrieval.

#### *Endometrial washing*

Briefly, the treatment consists in endometrial

irrigation inside the uterine cavity without any cell in co-culture, using the physiological solution or the culture medium admixed with melatonin with final concentration of  $10 \times 10^{-9}$  g/ml (preceding studies had  
5 been effected with one third and the half of the final concentration then maintained without giving the same results - not shown data).

After oocyte retrieval, (the time correspondent to the ovulation after the administration of hCG in the  
10 protocols of MAR) after having controlled the vaginal haemostasis, it has been jointed a sterile syringe from 2.5 ml to a single-lumen intrauterine catheter with apical opening and the culture medium (Blastocyst medium) or the physiological solution has  
15 been suctioned, modified through addition of melatonin up to a volume of 1.5 ml.

In the case of the physiological solution, the catheter can be pre-filled with physiological solution supplemented with melatonin.

20 The catheter has been introduced in the internal uterine orifice with the same steps through which the operator carries out the conventional embryo-transfer (ET).

Then, it has been slowly injected the saline solution  
25 supplemented with melatonin or the modified medium and, at the end of irrigation, the thickness of the endocavitary liquid stratum has been measured by transabdominal echography (washing and echography must be simultaneous, the liquid stratum arrives at

about 10 mm and then quickly disappears).

## RESULTS

### Clinical Study 1

The multi-centre *in vivo* study involved 863 patients  
5 subjected to assisted fertilization and to  
endometrial irrigation (echographic measurements of  
the liquid stratum) with melatonin and 3-4 days after  
that, they have been subjected to embryonic transfer.  
For the latter, the CI (confidence interval or trust  
10 interval) has been calculated through statistical  
analysis (IMPRUN. TXT) of linear regression with more  
variables of 95%.

The univariate analysis shows that the implantation  
of 3 embryos implies a significant increase, equal to  
15 about 3 times (OR=2,8, IF 95% 1,7-4,4) the chance of  
pregnancy.

A number of two embryos does not appear instead to be  
sufficient to increase the chance of pregnancy  
(OR=1,0, IF 95% 1,7-4,4).

20 On the other hand, also the optimal quality of the  
implanted embryos seems to be an important factor in  
increasing the chance of pregnancy (OR = 2.9, IF 95%  
1.8 - 4.7). In the univariate analysis, the same rate  
of relevance appears to be associated with the use of  
25 the irrigation procedure (OR = 2.2, IF 95% 1.6 -  
2.9).

The multivariate analysis allows to control the  
independent effect of each variable, therefore  
totally irrespective of the effect of the other

- applied procedures and of the mother's age. In this case, the use of the washing procedure doubles the chance of success of pregnancy (OR = 2.2, IF 95% 1.6 - 3.0).
- 5 A similar improvement appears to be associated with the implantation of three embryos, rather than the implantation of a single embryo (OR = 2.1, IF 95% 1.2 - 3.7), and to the use of embryos of optimal quality (OR = 1.9, IF 95% 1.1 - 3.3).
- 10 The use of all the three procedures as above reported seems to be therefore the best choice in the procedures of assisted fertilization.
- In conclusion, the *in vivo* studies have proved that the irrigation or the washing with melatonin of
- 15 endometrial cells allows to double the number of pregnancies with respect to the other two groups, which did not differentiated significantly.

#### Clinical Study 2

The single centre study involved 64 patients in the

20 group of control and 92 patients in the group of study.

The patients have been subjected to assisted reproduction and to endometrial washing (echographic measurements of the liquid stratum) with melatonin in

25 sterile saline solution, and 3-4 days after they have been subjected to embryonic transfer.

The primary endpoint of the study is the rate of clinical pregnancies (fetal heart) due to embryonic transfer (ET); the secondary endpoint is the rate of

ongoing pregnancies and abortions.

The rate of clinical pregnancies resulted to be equal to 37% in the group of study vs 17,2% in the group of control, whereas the rate of ongoing pregnancies  
5 resulted to be equal to 32,6% in the group of study vs 14,1% in the group of control.

The results have been reported in the following Table 1.

TABLE 1

		CONTROL		WASHING	
		N°	(%)	N°	(%)
N° ET		64		92	
Age (years $\pm$ SD)		38,8 $\pm$ 1,2		36 $\pm$ 4	
N° embryos transferred (Average $\pm$ SD)		2,7 $\pm$ 1,1		2,5 $\pm$ 1,08	
N° embryos Grade A (Average $\pm$ SD)		1,5 $\pm$ 1		1,3 $\pm$ 0.8	
N° embryos Grade B (Average $\pm$ SD)		0,9 $\pm$ 0,7		1,17 $\pm$ 0,8	
N° embryos Grade C (Average $\pm$ SD)		0,2 $\pm$ 0,5		0,1 $\pm$ 0,3	
Day ET (Average $\pm$ SD)		3,5 $\pm$ 0,5		3,5 $\pm$ 1,2	
BHCG Positive		12	18,8%	41	44,6%

(on total of ET)				
Clinical Pregnancy/ET	11	17,2%	34	37,0%
Ongoing Pregnancy /ET	9	14,1%	30	32,6%
Aborts	1	9,1%	4	11,8%

The endometrial washing carried out with the various solutions containing melatonin, allows to double the rate of clinical pregnancies and ongoing pregnancies vs the group of control in the infertile female population.

The data seem to confirm that the removal of endometrial exudate using the physiological solution with melatonin can create a physiological endometrial environment and improve implantation success.

This allows to obtain a valid and innovative means for increasing the implantation rates in assisted reproduction technologies (ART).

#### EXAMPLE 2: In vitro study

#### 15 MATERIALS AND METHODS

The melatonin and the culture medium used are the same as those used for the *in vivo* study.

#### *Electron Microscopy (SEM)*

It has been used a field emission at high resolution electron microscope, FE HITACHI S 4000 model, operating at 15-20 KW.

#### *Preparation of samples*

Soon after retrieval from the patient, small pieces of endometrial tissue of about 1 mm in size have been placed in a fixing solution composed of paraformaldehyde 1% and glutaraldehyde 0,5% in buffer  
5 cacodylate 0,1 M pH 7,2 for 3 hours.

After this fixing the pieces are washed in PBS 20 min x 3 times (1 hour total) and then the post fixing is carried out in a solution of osmium tetroxide 2% and potassium ferrocyanide 2,5%.

10 The preparation is kept in the dark for 3 hours to be then accurately washed rotating in PBS, with 4 replacements of 20 min each.

It follows dehydration of the samples of tissue with acetone in ascending succession with 3 replacements  
15 in 1 hour for each gradation 50%-70%-80%-90%-95% and 2 replacements with pure acetone.

At this stage it follows drying up to the critical point with liquid CO<sub>2</sub> in the apposite high-pressure device which substitutes acetone 10% with liquid CO<sub>2</sub>,  
20 it is then slowly brought to the critical point temperature and CO<sub>2</sub> evaporates. At this point the samples of tissue are perfectly anhydrous and can be arranged on the trays of the electron microscope.

The samples are fixed through a special electrically  
25 conductive double sided adhesive and for their arrangement two very thin needles are used and the preparation is ready to be observed with scanning electron microscope (SEM).

*Biological Samples*

Under prior informed consent, they have been placed under culture fragments of endometrium withdrawn from patients subjected to diagnostic hysteroscopy.

For each patient, two parts have been set: one part  
5 of the tissue has been placed under culture through a conventional Blastocyst Medium void of melatonin, the other part through a conventional Blastocyst medium supplemented with melatonin.

#### *Culture Set*

10 *In vitro* culture of endometrium (withdrawn through hysteroscopy from patients subjected to assisted pre-fertilization controls) with melatonin for 3-6 days and correlated visualization of pinopodes through electron microscopy (SEM).

#### 15 RESULTS

*In vitro* studies have shown that the co-culture from 3 to 5 days of endometrial tissue in test-tube with permanent addition of melatonin, involves an unquestionable increase of pinopodes (under electron  
20 microscopy (SEM)), irrespective of the age of the patient and of the menstrual cycle phase in spontaneous cycles or manipulated through pharmacological stimulation of super-ovulation, with respect to the culture of control. Such pinopodes are  
25 structures which are defined as the most important implantation marker which for each woman (with a variability of 5 days, that is, a woman can menstruate after 26 days with short cycle or menstruate after 33 days) is of 48 hours. In fact,



two thirds of success of becoming pregnant depends on the correct moment of implantation, the other third depends on the quality of the embryo.

The examination under the electron microscope SEM has  
5 shown that the addition of melatonin leads to a complete morphological expression of pinopodes as illustrated in Figures 1-4.

This outlines the importance of pinopodes during the implantation window and the increase of their  
10 expression induced in culture through mediums supplemented with melatonin.

EXAMPLE 3: Study on the effects of melatonin and analogues on oxidation and apoptosis of endometrial cell line HEC-1-A

15 *Tested Compounds*

The analogues of melatonin which has been used for comparison with melatonin (M5250), are agomelatine (A1362) and 6-hydroxy melatonin (H0627), all purchased from Sigma Aldrich.

20 They were used in different concentrations (i.e. 40 nM, 80 nM, 170 nM and 340 nM, corresponding respectively to  $9.4 \times 10^{-9}$  g/ml,  $18 \times 10^{-9}$  g/ml,  $40 \times 10^{-9}$  g/ml and  $80 \times 10^{-9}$  g/ml).

*Cell Line*

25 HEC-1A cells were obtained by ATCC and culture following instructions of the provider.

HEC-1-A is an epithelial stabilized cell line isolated by H. Kuramoto from adenocarcinoma patient (21), which provide a valuable model to study endometrial

epithelial cell in vitro (22).

*Antioxidant activity assay*

The *HEC-1-A* cell line was cultured with 170 nM (40 ng/ml) of Melatonin, 6-hydroxy-melatonin and  
5 agomelatine and two different concentrations of  $H_2O_2$  (20 and 200 mcg/ml).

Reactive Oxidative Substances (ROS) were measured by FACS as previously described by Italiano et al. (23) in two independent replicates.

10 *Results*

Melatonin and the two analogues asserted the same anti-oxidative effect, on the *in-vitro* cell line model, at the  $H_2O_2$  concentration of 20 mcg/ml.

When the  $H_2O_2$  concentration is increased up to 200  
15 mcg/ml melatonin and agomelatine continued to protect cell line from oxidation, whereas the 6-hydroxy-melatonin does not. However, increasing the 6-hydroxy-melatonin dose up to 340 nM the anti-oxidative effect of this analogue is reached (data not shown).

20 Results are illustrated in the chart of Figure 5.

These results are in line with data published by Duan et al. (24) on the anti-oxidative effect of melatonin in a different cell model and demonstrate that melatonin and analogues assayed have a similar effect  
25 at least when 20 mcg/ml of  $H_2O_2$  is used on endometrial cell line.

*Apoptosis assay*

The apoptosis (%cell death) of the endometrial cells is a physiological phenomenon generally observed at 10-20%

rate during cell culture.

In order to determine if melatonin and its analogs induce cell death in this specific experimental context, we have treated the HEC-1-A cell line with 340  
5 nM (80 ng/ml), 170 nM (40 ng/ml) and 80 nM (18,8 ng/ml) of melatonin, 6-hydroxy-melatonin and agomelatine, respectively, for 24 hours.

Apoptosis levels were assayed by Propidium Iodide staining and FACS analysis. Percentage of sub-G1 events  
10 is shown for one of two experiments performed.

#### *Results*

In all the concentrations tested, Melatonin exerts a protective effect on apoptosis. Agomelatin has a protective effect at 170 and 340 nM, while no effect on  
15 apoptosis is shown by 6-hydroxy-melatonin up to concentration of 170 nM. Results are shown in Figure 6.

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## CLAIMS

1. N-acetyl-5-methoxy tryptamine or an analogue thereof, for use in the medical or veterinary field in assisted reproduction for the prevention of  
5 implantation failure into the uterus by topical administration of an effective amount in a mammalian subject female in need of such treatment.
2. N-acetyl-5-methoxy tryptamine or an analogue thereof, according to claim 1, wherein said analogue is  
10 selected from agomelatine, 6-hydroxymelatonin, serotonin, 5 hydroxytryptophan or their derivatives.
3. N-acetyl-5-methoxy tryptamine or an analogue thereof, according to any one of claims 1-2, wherein said female mammalian subject is a woman suffering from  
15 infertility or polyabortion.
4. N-acetyl-5-methoxy tryptamine or an analogue thereof, according to any one of claims 1-3, wherein said topical administration is via endometrial irrigation or uterine washing or endometrial washing.
- 20 5. N-acetyl-5-methoxy tryptamine or an analogue thereof, according to any one of claims 1-4, wherein said topical administration is carried out in a single administration at the time of oocyte retrieval.
6. N-acetyl-5-methoxy tryptamine or an analogue  
25 thereof, according to any one of claims 1-5, wherein said N-acetyl-5-methoxy tryptamine or analogue thereof is present in a concentration ranging from  $4 \times 10^{-9}$  g/ml to  $25 \times 10^{-9}$  g/ml, preferably greater than or equal to  $10 \times 10^{-9}$  g/ml.

7. N-acetyl-5-methoxy tryptamine or an analogue thereof, according to any one of claims 1-6, for use with a contemporaneous systemic administration of N-acetyl-5-methoxy tryptamine, HCG or progesterone, or a  
5 combination thereof, from the day of oocyte retrieval.

8. N-acetyl-5-methoxy tryptamine or an analogue thereof, according to any one of the preceding claims, wherein the assisted reproduction techniques are selected from the group consisting of planned  
10 copulation; intrauterine insemination (IUI); in vitro insemination and embryo transfer (FIVET); in vitro fertilization (IVF); intracytoplasmic sperm injection (ICSI), intracytoplasmic morphologically selected sperm injection (IMSI) techniques and Tesa-Tese (Testicular  
15 Sperm Aspiration-Extraction).

9. Composition suitable for topical administration comprising N-acetyl-5-methoxy tryptamine or an analogue thereof, or a combination thereof as active ingredient, preferably N-acetyl-5-methoxy tryptamine,  
20 in an effective amount in a mammalian subject female in need of such treatment, along with one or more physiologically acceptable excipients or adjuvants, for use in the medical or veterinary field in the assisted reproduction for the prevention of  
25 implantation failure into the uterus.

10. Composition according to claim 9, wherein said active ingredient is present in a concentration ranging from  $4 \times 10^{-9}$  g/ml to  $25 \times 10^{-9}$  g/ml, preferably greater than or equal to  $10 \times 10^{-9}$  g/ml.

11. Composition according to any one of claims 9-10, wherein said active ingredient is formulated as an endometrial irrigation or uterine washing or endometrial washing in a medium for cell culture to a  
5 final concentration ranging from  $4 \times 10^{-9}$  g/ml to  $25 \times 10^{-9}$  g/ml, preferably greater than or equal to  $10 \times 10^{-9}$  g/ml.

12. Composition according to claim 11, wherein said culture medium for blastocyst, comprises the following  
10 components:

- source of D-glucose;
- Antibiotic, preferably gentamicin;
- Human serum albumin;
- Essential and non-essential amino acids,  
15 preferably L-taurine,
- buffer salts preferably selected between:
  - calcium salts: calcium lactate, calcium pantothenate;
  - sodium salts: sodium chloride, sodium bicarbonate  
20 and sodium pyruvate;
  - potassium salts: potassium chloride, potassium phosphate;
  - magnesium salts: magnesium chloride, magnesium sulfate;
  - 25 - Water;

and has a pH between 7.5 and 7.8.

13. Composition according to claim 12, wherein said culture medium is the Sydney IVF ® Blastocyst medium.

14. Composition according to any one of claims 9-10,



wherein said active ingredient in the above mentioned composition is formulated as endometrial irrigation or uterine washing or endometrial washing in physiological solution.

5 15. Composition according to claim 14, characterized in that it further comprises essential and non-essential amino acids and buffer salts to maintain pH between 7-8, preferably 7.5-7.8.

16. Medical device for uterine washing comprising a  
10 sterile container pre-filled or to be filled with a composition according to any one of claims 9-15.

17. Medical device according to claim 16, wherein the sterile container is disposable.

18. Medical device according to claim 16 or 17,  
15 wherein the sterile container is a syringe, a dispenser or a cartridge for self-injection devices.

19. Medical device according to anyone of the preceding claims, further comprising a sterile intrauterine catheter.

20 20. Medical device according to anyone of claims 16-19, comprising a disposable sterile container pre-filled with a formulation based on a physiological solution supplemented with melatonin, amino acids and buffer salts to maintain pH between 7-8, preferably  
25 7.5-7.8.

21. Medical device according to claim 20, wherein said formulation comprises essential and non-essential amino acids and buffer salts preferably selected between calcium, potassium and magnesium

salts, and mixtures thereof.

22. Medical device according to any one of claims 16-21, wherein melatonin has a concentration comprised between  $4 \times 10^{-9}$  g/ml and  $25 \times 10^{-9}$  g/ml, preferably  
5 greater than or equal to  $10 \times 10^{-9}$  g/ml.

23. Medical device according to any one of claims 16-22, wherein said disposable sterile container is pre-filled with 1.5 ml volume of physiological solution comprising:

- 10 -  $10 \times 10^{-9}$  g/ml of melatonin  
- essential and non-essential amino acids  
- calcium salts  
- potassium salts  
- magnesium salts.

15 24. Medical device according to any one of claims 16-23, for use in the medical or veterinary field in the assisted reproduction for the prevention of implantation failure into the uterus.

25. Cell culture medium *in vitro* or *in vivo*,  
20 comprising:

- source of D-glucose;
- Antibiotic, preferably gentamicin;
- Human serum albumin;
- Essential and non-essential amino acids,  
25 preferably L-aurine,
- buffer salts selected between:
  - Calcium salts: calcium lactate, calcium pantothenate;
  - Sodium salts: sodium chloride, sodium bicarbonate

and sodium pyruvate;

- Potassium salts: potassium chloride, potassium phosphate;

- Magnesium salts: magnesium chloride, magnesium sulphate;

and mixtures thereof;

- water;

characterized in that it further comprises N-acetyl-5-methoxy tryptamine or an analogue thereof, or a combination thereof, wherein said active ingredient is present in a concentration ranging from  $4 \times 10^{-9}$  g/ml to  $25 \times 10^{-9}$  g/ml, preferably greater than or equal to  $10 \times 10^{-9}$  g/ml.

26. Use of the cell culture medium according to claim 25, for the growth of pinopodes and the obtaining of an *in vitro* model study.

1/4

FIG.1

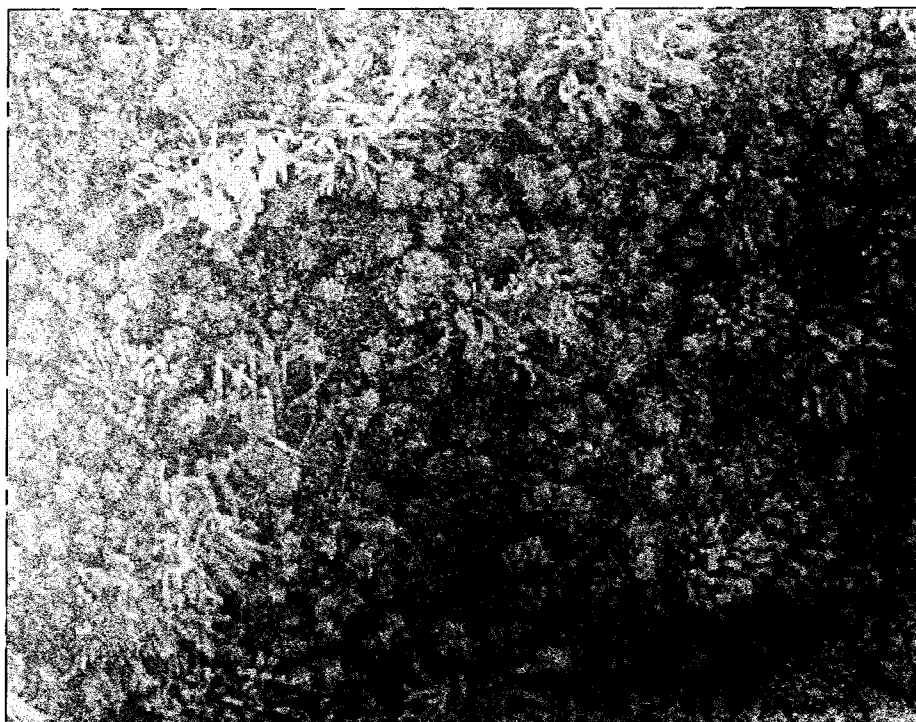
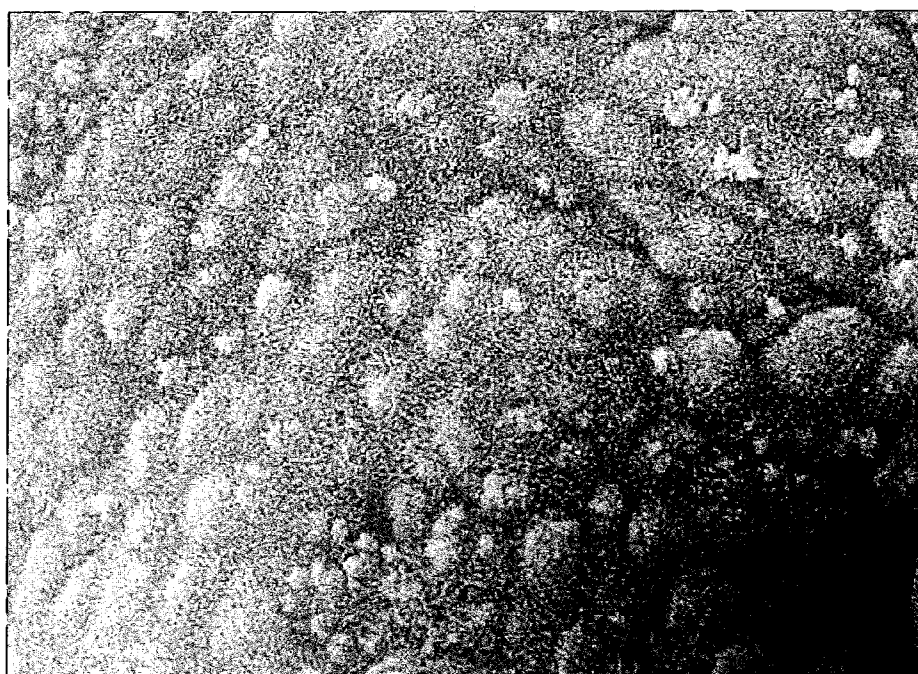


FIG.2



2/4

FIG.3

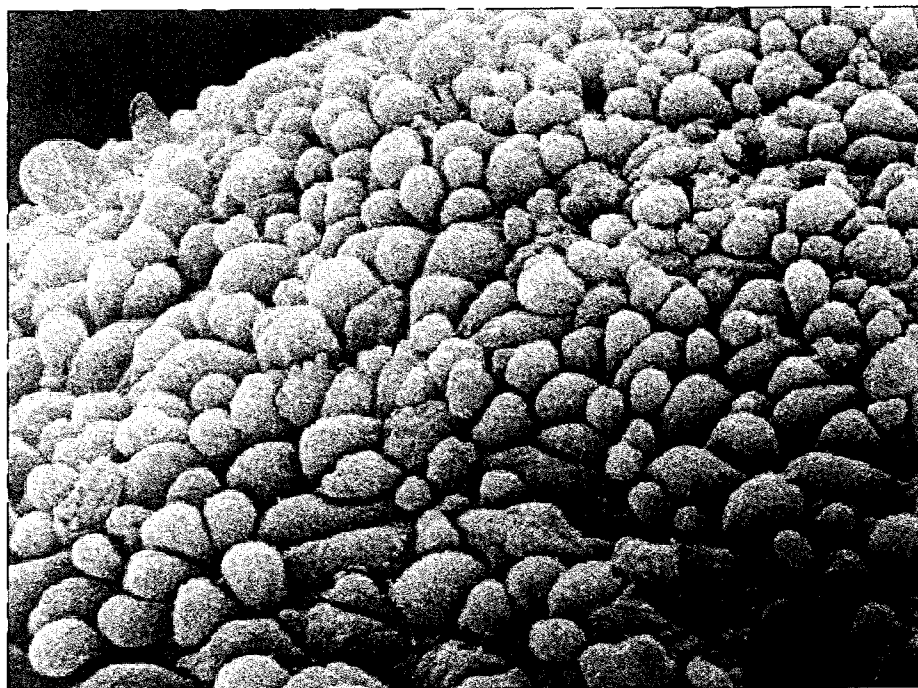
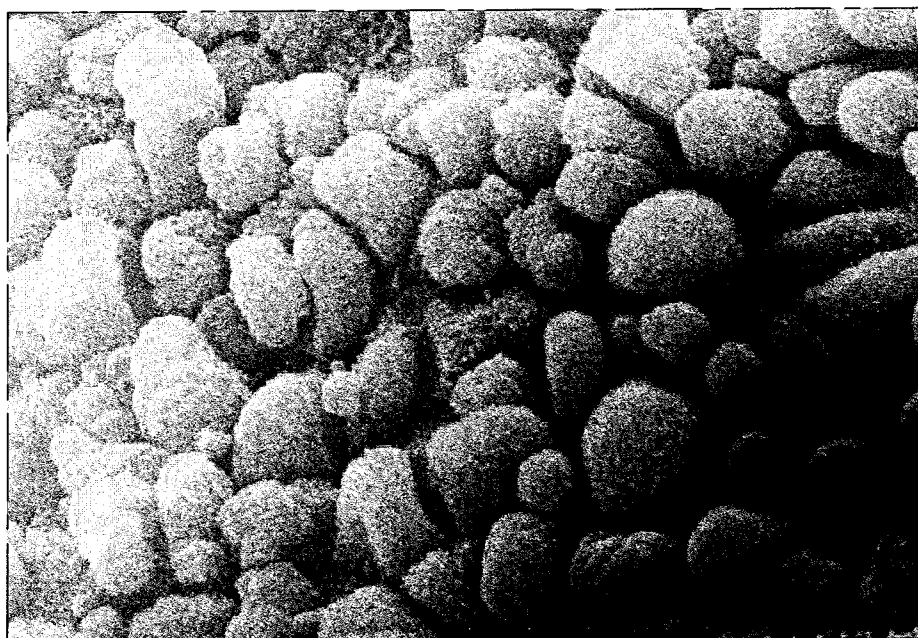


FIG.4



3/4

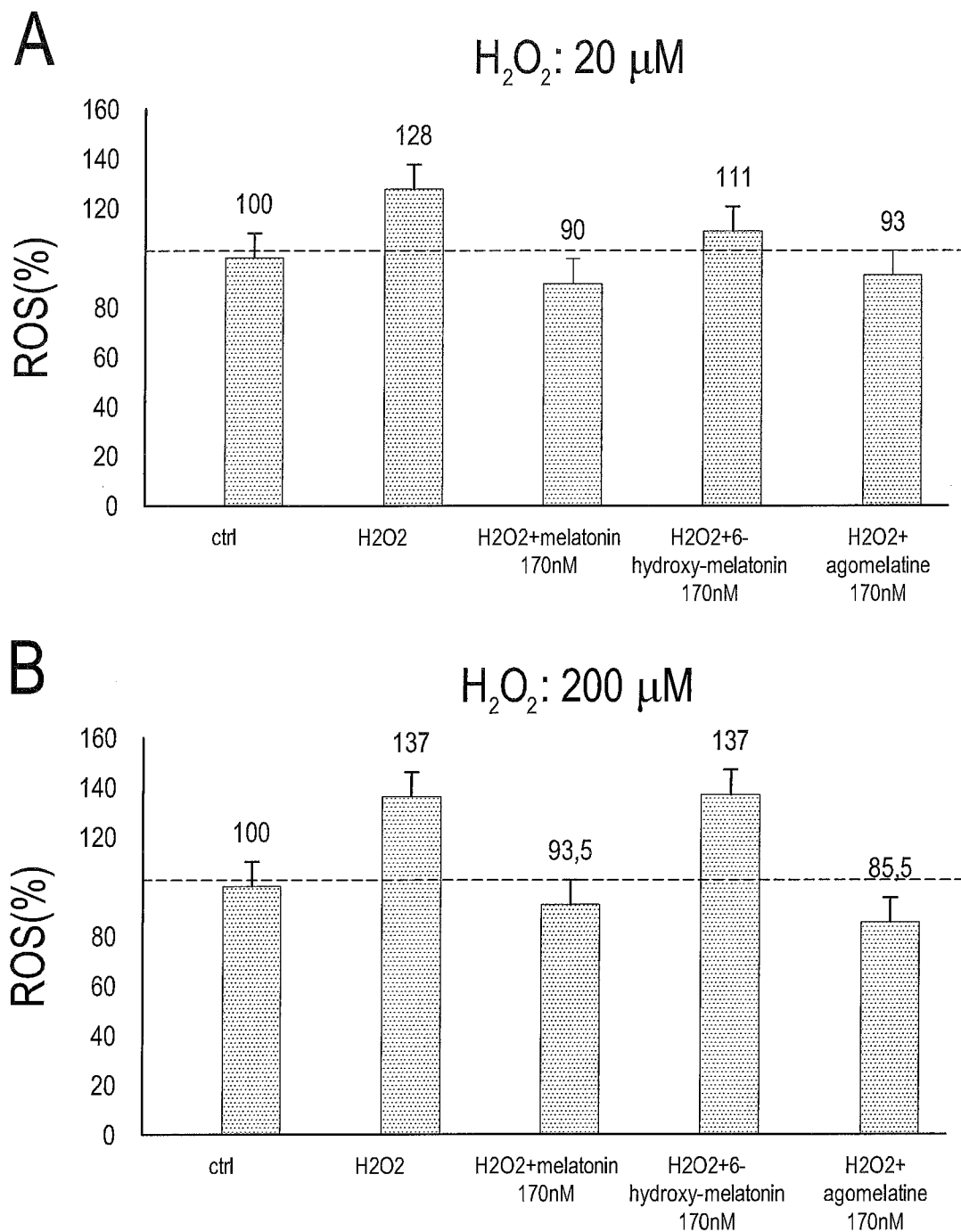


FIG.5

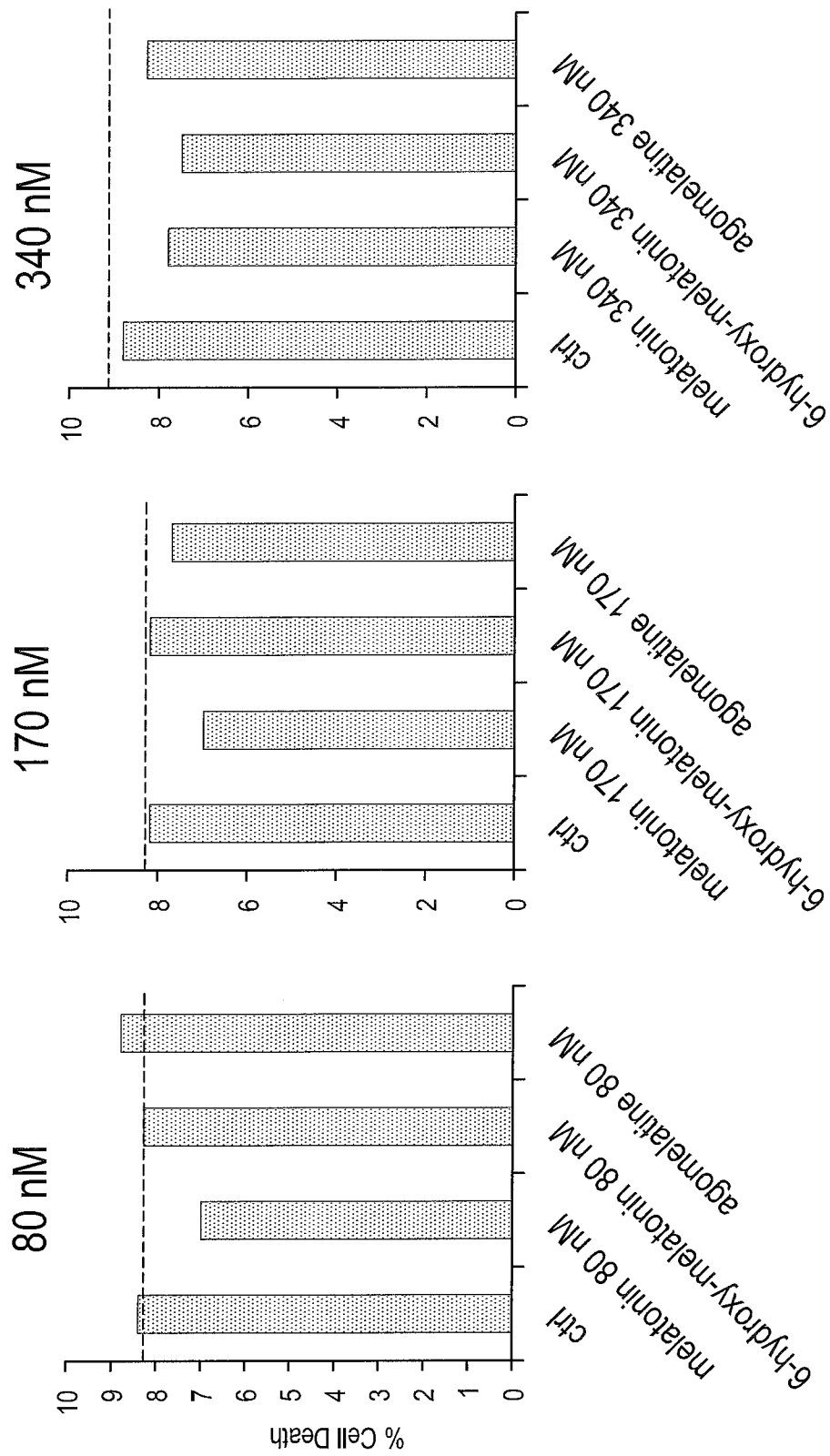


FIG.6

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/EP2013/060872

## Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-15, 25, 26(completely); 16-24(partially)

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.



# INTERNATIONAL SEARCH REPORT

International application No  
PCT/EP2013/060872

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> INV. A61K31/165 A61K31/4045 C12N5/00 A61P15/08 A61K9/00 A61B17/42 A61K9/08 ADD. According to International Patent Classification (IPC) or to both national classification and IPC											
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) A61K C12N A61B 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) EPO-Internal, WPI Data, BIOSIS, EMBASE											
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b> <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%;">Category*</th> <th style="width: 70%;">Citation of document, with indication, where appropriate, of the relevant passages</th> <th style="width: 20%;">Relevant to claim No.</th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top;">X</td> <td style="vertical-align: top;">CN 102 250 832 A (UNIV CHINA AGRICULTURAL) 23 November 2011 (2011-11-23) abstract</td> <td style="text-align: center; vertical-align: top;">25</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">X</td> <td style="vertical-align: top;">           -----            ISHIZUKA BUNPEI ET AL: "The effect of melatonin on in vitro fertilization and embryo development in mice",            JOURNAL OF PINEAL RESEARCH,            vol. 28, no. 1, January 2000 (2000-01),            pages 48-51, XP002690679,            ISSN: 0742-3098            abstract            page 49, right-hand column, paragraph 2 -            page 50, left-hand column, last paragraph            -----            -/--         </td> <td style="text-align: center; vertical-align: top;">25</td> </tr> </tbody> </table>			Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.	X	CN 102 250 832 A (UNIV CHINA AGRICULTURAL) 23 November 2011 (2011-11-23) abstract	25	X	----- ISHIZUKA BUNPEI ET AL: "The effect of melatonin on in vitro fertilization and embryo development in mice", JOURNAL OF PINEAL RESEARCH, vol. 28, no. 1, January 2000 (2000-01), pages 48-51, XP002690679, ISSN: 0742-3098 abstract page 49, right-hand column, paragraph 2 - page 50, left-hand column, last paragraph ----- -/--	25
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.									
X	CN 102 250 832 A (UNIV CHINA AGRICULTURAL) 23 November 2011 (2011-11-23) abstract	25									
X	----- ISHIZUKA BUNPEI ET AL: "The effect of melatonin on in vitro fertilization and embryo development in mice", JOURNAL OF PINEAL RESEARCH, vol. 28, no. 1, January 2000 (2000-01), pages 48-51, XP002690679, ISSN: 0742-3098 abstract page 49, right-hand column, paragraph 2 - page 50, left-hand column, last paragraph ----- -/--	25									
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C.         </div> <div style="width: 45%;"> <input checked="" type="checkbox"/> See patent family annex.         </div> </div>											
<table style="width: 100%;"> <tr> <td style="width: 50%; vertical-align: top;">           * Special categories of cited documents :             "A" document defining the general state of the art which is not considered to be of particular relevance            "E" earlier application or patent but published on or after the international filing date            "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)            "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         </td> <td style="width: 50%; vertical-align: top;">           "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             "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            "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             "&amp;" document member of the same patent family         </td> </tr> </table>			* Special categories of cited documents :  "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "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) "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	"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  "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 "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  "&" document member of the same patent family							
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Date of the actual completion of the international search  <div style="text-align: center; font-size: 1.2em;">2 October 2013</div>		Date of mailing of the international search report  <div style="text-align: center; font-size: 1.2em;">10/10/2013</div>									
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer  <div style="text-align: center; font-size: 1.2em;">Bazzanini, Rita</div>									

## INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2013/060872

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	N. RODRIGUEZ-OSORIO ET AL: "Melatonin increases cleavage rate of porcine preimplantation embryos in vitro", JOURNAL OF PINEAL RESEARCH, vol. 43, no. 3, 1 October 2007 (2007-10-01), pages 283-288, XP055050322, ISSN: 0742-3098, DOI: 10.1111/j.1600-079X.2007.00475.x abstract page 284, left-hand column, paragraph 2-5 page 285, right-hand column, paragraph 2 - page 287, left-hand column, paragraph 1 figure 1 tables 2,3	25
A	----- JIHO CHOI ET AL: "Anti-apoptotic effect of melatonin on preimplantation development of porcine parthenogenetic embryos", MOLECULAR REPRODUCTION AND DEVELOPMENT, vol. 75, no. 7, 1 July 2008 (2008-07-01), pages 1127-1135, XP055049357, ISSN: 1040-452X, DOI: 10.1002/mrd.20861 abstract page 1128, right-hand column, paragraphs 1,5 page 1129, left-hand column, paragraph 2 page 1130, left-hand column, paragraph 2 - page 1131, right-hand column, paragraph 1	25
A	----- UNFER VITTORIO ET AL: "Effect of a supplementation with myo-inositol plus melatonin on oocyte quality in women who failed to conceive in previous in vitro fertilization cycles for poor oocyte quality: a prospective, longitudinal, cohort study", GYNECOLOGICAL ENDOCRINOLOGY : THE OFFICIAL JOURNAL OF THE INTERNATIONAL SOCIETY OF GYNECOLOGICAL ENDOCRINOLOGY, LONDON : INFORMA HEALTHCARE, GB, vol. 27, no. 11, 1 November 2011 (2011-11-01), pages 857-861, XP008159196, ISSN: 1473-0766, DOI: 10.3109/09513590.2011.564687 the whole document -----	1-15

## INTERNATIONAL SEARCH REPORT

### Information on patent family members

International application No

PCT/EP2013/060872

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
CN 102250832	A	23-11-2011	NONE
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**FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210**

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-15, 25, 26(completely); 16-24(partially)

N-acetyl-5-methoxytryptamine (melatonin) for use in the medical or veterinary field in the assisted reproduction for the prevention of implantation failure into the uterus, by topical administration of an effective amount in a mammalian subject female in need of such treatment. Composition suitable for topical administration comprising N-acetyl-5-methoxytryptamine in a mammalian subject female, for use in the medical or veterinary field in the assisted reproduction for the prevention of implantation failure into the uterus. Medical device for uterine washing comprising a sterile container pre-filled with a composition according to any one of claims 9-15. Cell culture medium comprising N-acetyl-5-methoxytryptamine according to claim 25, and use thereof according to claim 26.

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2. claims: 16-24(partially)

Medical device for uterine washing comprising a sterile container to be filled with a composition according to any one of claims 9-15.

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