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(54) Title: METHOD FOR PRESERVATION FOR GENDER SPECIFIC SELECTION OF MAMMALIAN SPERMATOZOA

(57) Abstract: The present invention relates to preservatives for mammalian spermatozoa, independent from any pre-treatment, a novel method for preserving spermatozoa and to preserved spermatozoa preparations. The novel preservatives improve the quality, e.g. the motility and fertility of spermatozoa obtained from male mammals, both for fresh and frozen spermatozoa preparations. The preservation method and preservatives can also be applied to spermatozoa, sorted into X- and Y- chromosome bearing populations. In another aspect, the present invention describes an improved process for sorting mammalian spermatozoa into X- and Y-chromosome bearing fractions and to the spermatozoa fractions obtained by the process, both using the effects of the novel preservatives. When sorting spermatozoa into X- and Y-chromosome bearing species, measurement of their DNA content and the subsequent directing into different collection vessels are apt to induce damages to the spermatozoa cells. The improved process yields spermatozoa cells that have suffered less damages, that is reflected in an improved fertility, by utilizing the effects of the preservatives according to the present invention.



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METHOD FOR PRESERVATION AND FOR GENDER SPECIFIC SELECTION OF MAMMALIAN SPERMATOZOA

The present invention relates to preservatives for mammalian spermatozoa independent from any pre-treatment, a novel method for preserving spermatozoa and to preserved sperm preparations. The novel preservatives improve the quality, e.g. the motility and fertility of spermatozoa obtained from male mammals, both for fresh and frozen sperm preparations. The preservation method and preservatives can also be applied to spermatozoa sorted into X- and Y- chromosome bearing populations.

Nowadays, for producing livestock, artificial insemination is used in the attempt to maximize the utility of spermatozoa produced by males bearing characteristics preferred for breeding. The present invention is not limited to spermatozoa of specific origin; exemplary mammalian species include cattle, swine, sheep, camel and horse. However, the present invention can also be applied to spermatozoa obtained from man.

In the interval between collecting spermatozoa and insemination, spermatozoa can be stored under cooling conditions as liquid semen, or under frozen conditions as frozen semen. Especially without freezing, the viability of spermatozoa, i.e. the fertility rate obtained in artificial insemination decreases with longer storage periods. Along with decreasing motility and decreasing integrity of the acrosome membrane, both indicating a loss of vitality, fertility of the spermatozoa decreases.

Furthermore, the present invention relates to an improved process for sorting mammalian spermatozoa into X- and Y- chromosome bearing fractions and to the sperm fractions obtained by the process, both using the effects of the novel preservatives. When sorting spermatozoa into X- and Y- chromosome bearing species, measurement of their DNA content and the subsequent directing into different collection vessels are apt to induce damages to the spermatozoa. The improved process yields spermatozoa that have suffered less damages that is reflected in an improved fertility, by utilizing the effects of the preservatives according to the present invention.

The present invention relates to novel preservatives for spermatozoa to prolong their viability and extend the storage period as well as to improvements for the sorting process of spermatozoa according to their DNA content, utilizing the effect of the novel preservative on spermatozoa.

State of the art

For preservation of non-sorted liquid semen, it is known to extend the viability of spermatozoa by the presence of antioxidant compounds as well as substances eliminating aggressive or destructive oxygen species. For the purposes of the invention, these substances include enzymes. An example is catalase, an enzyme converting hydrogen peroxide to water and oxygen or, alternatively, converting hydrogen peroxide to water while oxidizing methanol or ethanol. Further known antioxidant enzymes are superoxide dismutase (SOD), glutathione reductase (GR), and glutathione peroxidase (GPx), as well as mercapto ethanol and sodium pyruvate.

In order to pre-determine the sex of mammalian offspring during breeding, sperm fractions are produced from naturally collected spermatozoa which are enriched for X- and Y-chromosome bearing species, respectively. The methods for fractionation, also called sorting, are based on the differentiation according to the different total DNA content of the spermatozoa being smaller for the Y-chromosome. A frequently employed method for sperm fractionation uses fluorescence activated cell sorting (FACS) of stained spermatozoa. It is known to sort mammalian spermatozoa into fractions predominantly containing X- or Y-chromosome bearing sperm populations using FACS, for example from US patent 5,135,759 to Johnson. This method detects and measures the DNA content of spermatozoa that have been treated with a fluorescent dye, namely Hoechst bisbenzimidazole H 33342 fluorochrome, which reversibly stains DNA in living cells. Prior to the actual measurement, spermatozoa are separated into single droplets, predominantly formed by a sheath fluid encapsulating each cell when passing through a bevelled sample injection tip, which is vibrated for generating droplets. These single droplets, each containing one sperm cell, are passed through a laser beam, acting as the excitation source for the fluorescent dye.

According to US 5,135,759, a first optical detector, arranged in the pathway of the excitation laser, detects the light emitted from the flat surface of the spermatozoa. Its intensity signal is correlated with the DNA content for X- and Y-chromosome bearing cells. Cells are diverted into separate containers by applying opposite electrostatic forces according to their DNA content. The hydrodynamic forces within the bevelled tip identically orient sperm nuclei. This identical orientation is essential for measuring their fluorescence quantitatively because the flat shape of the sperm nuclei leads to different signal intensities according to the orientation of the sperm nuclei in relation to the excitation laser and the optical detector. Therefore, an additional second optical detector arranged at 90° to the first optical detector serves to measure the fluorescence intensity indicating the orientation of the sperm nuclei. Sperm nuclei, which are not oriented accurately, are rejected, leading to substantial losses. Because of the stress induced by the sorting process, like UV irradiation of the laser, sperm nuclei cannot be subjected to a second round of sorting without deteriorating their viability to unacceptably low levels.

When sorting flat ovoid sperm heads only, i.e. spermatozoa whose tails have been removed, for example by ultrasonication, more than 80% of sperm nuclei are identically oriented by the hydrodynamic forces within the bevelled tip.

However, when using polyfunctional spermatozoa that is intact spermatozoa having tails, spermatozoa movements complicate the orientation. According to US 5,135,759, a hydrodynamic orientation of intact spermatozoa cannot be achieved. Maloriented spermatozoa are rejected according to the signal obtained from the second optical detector, measuring a fluorescence signal indicative of the orientation. The proportion of properly oriented spermatozoa amounts to 15 to 20% for intact spermatozoa, which can be measured accurately and sorted, whereas the remaining 80 to 85% are rejected when using a flow rate of approximately 25,000 events per second. For improving the rate of proper orientation of spermatozoa, the nozzle geometry can be altered according to US 5 985 216 or according to US 2002/0129669 A1.

It has been found by the present inventors that the viability of polyfunctional bull spermatozoa sorted according to US 5,135,759 is at maximum for 6 hours sufficiently viable for artificial insemination when stored at 5 – 7°C in TALP medium (10 mM phosphate buffered saline at pH 6.9, containing 0.1% by weight bovine serum albumin) after removal of the sheath fluid by gentle centrifugation. Longer storage under these conditions, which are similar to the conditions in the female genital tract, inevitably leads to drastically reduced motility of spermatozoa, insufficient for artificial insemination. Furthermore, the sorting process of US 5,135,759 results in damaged acrosomes of the spermatozoa, which may also contribute to the reduction of fertility observed.

Objects of the invention

In a first aspect, the present invention relates to preservatives for mammalian spermatozoa, which are maintained under cooling conditions, for example at 3 to 15 °C, preferably at 5 to 10°C, or under freezing conditions, e.g. at –10 to –196 °C or at –20 to –70 °C. It is an object of the present invention to provide novel preparations of mammalian spermatozoa, independent from any prior treatment process, to maintain their viability and fertility for artificial insemination or for other biotechnological methods like *in vitro* fertilization or single sperm injection into fertilizable oocytes, over substantially longer periods under cooling conditions or after freezing and thawing. Accordingly, the present invention also seeks to provide the novel use of substances as preservatives for unsorted and sorted sperm preparations stored at room temperature, or under cooling or freezing conditions.

In a second aspect, the present invention aims at providing an improved fractionation process for spermatozoa, which process induces less damages to the spermatozoa during FACS fractionation. Furthermore, it is an object of the invention to provide novel sperm preparations enriched for X- or Y-chromosome bearing populations maintaining motility and fertility over substantially longer periods when stored at room temperature or under cooling conditions or after freezing and thawing. These sorted sperm preparations are obtainable by the sorting process according to the present invention.

In a third aspect, the present invention seeks to improve the efficiency of the FACS sorting mammalian spermatozoa. In view of the disadvantages of the known method for sorting spermatozoa using FACS of US 5,135,759, it is also desirable to improve the yield of the sorting process, avoiding high percentages of rejected spermatozoa. In this respect, the present invention relates to intact and fertile spermatozoa, which can be used for artificial insemination or other biotechnological methods.

General description of the invention

In accordance with the above objects, the present invention provides novel preservatives for mammalian sperm preparations, whether in the form of liquid or frozen preparations, which preservatives are selected from the immobilising agents. The immobilising agents of the present invention reversibly immobilize mammalian spermatozoa and preferably are selected from fluorides, for example sodium fluoride, potassium fluoride, ammonium fluoride, magnesium fluoride, calcium fluoride and further fluorides of mono- or divalent cations. In a preferred embodiment, the present invention uses a combination of immobilising agents with antioxidants for preserving mammalian spermatozoa. Generally, a range of the final concentration of immobilizing agent, especially of fluoride ions in the range of 0.1 to 100 mM, preferably of 10 nM to 10 mM was found suitable. It was found that the optimum concentration of the preferred immobilizing agent, namely a fluoride, differed between species and for individuals. The optimum concentration is species specific and could generally be determined as the concentration yielding an immobilization of at least 90 % of spermatozoa in microscopic observation, preferably of virtually all spermatozoa. Accordingly, the present invention also relates to the use of immobilising agents, preferably in combination with antioxidants, for preserving mammalian sperm preparations.

For preservation according to the invention, mammalian sperm preparations are suitable independent of their origin or pre-treatment, for example fresh semen, frozen semen, both optionally sorted into X- and Y- chromosome bearing fractions.

Prior to artificial insemination or *in vitro* fertilization, the motility is preferentially restored in these sperm preparations by separating or removing the immobilizing agent from the spermatozoa. For removal, sperm preparations can be centrifuged and cells collected to exchange the medium for fresh medium not containing immobilizing agents. As an alternative, immobilizing agent can be removed from the spermatozoa by adsorption, for example by adding a suitable ion exchange resin to the sperm preparation, or filtration, e.g. cross-flow filtration. As one precondition, the immobilizing agents of the present invention need to provide for a reversible immobilization of spermatozoa in order to restore their motility necessary for fertility.

However, sperm preparations containing an immobilizing agent according to the invention may also be utilized for artificial insemination without prior removal of the immobilizing agent. It is assumed that the immobilizing agent is sufficiently diluted or diffused within the female genital tract to restore motility of the spermatozoa.

Generally, artificial insemination can be practised with spermatozoa sorted and/or stored in the presence of an immobilizing agent, optionally with additional antioxidant, using known insemination devices and methods at species specific times in relation to ovulation. Similarly, spermatozoa treated according to the invention may be utilized for *in vitro* fertilization (IVF) or single sperm injection (ICSI) methods, including specific treatments like centrifugation, dilution and capacitation.

The use of immobilizing agents according to the invention is independent from specific extender fluids and media and hence known species specific extenders and media for spermatozoa may be used in combination with the inventive immobilizing agents.

It is assumed that fluorides used as immobilizing agents exert their impact on spermatozoa either by influencing the metabolism of calcium or by influencing the concentration of calcium available to the cells. Accordingly, within the terms of this disclosure, preferred immobilising agents are substances reversibly reducing the motility of spermatozoa. In

addition to or in alternative to fluorides, further compounds can be used for immobilisation of spermatozoa for the purposes of preservation and/or sorting sperm preparations as long as the compound reversibly immobilizes the spermatozoa, does not interfere with fertility and does not cause damages to the spermatozoa.

It is assumed by the present inventors that the preserving effect of immobilizing agents under cooling or freezing conditions on all motile sperm preparations exploits the reduction of energy consumption of spermatozoa during storage as a direct consequence of immobilisation.

The use of antioxidants in addition to the immobilising agents for preserving spermatozoa further increases viability and fertility of the spermatozoa. It is assumed that the beneficial effect of antioxidants is based on the elimination of radicals and, especially oxygen radicals that arise from e.g. the hydrolysis of fatty acids during storage.

It has furthermore been found by the present inventors that immobilising agents can advantageously be used to improve the fractionation of sperm into X- and Y- chromosome bearing fractions. Accordingly, the present invention provides an improvement to the known sorting process using FACS characterized in immobilising polyfunctional spermatozoa at least during the actual sorting process as described in relation to US 5135759. As a result of the immobilising agent, it is now possible to accurately orient spermatozoa using the fluid dynamics of a bevelled tip.

For flowcytometrical sorting, the immobilizing agent contacts the spermatozoa before their orientation within the droplet forming nozzle of the FACS apparatus. Preferably, the immobilizing agent is comprised in the medium containing the spermatozoa intended for sorting. More preferably, fluorides as the immobilizing agent are comprised in the medium containing the spermatozoa from the time of staining with Hoechst bisbenzimidazole H 33342 and during the sorting process, at least until they are fractionated. After fractionation, spermatozoa may be received in a collection fluid, e.g. TEST-yolk extender, preferably also comprising immobilizing agent in the case when a storage period occurs before insemination or IVF, ICSI.

In the alternative to the aforementioned immobilising agents, for improving the sorting process using FACS, the quality of the resultant fractionated spermatozoa is improved by the presence of an antioxidant in contact with the spermatozoa during the sorting process in alternative to, preferably in addition to the immobilising agent.

Accordingly, the present invention in a second aspect provides a novel sorting process of mammalian spermatozoa into fractions, predominantly containing X- or Y- chromosome bearing species, characterized in contacting the spermatozoa at least during the sorting process with an immobilizing agent and/or an antioxidant. Subsequently, the present invention also provides the use of immobilising agents and/or antioxidants for use in a sorting process using FACS providing sperm preparations enriched for X- or Y- chromosome bearing species. In addition to the medium containing the spermatozoa comprising the immobilizing agent and/or antioxidant, the sheath fluid used for the sorting process may also comprise the immobilizing agent and/or antioxidant.

In a very preferred embodiment of the present invention, sperm preparations are provided that are fractionated into X- or Y- chromosome bearing species by the inventive process using at least one immobilizing agent and/or antioxidant during the sorting process which comprise a storage medium also containing a reversibly immobilizing agent and/or antioxidant.

It has furthermore been found that the fractions obtainable by the inventive process show improved vitality and fertility characteristics in comparison to sperm fractions obtainable under identical conditions by the FACS sorting process with no immobilizing agent and/or antioxidant present during the sorting. As a further advantage, it has been found that the sorting process can be performed at higher sorting rates of spermatozoa containing droplets, which translates into an improved performance of the sorting process.

Therefore, both the use of immobilizing agents as preservatives for sorted or unsorted sperm preparations and the use of immobilizing agents for the sorting process of spermatozoa by FACS is based on the same impact of immobilizing agents on the spermatozoa. This immobilizing effect on the one hand allows for orientation of functionally complete spermatozoa by fluid dynamics and prevents their subsequent rotation, enabling an accurate measurement of the fluorescence signal emitted from the stained DNA. On the other hand, the

immobilizing effect reduces the movements of spermatozoa, which results in a reduction of the energy consumption and, hence, in a preserving effect.

The sorting process of the invention uses the apparatus described in US 5,135,759 and reduces the loss due to malorientation of spermatozoa during sorting. The reduction or minimization of malorientation of spermatozoa is achieved by immobilizing intact spermatozoa at least for the time when exiting a droplet forming tip and passing before a detector measuring the intensity of emitted light.

The inventive use of immobilising agents allows to improve the yield of spermatozoa sorting, resulting in a significantly enlarged fraction of spermatozoa separated into X- and Y-chromosome bearing fractions in comparison to US 5,135,759.

The novel sorting process advantageously allows the orientation of intact spermatozoa by fluid dynamics, e.g. by the geometry and arrangement of the bevelled exit tip generating droplets which contain single spermatozoa. Because of the immobilizing effect, spermatozoa cannot move during or after orientation and, accordingly, maintain their uniform orientation. Therefore, a sufficiently reduced percentage of spermatozoa compared to US 5,135,759 is improperly oriented for detection and can optionally be rejected before entering the sorted sperm fractions.

It is a further advantage of the present invention that the sorting process can be performed at higher flow rates of spermatozoa containing droplets, which translates into an improved performance of the sorting process. As another aspect of the improved sorting process of the invention, a higher accuracy can be obtained for sorting, giving a higher rate of homogeneity of the sorted X- and Y-chromosome bearing sperm fractions.

As a further effect of the improved sorting process, it has been found that the sorted spermatozoa have suffered less damages compared to the process of US 5,135,759 at similar flow rates. In detail, the acrosome of spermatozoa sorted according to the present invention has less detectable damages, which indicates a higher viability and fertility. Also, higher flow rates during detection of the fluorescence signal emitted from the dye staining the DNA under irradiation by a UV-laser lead to a reduction of UV-damage to the DNA.

Accordingly, the sperm fractions obtainable by the sorting process according to the invention using an immobilizing agent are characterized in higher viability and fertility due to the reduced acrosome damage and less DNA damage induced by UV due to the reduced exposure time. These characteristics are reflected in the longer viability of sperm preparations sorted according to the invention and in their higher fertility.

When staining mammalian spermatozoa with the fluorescent dye Hoechst bisbenzimidazole H 33342 it has been found that the staining efficiency varies between samples of spermatozoa collected from one male mammal. Furthermore, when staining spermatozoa different results have been observed for the spermatozoa obtained from different male individuals of the same species, for example between different bulls. In detail, it has been found that individual bulls produce semen that cannot be adequately stained with bisbenzimidazole to allow for the FACS-based sorting process.

Surprisingly, it has been found by the present inventors that staining of spermatozoa with Hoechst bisbenzimidazole H 33342 is enhanced by the presence of a fluoride containing compound, preferably sodium fluoride or potassium fluoride, in the medium used for staining spermatozoa with the bisbenzimidazole. When staining spermatozoa with bisbenzimidazole in presence of a fluoride containing compound, the differences in staining properties of sperm collected at different times from one male individual are compensated for. As a further advantage, the sperm obtained from male individuals that could hitherto not be stained with Hoechst bisbenzimidazole H 33342, or stained only to an extent insufficient for FACS sorting, for example certain bulls, can now be stained sufficiently when using fluoride containing compounds during the staining with Hoechst bisbenzimidazole H 33342. Accordingly, the FACS assisted sorting into X- or Y- chromosome bearing fractions of sperm obtained from for example bulls that could hitherto not be stained with Hoechst bisbenzimidazole H 33342, can now be achieved.

Detailed description of the invention

The invention is now described with reference to the accompanying figures, wherein

- Figure 1 is a graph showing the motility of mammalian spermatozoa after freezing and thawing subjected to sorting without immobilizing agent and without antioxidant (●) in comparison to an unsorted control (▲) over an incubation period at 37 °C,
- Figure 2 is a graph showing the acrosome deficiencies and morphological abnormalities of mammalian spermatozoa subjected to sorting without immobilizing agent and without antioxidant in comparison to an unsorted control sample,
- Figure 3 is a graph showing the motility of mammalian spermatozoa subjected to sorting without immobilizing agent or antioxidant (▲), in the presence of immobilizing agent in combination with antioxidant (■) and an unsorted control (●) with subsequent freezing and thawing observed for an incubation period at 37 °C,
- Figure 4 is a graph showing the acrosome deficiencies and morphological abnormalities of mammalian spermatozoa subjected to sorting according to the invention using an immobilizing agent in combination with an antioxidant (n) in comparison to samples without immobilizing agent and without antioxidant (o) as well as an unsorted sample (cont), each with subsequent freezing and thawing,
- Figure 5 is a graph showing the proportion of membrane intact, uncapacitated mammalian spermatozoa, sorted according to the invention using an immobilizing agent in combination with an antioxidant (n) in comparison to samples without immobilizing agent and without antioxidant (o) and unsorted control (cont), with subsequent freezing and thawing after an incubation period of 24 h at 37 °C,
- Figure 6 is a graph showing the motility of mammalian spermatozoa subjected to sorting according to the invention using an immobilizing agent in combination with an antioxidant (▲) in comparison to a sample without immobilizing agent but with antioxidant (●) and unsorted control (■) with subsequent freezing and thawing after into an incubation period of 24 hours at 37 °C.
- Figure 7 is a graph showing the acrosome deficiencies and morphological abnormalities of mammalian spermatozoa after sorting according to the invention using an immobilizing agent (NF) or antioxidant (AO) in comparison to samples without immobilizing agent or antioxidant (C1) , and

- Figure 8 is a graph showing the proportion of membrane intact, uncapacitated mammalian spermatozoa, sorted according to the invention using an immobilizing agent (s-NF) or an antioxidant (s-AO) in comparison to samples without immobilizing agent and without antioxidant (C) after freezing and thawing after a subsequent incubation period of 24 hours at 37 °C.

In the following examples, mammalian spermatozoa are represented by semen obtained from bulls, using aliquots of one sample ($n \geq 6$) for comparative tests. Unless indicated otherwise, polyfunctional spermatozoa were used. Similar effects were found for mammalian spermatozoa of different species. For dilution, TRIS medium was used as an extender, unless indicated otherwise. It is generally known that species specific extender fluids are used in order to meet specific requirements, however, the present invention is not dependent on a specific extender fluid or sheath fluid as long as that does not counteract the effects of the immobilizing agent and/or the antioxidant.

Examples are given for fresh and frozen, sorted and unsorted sperm preparations. More detailed data of results are given for preparations that were sorted and subsequently frozen as these are more sensitive to loss of vitality and fertility than fresh semen preparations. The data presented demonstrate that even with the more sensitive sorted and frozen preparations, major improvements in semen quality and fertility are obtained according to the invention.

For the sorting experiments, a flow rate of at least 25,000 events /min were used on a FACS sorting apparatus (model MOFLO, Dakocytomation, Fort Collins, Colorado, USA, equipped with a nozzle model sx according to US2002/0129669 A1) in TRIS medium.

In the following examples, capacitation was determined as FITC-PNA positive spermatozoa. Morphological abnormalities and acrosome deficiencies were determined microscopically and motility was estimated microscopically.

Example 1: Preserving fresh mammalian semen using an immobilizing agent

To freshly obtained bull semen, diluted in TRIS medium, immobilizing agent (sodium fluoride) was added to a final concentration of 7.5 mM. However, it was found that the

optimum concentration of the preferred immobilizing agent, namely a fluoride, differed between individuals.

Fresh semen in extender fluid containing 7.5 mM sodium fluoride was stored for 24 hours at 5 °C or 15 °C, respectively, then the immobilizing agent was removed by gentle centrifugation, optionally washed in fresh extender fluid, and resuspended in fresh extender fluid. As a control, a parallel aliquot without immobilizing agent was stored under otherwise identical conditions. Motility was observed microscopically at 0 hours and after 3 to 12 hours at 37 °C.

It was found that viability of spermatozoa could be restored by removal of the immobilising agent and that spermatozoa were still fertile, resulting in pregnancies, even after 7 days of storage in the presence of immobilizing agents at 5 °C.

Improved results were found when immobilizing agent was added to the extender fluid in addition to the antioxidant.

Example 2: Preserving fresh sorted mammalian spermatozoa using an immobilizing agent

The assay of Example 1 was also performed using sperm fractions enriched for X- or Y-chromosome bearing fractions produced from fresh semen according to Comparative example 1, described below, using no immobilizing agent during the sorting process.

Storage was under cooling conditions at 5 °C or 15 °C, in presence of 7.5 mM sodium fluoride. A control was treated in parallel without immobilizing agent added. Spermatozoa were collected by gentle centrifugation, transferred into fresh TRIS medium and incubated at 37 °C. At the beginning (0 h) and after 3 and 6 hours, respectively, aliquots were removed and the percentage of motile and morphologically intact spermatozoa was examined microscopically.

It was found that the motility after removal of the immobilizing agent was restored to approximately the level before addition of the immobilizing agent. Further, during the subsequent incubation at 37 °C motility was maintained in the sample that had been stored in presence of the immobilizing agent at significantly higher levels and over a longer period of time when compared to the control sample. In the comparative tests of Examples 1 and 2, the

beneficial effect of the immobilizing agent on the extended storage period for fresh semen, whether unsorted or sorted could be demonstrated.

Improved results were found when antioxidant was added to the extender fluid in addition to the immobilizing agent.

Example 3: Preserving frozen mammalian semen using an immobilizing agent

In order to assess the suitability of immobilizing agents for preserving frozen mammalian semen, Example 1 was repeated identically except for the storage conditions, which were instead gradual freezing of the semen preparation in presence of an immobilizing agent (sodium fluoride) down to -196°C in liquid nitrogen.

Following a storage period of at least 10 days, semen samples were thawed in a 37°C water bath for 30 seconds. Motility was observed as described in Example 1 and it was found that over the incubation period at 37°C the motility of spermatozoa frozen in the presence of an immobilizing agent gradually increased over a period of at least 20 minutes but was significantly lower than for controls without immobilizing agent. These observations can be interpreted as a sign for the energy saving effect of the immobilizing agent. Morphology in contrast was superior to samples frozen without immobilizing agent or antioxidant.

Fertility results did not differ from normal insemination (standard frozen, i.e. unsorted and without immobilizing agent, using 20,000,000 spermatozoa) when using only one tenth of spermatozoa for artificial examination stored frozen according to the invention (2,000,000 frozen spermatozoa in presence of immobilizing agent). This demonstrates that even without removing the immobilizing agent before artificial insemination, the fertility of semen preparations stored under freezing conditions according to the invention, i.e. in the presence of an immobilizing agent, is comparable to normal fresh semen according to the state of art. It is assumed that the immobilizing agent diffuses from the spermatozoa after artificial insemination within the female genital tract.

Example 4: Preserving frozen mammalian sorted spermatozoa using an immobilizing agent

In order to assess the suitability of immobilizing agents for preserving frozen mammalian sperm fractions that have been sorted according to US 5 135 759, Example 3 was repeated identically except using sperm fractions enriched for X- or Y-chromosome bearing spermatozoa produced from fresh semen according to Comparative Example 1, described below, using no immobilizing agent or antioxidant during the sorting process. For control purposes, aliquots of the sorted fractions were treated identically except no immobilizing agent was contained in the storage medium.

It was found that the presence of an immobilizing agent during the storage of sorted spermatozoa at freezing conditions improved their motility and viability when thawed with the immobilizing agent removed.

Comparative Example 1: Sorting polyfunctional bull spermatozoa into X- and Y-chromosome bearing fractions according to US 5 135 759

Bull semen as an example for mammalian sperm was obtained using any known method, diluted and stained with Hoechst bisbenzimidazole H 33342. Stained spermatozoa contained in extender fluid were sorted by FACS as described in US 5 135 759 into TEST yolk medium.

As a control sample, an aliquot of the semen was removed before sorting and used for comparison without being subjected to sorting.

After sorting, in the case of the control sample without sorting, spermatozoa were transferred into fresh TRIS medium and incubated at 37 °C. At the beginning (0 h) and after 3 and 6 hours, respectively, aliquots were removed and the percentage of motile spermatozoa was examined microscopically. The results are depicted in Figure 1, showing that spermatozoa sorted according to US 5 135 759 (▲) have a reduced motility compared to the unsorted control (●), indicating a loss of vitality and fertility due to sorting.

For practical purposes, a percentage of 50 % motile spermatozoa is regarded as the minimum for frozen semen preparations. This percentage is reached by the sorted sample after a significantly shorter incubation period at 37 °C than by the unsorted sample.

In addition to determining the motility, the effects of sorting onto the integrity of spermatozoa was examined by checking their acrosome deficiency and morphology. Abnormalities in morphology and acrosome integrity were determined microscopically. The results are depicted in Figure 2 and show an increase of acrosome deficiency (ACR) and of morphological abnormalities (MAS) for conventionally sorted spermatozoa (sort) in comparison to the unsorted control sample (cont).

Example 5: Sorting mammalian spermatozoa in presence of an immobilizing agent

Polyfunctional bull semen was sorted as described in Comparative Example 1, except supplementing the extender fluid (TRIS medium) with an immobilizing agent (7.5 mM sodium fluoride) and antioxidant (1058 U/L catalase).

The purity of the X- and Y-chromosome bearing fractions obtained was checked by sorting these fractions again after removing the sperm tails by ultra-sonication. It was found that each fraction contained at least 92 % of the desired species, and due to malorientation and coincidence or abortion of cells the total yield of spermatozoa contained in each fraction amounted to 20 % of the number of spermatozoa used for sorting, equal to a rejection rate due to malorientation of 50 – 65 %.

Example 6: Sorting and freezing mammalian spermatozoa in presence of an immobilizing agent and antioxidant

In order to assess the influence of an immobilizing agent present during the sorting procedure according to Comparative Example 1, sodium fluoride at a final concentration of 7.5 mM was added to the TRIS medium used for staining before sorting one semen sample. A control sample without immobilizing agent was sorted under identical conditions using the FACS sorting process as described above. After sorting, the sperm fractions enriched for X- or Y-chromosome bearing fractions sorted in presence of an immobilizing agent, without immobilizing agent and a background control sample were changed to TRIS medium with antioxidant and frozen at -196 °C following established protocols to avoid damaging effects of freezing, then thawed by incubating in a water bath of 38 °C.

In Figure 3, the effects of the immobilizing agent on motility are shown for sorting in presence of an immobilizing agent and antioxidant (■), without immobilizing agent or antioxidant (▲) and an unsorted control without immobilizing agent (●). After thawing, samples were incubated in a volume of TRIS medium at 37 °C while aliquots were removed at the beginning (0 h) and after 6, 12 and 24 h, respectively, for microscopic evaluation of their motility.

This comparison demonstrates firstly that the spermatozoa sorted in the presence of an immobilizing agent have a higher percentage of motile cells than those sorted without immobilizing agent immediately after thawing, indicating the effect of the immobilizing agent to reduce the degradation of sperm vitality during the sorting procedure. Secondly, the reduction of sperm motility during the incubation period at 37 °C is reduced for spermatozoa sorted in presence of an immobilizing agent. This effect of the immobilizing agent is so pronounced that spermatozoa sorted in presence of the immobilizing agent maintain a higher motility than the unsorted background control sample. Thirdly, these observations demonstrate that motility is restored after sorting by removing the immobilizing agent. Removal of the immobilizing agent is exemplified here by changing the medium to not comprise immobilizing agent.

The influence of the sorting and freezing in presence of an immobilizing agent was further assessed by examining the acrosome deficiencies (ACR) by reaction with PNA and morphological abnormalities of spermatozoa. Results are depicted in Figure 4, showing the spermatozoa sorted and frozen according to US 5135759 (o), in the presence of an immobilizing agent (7.5 mM sodium fluoride) in combination with antioxidant (n) (1058 U/L catalase) as well as a control of unsorted spermatozoa, frozen without immobilising agent or antioxidant (cont). These observations demonstrate that the acrosome damages for the semen samples sorted in the presence of an immobilizing agent in combination with an antioxidant in comparison to the sample sorted without immobilizing agent and even in comparison to a sample that had not been subjected to the sorting process, but only frozen and thawed, are reduced for the spermatozoa sorted according to the inventive process. In accordance with these findings, also the morphological abnormalities are reduced for the spermatozoa sorted according to the invention, i.e. in the presence of an immobilizing agent and antioxidant, in comparison to conventionally sorted spermatozoa and even in comparison to spermatozoa that had not been subjected to the sorting process.

Figure 5 shows the results from examining the stability of the percentage of membrane intact uncapacitated spermatozoa (as determined by the affinity for the lectin PNA (*Arachis hypogaea* (peanut)) agglutinin) over an incubation period at 37 °C. This demonstrates that the reduced incidence of membrane deficiencies due to the sorting, i.e. the increased percentage of membrane intact spermatozoa sorted in the presence of an immobilizing agent, only moderately deteriorates over the incubation period of 24 hours. In contrast to the spermatozoa sorted according to the present invention with an immobilizing agent (n), conventionally sorted spermatozoa, i.e. without immobilizing agent (o), as well as unsorted aliquots (cont) after freezing and thawing show an increased incidence of membrane deficiencies, i.e. a reduction of the percentage of membrane intact spermatozoa over the course of the incubation period at 37 °C.

These data demonstrate that the sorting process using an immobilizing agent produces sperm fractions with significantly reduced membrane deficiencies, i.e. an improved fertility even after freezing and thawing of the sorted fractions. This beneficial effect of the sorting process according to the invention on the resultant sperm fractions are even more pronounced after an incubation at 37 °C, additionally indicating an improved fertility.

Similar results for an improved motility and morphology were obtained when substituting the above combination of immobilizing agent and antioxidant for at least one antioxidant (e.g. catalase, sodium pyruvate or mercapto ethanol), or alternatively for an immobilizing agent during the sorting procedure, although to a smaller extent.

Example 7: Sorting and freezing mammalian spermatozoa in presence of an immobilizing agent or antioxidant

In order to test the effect of immobilizing agents or antioxidants on the quality of mammalian spermatozoa sorted according to their relative DNA content, bull spermatozoa sorted in the presence of either immobilizing agent in combination with antioxidant or antioxidant without immobilizing agent were assessed for acrosome deficiencies (ACR) and morphological abnormalities (MAS) as well as for capacitation.

Fresh bull spermatozoa labelled with Hoechst bisbenzimidazole H 33342 were sorted in the presence of antioxidant or the presence of an immobilizing agent or without additive to the

TRIS medium. Following sorting, fractions were frozen at -196 °C for at least ten days, followed by warming for 30 seconds in a 37 °C water bath and, changed to fresh TRIS medium without immobilizing agents and incubated for 24 hours at 37 °C. Examination for integrity of the acrosome was by staining with the agglutinin PNA.

Motility results are given in Figure 6, indicating the superior degree of motile spermatozoa when processed in the presence of the immobilizing agent in combination with the antioxidant (▲) as compared to the group supplemented with the antioxidant (■) alone or the control group (●) sorted without immobilizing agent or antioxidant.

The results from examining acrosome deficiencies (ACR) and morphological abnormalities (MAS) are represented in Figure 7 with NF denoting the sample with immobilizing agent (sodium fluoride, final concentration of 7.5 mM), AO denoting the sample with antioxidant (catalase, final concentration of 1058 U/L), and C1 denoting a control aliquot without any additive.

The results depicted in Figure 7 indicate that spermatozoa sorted in the presence of the immobilizing agent suffer significantly less acrosome damages in comparison to spermatozoa sorted in the presence of an antioxidant only or in comparison to an aliquot sorted without immobilizing agent and antioxidant, respectively. In agreement with the reduction of acrosome damages effected by the presence of an immobilizing agent during the sorting process, significantly reduced morphological abnormalities have been determined in spermatozoa sorted in the presence of an immobilizing agent and the antioxidant.

As a further analysis, the percentage of capacitated spermatozoa was determined with results shown in Figure 8. The percentage values of membrane intact uncapacitated spermatozoa demonstrate that a higher percentage of membrane intact uncapacitated spermatozoa are contained in the fractions sorted in the presence of immobilizing agent (s-NF, 7.5 mM sodium fluoride) than in the fractions sorted in the presence of antioxidant alone (s-AO, 1058 U/L catalase) and than in the control aliquot (c, unsorted control).

The single effect of the immobilizing agent on the quality of sorted spermatozoa is in accordance with the results of Example 6, showing that the morphological abnormalities (MAS) and acrosome deficiencies (ACR) are reduced for the spermatozoa due to the presence

of an immobilizing agent during the sorting process, and even in comparison to spermatozoa that had not been subjected to the sorting process.

Example 8: Fertility of spermatozoa sorted in presence of immobilizing agent

In addition to the above *in vitro* examination of spermatozoa sorted in the presence of immobilizing agent, their fertility was assessed in artificial insemination of heifers and cows using 2 million intact polyfunctional spermatozoa per straw.

The following mean results were obtained with at least 10 animals per sperm preparation:

Table 1: Pregnancy results

	Preparation of spermatozoa	According to	Pregnancy
Frozen semen, conventionally sorted	Sorting by FACS without immobilizing agent or antioxidant, collecting in TEST yolk medium without immobilizing agent or antioxidant, medium change to TRIS freezing extender, freezing at -196°C , thawing to 37°C	Comparative Example 1	21.1%
Frozen semen, sorted according to the invention	Sorting by FACS with immobilizing agent (7.5 mM sodium fluoride) and catalase, collecting in TEST yolk medium without immobilizing agent, medium change to TRIS freezing extender supplemented with antioxidant (1058 U/L catalase) and immobilizing agent (7.5 mM sodium fluoride), freezing at -196°C , thawing to 37°C	Example 6	60.6%
Unsorted semen, conventionally frozen	Control: unsorted semen, frozen in TRIS freezing extender at -196°C , thawing to 37°C		56.9%

Table 1 continued:

Fresh semen, sorted according to the invention	Sorting by FACS with immobilizing agent (7.5 mM sodium fluoride) and antioxidant (1058 U/L catalase), collecting in TEST yolk medium, medium change to TRIS extender with antioxidant (1058 U/L catalase), storage at 15 °C until artificial insemination (tested for 72 hours)	Example 2	64.7%
Conventional fresh semen	Stored at 5 °C for 12 hours		72.2%

The pregnancy results given in table 1 show that for frozen preparations, semen sorted according to the invention in the presence of immobilizing agent can lead to an approximately threefold increase of the pregnancy rate over conventionally sorted semen. In comparison to unsorted semen, that was conventionally frozen, the inventive sorting process yields semen producing even higher pregnancy rates.

For the fresh semen preparations, spermatozoa sorted according to the invention in the presence of immobilizing agent and stored at 15 °C according to the invention in the presence of antioxidant yield pregnancy rates in the same range as conventional unsorted semen.

These results demonstrate that mammalian spermatozoa sorted according to the invention in presence of an immobilizing agent and antioxidant have a high fertility in comparison to spermatozoa sorted without immobilizing agent, using a FACS sorting apparatus according to US 5 135 759.

Claims

1. Preservative for mammalian spermatozoa, characterized in comprising at least one reversibly immobilizing agent.
2. Preservative for mammalian spermatozoa according to claim 1, characterized in that the immobilizing agent is selected from fluoride containing compounds.
3. Preservative for mammalian spermatozoa according to claim 2, characterized in that the fluoride containing compound is selected from fluorides of mono- and divalent cations, especially from sodium fluoride, potassium fluoride, ammonium fluoride, calcium fluoride and magnesium fluoride.
4. Preservative according to one of the preceding claims, characterized in that the preservative furthermore comprises at least one antioxidant.
5. Preservative according to claim 4, characterized in that the antioxidant is selected from catalase, superoxide dismutase, glutathione reductase, glutathione peroxidase, pyruvate and mercapto ethanol.
6. Process for preserving mammalian spermatozoa, characterized by contacting the spermatozoa with a preservative according to one of the preceding claims.
7. Mammalian sperm preparation, characterized in comprising a preservative according to one of claims 1 to 6.
8. Mammalian sperm preparation according to claim 7, selected from liquid semen or frozen semen.
9. Mammalian sperm preparation according to claim 7 or 8, characterized in that the mammalian sperm is a fraction enriched for X- or Y- chromosome bearing species.
10. Agent reversibly immobilizing mammalian spermatozoa for use as a preservative for mammalian spermatozoa.
11. Agent according to claim 10, characterized in that the agent is a fluoride containing compound, preferably selected from fluorides of mono- and divalent cations, especially from sodium fluoride, potassium fluoride, ammonium fluoride, calcium fluoride and magnesium fluoride.

12. Process for sorting mammalian spermatozoa comprising the steps of

- a. staining intact, viable spermatozoa collected from a male mammal with a fluorescent dye capable of selectively staining DNA in living cells,
- b. orienting the spermatozoa within an electrically conductive and isotonic sheath fluid,
- c. separating the spermatozoa into droplets comprising the sheath fluid to contain single spermatozoa,
- d. passing the droplets through the path of an excitation light source, measuring the relative intensity of fluorescence emitted from the spermatozoa containing droplet,
- e. separating the spermatozoa containing droplets according to the intensity of the fluorescence measured,
- f. characterized by treating the spermatozoa with a reversibly immobilizing agent and/or antioxidant.

13. Process according to claim 12,

- g. characterized in removing the immobilizing agent after sorting.

14. Process according to claim 12 or 13, characterized in that the immobilising agent is selected from fluoride containing compounds.

15. Process according to claim 14, characterized in that the fluoride containing compound is selected from fluorides of mono- and divalent cations, especially from sodium fluoride, potassium fluoride, ammonium fluoride, calcium fluoride and magnesium fluoride.

16. Process according to one of claims 12 to 15, characterized in that the antioxidant is selected from catalase, superoxide dismutase, glutathione reductase, glutathione peroxidase, pyruvate and mercapto ethanol.

17. Preparation of sorted mammalian spermatozoa, obtainable by a process according to claims 12 to 16.

18. Preparation of sorted mammalian spermatozoa according to claim 17, characterized by a content of a preservative according to one of claims 1 to 5.

19. Fluoride containing compound for use as an immobilizing agent in a process according to claims 12 to 16.
20. Process for staining mammalian spermatozoa cells with Hoechst bisbenzimidazole H 33342, characterized by the presence of a fluoride containing compound.
21. Fluoride containing compound according to claim 19 or 20, characterized in that the fluoride containing compound is selected from fluorides of mono- and divalent cations, especially from sodium fluoride, potassium fluoride, ammonium fluoride, calcium fluoride and magnesium fluoride.
22. Antioxidant for use in a process according to claims 12 to 16.
23. Antioxidant according to claim 22, characterized in that the antioxidant is selected from catalase, superoxide dismutase, glutathione reductase, glutathione peroxidase, pyruvate and mercapto ethanol.

Figure 1

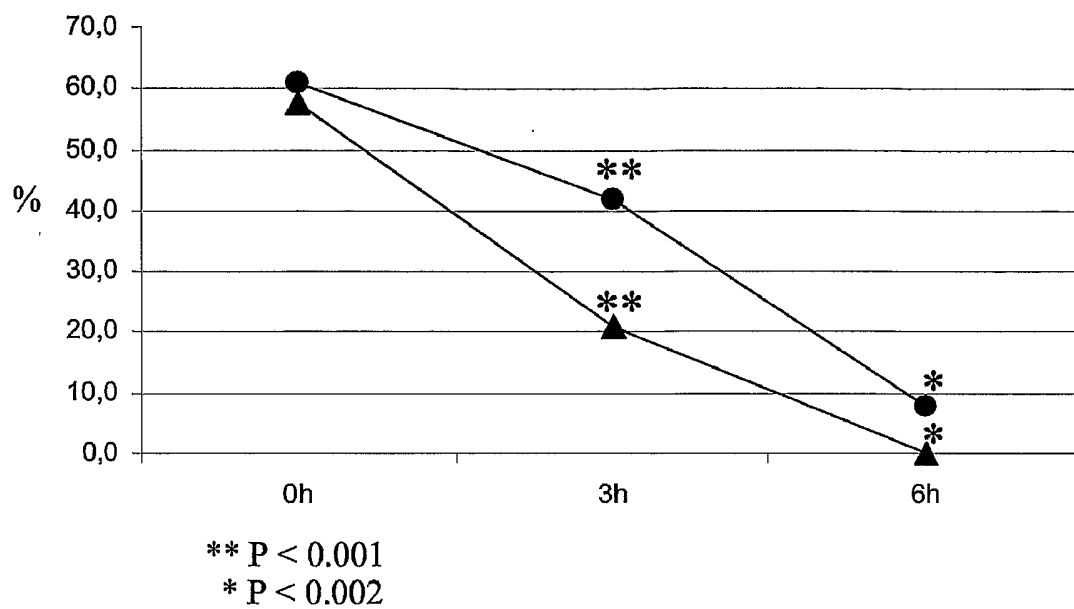


Figure 2

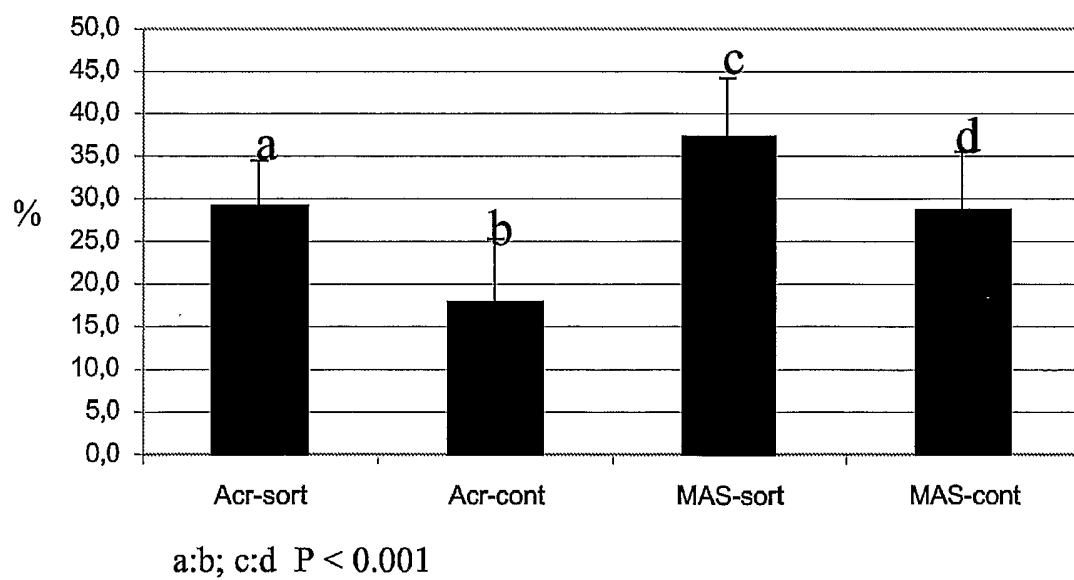


Figure 3

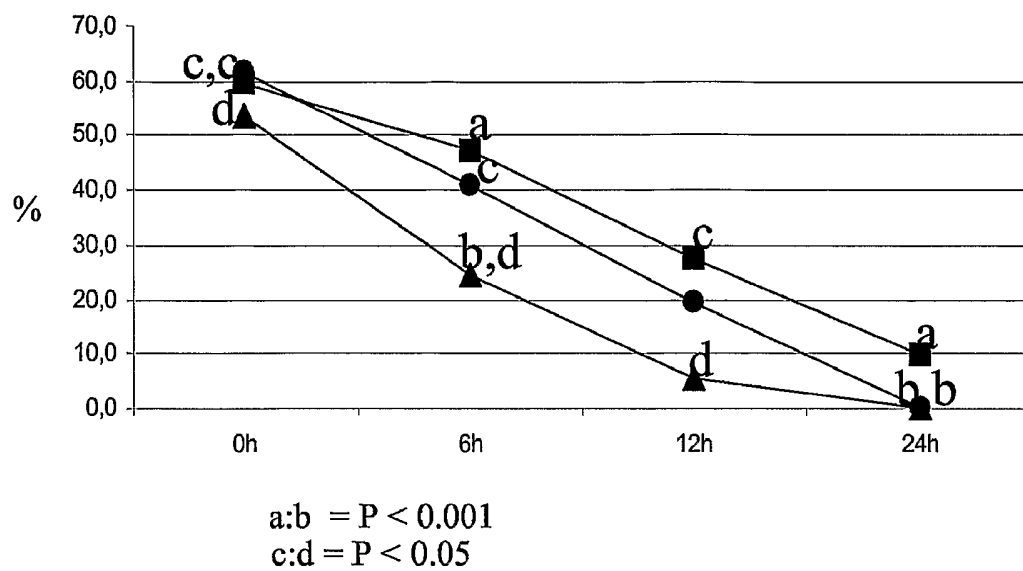


Figure 4

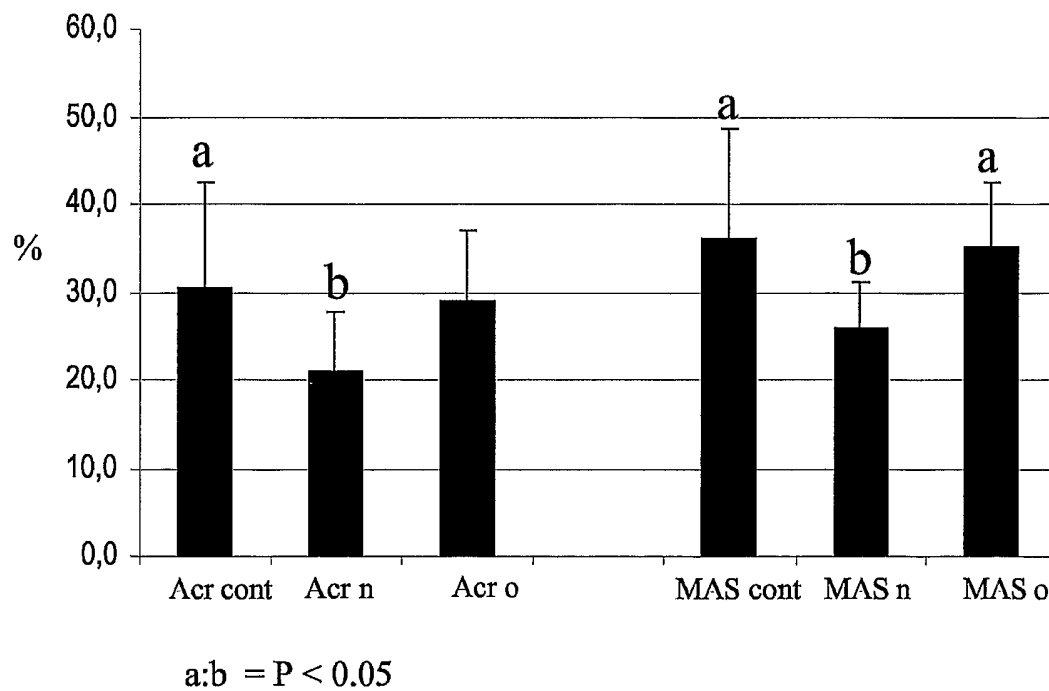
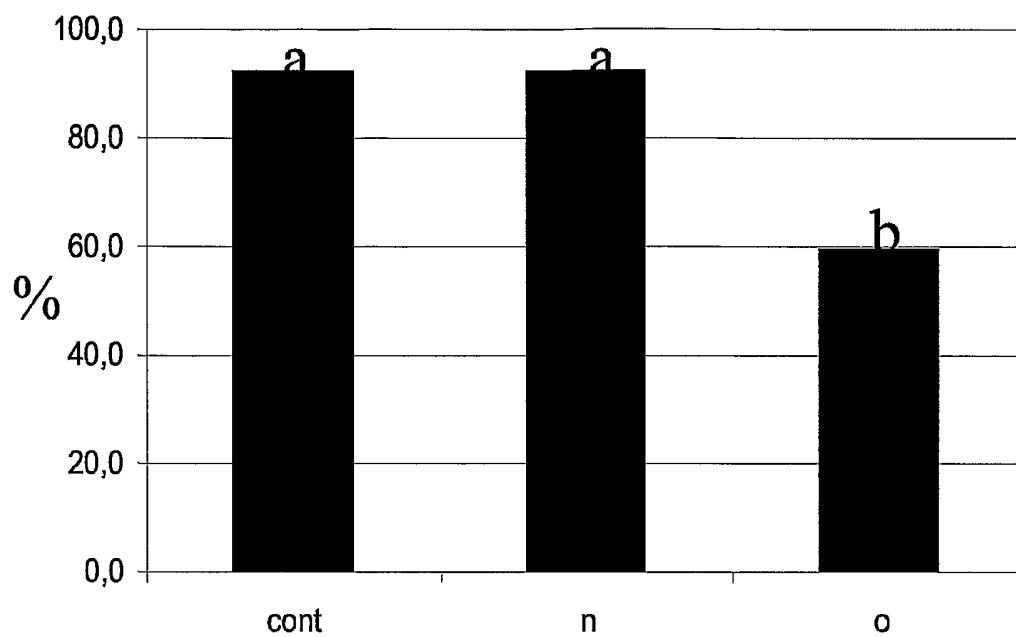
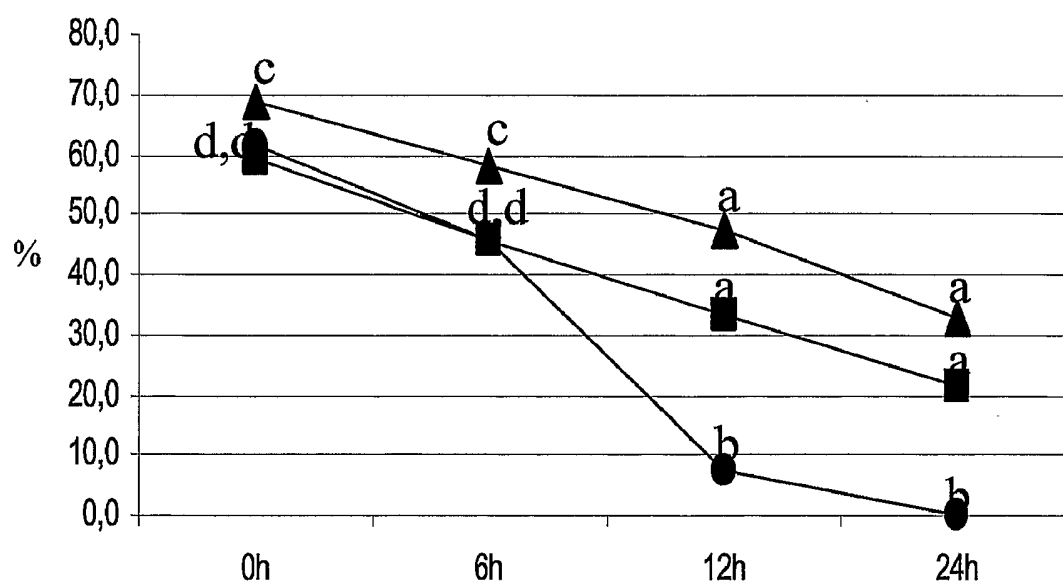


Figure 5



a:b $p < 0.001$

Figure 6



a:b = $P < 0.001$

c:d = $P < 0.05$

Figure 7

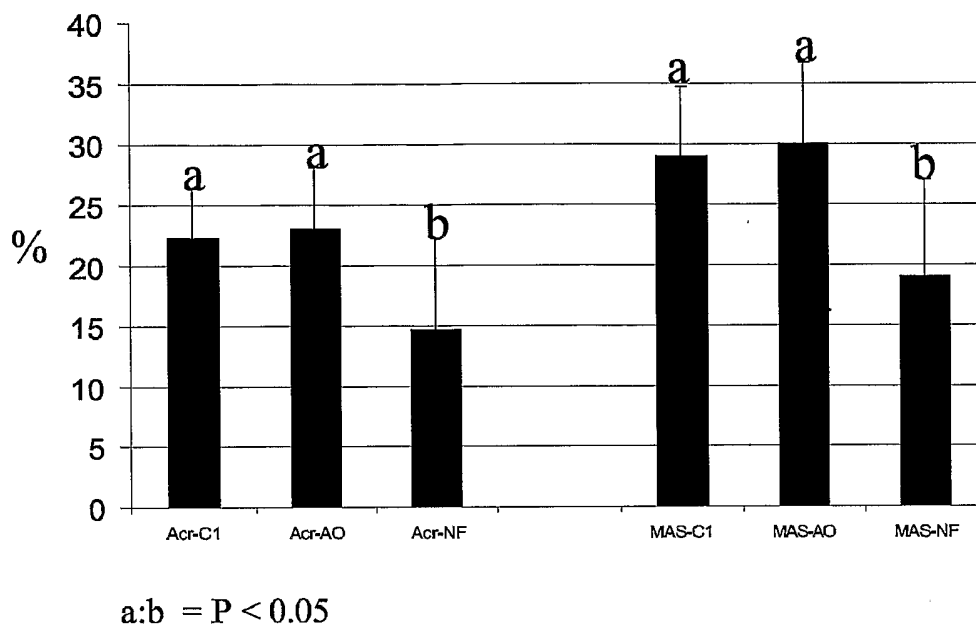
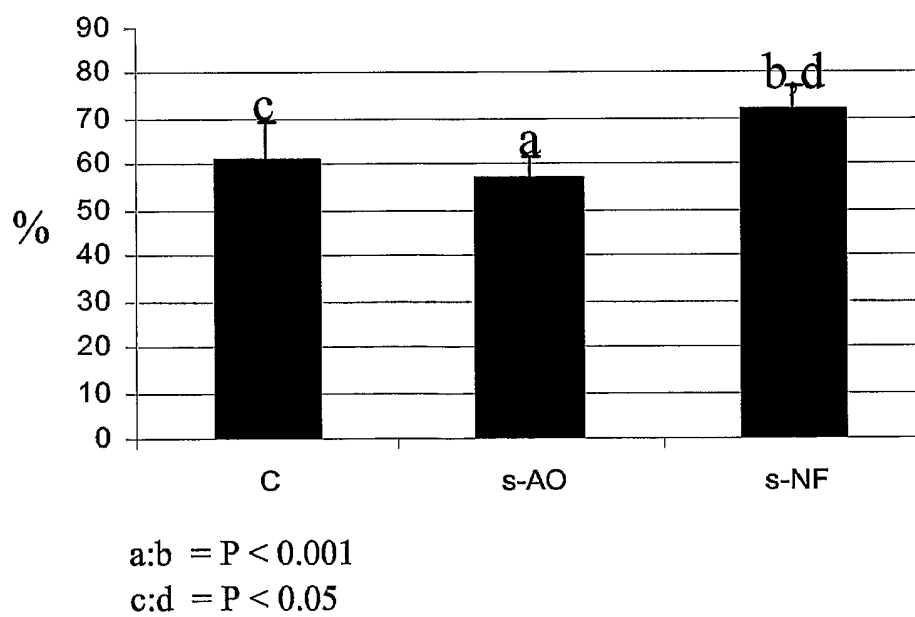


Figure 8



INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2004/052217

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A01N1/02 G01N1/30

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A01N C12N G01N A61K

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 practical, search terms used)

EPO-Internal, WPI Data, BIOSIS, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2004/052217

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INTERNATIONAL SEARCH REPORT

International Application No
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Information on patent family members

International Application No

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