Abstract: The present invention relates to a method and a use, wherein the extract of Morinda citrifolia (noni) fruit juice is used in the field of spermatology to improve the preservation of spermatozoa. The present invention provides the use of noni extract devoid filamentous components obtained after the centrifugation or sedimentation and neutralization of fruit juice by the addition of the solution obtained to fresh, diluted or frozen/thawed semen before the storage, equilibration or freezing, or after the freezing/thawing. The noni supplement decreases the degree of membrane damage and the number of damaged spermatozoa, and results in better quality inseminating material after the freezing/thawing or during the storage of the semen, in which the number of live, motile spermatozoa with intact-membranes, is higher.
Use of Morinda citrifolia (noni) extract in the field of spermatology and method for improving sperm preservation

Field of the invention
The present invention relates to a method and a use, wherein the extract of Morinda citrifolia (noni) fruit juice is used in the field of spermatology to improve the preservation of spermatozoa.

State of the art

Morinda citrifolia is a member of Rubiaceae. Morinda citrifolia (Tahitian name: noni) is used for millennia in tropical cultures' folk medicine to treat diverse illnesses from tumors to

According to the research of Dr. Ralph Heinicke, the beneficial effects of noni are based on the alkaloid xeronine (or rather its precursor, proxeronine, which is abundant in the fruit) [US 4,543,212. (Patent filed 1983, RALPH HEINICKE Xeronine, A new Alkaloid, Useful in Medical, Food, And Industrial Fields.) Xeronine, or the precursor proxeronine is present in living organisms in extremely low concentrations, in an amount well below the detection limit of analytical chemical reactions. It is present not only in plants, but, according to the hypothesis of Dr. Heinicke, in every human and animal cell, where it helps to preserve the structural integrity of protein molecules, and make them fold into their proper conformations. Approximately a hundred essential nutrients are present in Morinda citrifolia (vitamins, enzymes, minerals, carbohydrates, amino acids and trace elements), as well as it contains proxeronine, proxeroninase, bromelain, serotonin, melatonin, dammacanthal in high concentrations, the physiological effect of which are realized after oral ingestion into the body. The publications thus far focused on the botanical, medicinal, pharmacological effects of Morinda citrifolia.

During the sperm freezing process, the semen plasma is removed by centrifugation in order to limit the amount of compounds diminishing the lifespan of spermatozoa (in case of stallion or boar sperm). Other useful components, such as enzyme systems, buffers and antioxidants are also removed in this way. Previous experiments demonstrated that frozen sperm without seminal plasma causes endometritis in greater proportions after insemination than fresh sperm in problematic mares. [Katila T. Sperm-uterine interactions: a review Animal Reproduction Science 68 (2001) 267-272]. Since Morinda citrifolia has intensive anti-inflammatory and antibacterial effects, it can be expected to decrease the inflammatory response of the uterus as well as to increase the pregnancy rates when the semen dose is supplemented with noni fruit extract.
The use of frozen sperm in some species (e.g. cattle) is widespread, while its use in the case of other species — such as horse — is limited due to unacceptable quality, as well as the fact that fertilization with frozen sperm requires several ultrasound examinations on mares and the more precise determination of the time of ovulation. Furthermore, the pregnancy rates lag behind the results seen with fresh or chilled sperm inseminations. In the first case, especially with the use of poorly freezable bull sperm, in the latter case in general, it is particularly important that the frozen semen material to contain more viable spermatozoa with long lifespan. In other species (such as swine), frozen sperm is used in little percentage during breeding, because of the low number of viable spermatozoa with intact membranes.

It was now found that the addition of *Morinda citrifolia* fruit juice to diluted and frozen/thawed semen can increase the viability of the spermatozoa. Since both the fruit of *Morinda citrifolia* and the fruit juice prepared from it comprise a mixture of numerous biologically active ingredients, it does not follow from the beneficial effects of the orally ingested fruit and fruit juice that the composition has positive effect on the spermatozoa under *in vitro* spermatological conditions. The product as used has an acidic pH (pH 3.4), therefore it cannot even be used in its original state to be added to the semen or diluted semen, because of the damage to the proteins and other effects due to its acidic nature.

During my experiments it was found that the addition of the neutralized supernatant of *Morinda citrifolia* fruit extract to semen decreases the level of membrane damage and the proportion of damaged cells, thus providing a better quality inseminating material with higher proportion of the live, intact, motile spermatozoa after freezing/thawing or during the storage of the semen, which could improve the efficiency of the insemination, therefore could increase the pregnancy rates.

The present invention provides a use and method to improve the viability and increase the storability of spermatozoa. The use of *Morinda citrifolia* fruit juice in the field of spermatology improves the storability of spermatozoa.

The present invention provides a method for improving the storability of spermatozoa, comprising centrifugation or sedimentation and neutralization of the fruit juice obtained from *Morinda citrifolia*, then the addition of the solution thus obtained which is free from filamentous components to fresh, diluted or frozen/thawed semen in a predetermined ratio depending on the species and cell concentration.

The present invention provides a method for improving the storability of spermatozoa, comprising centrifugation or sedimentation and neutralization of the fruit juice obtained from *Morinda citrifolia*, then the addition of the solution thus obtained which is free from filamentous components to the semen after diluting it with a freezing extender and before equilibration and freezing.

The present invention provides a method for improving the storability of spermatozoa, comprising centrifugation or sedimentation and neutralization of the fruit juice obtained from *Morinda citrifolia*, then the addition of the solution thus obtained which is free from filamentous components to the semen after freezing/thawing.

The present invention provides a method for improving the storability of spermatozoa, comprising centrifugation or sedimentation and neutralization of the fruit juice obtained from *Morinda citrifolia*, then the addition of the solution thus obtained which is free from filamentous components to the fresh diluted semen before chilled storage.

The present invention provides the use of noni extract having a pH of 6.6 to 7.4 obtained after the centrifugation or sedimentation and neutralization of fruit juice obtained from the mashed *Morinda citrifolia* fruit to improve the storability of spermatozoa by the
addition of the solution obtained to fresh, diluted or frozen/thawed semen before storage, equilibration or freezing, or after the freezing/thawing.

The following examples are intended to illustrate the invention in more detail.

**Example 1**

*The use of Morinda citrifolia supplement in frozen/thawed stallion semen*

Quality assessment of frozen/thawed semen is performed by routine techniques after 24 hours storage at 5 °C or 1 hour at 38 °C. The ratio of live intact cells in the case of stallion semen decreases dramatically with time from the value of 30-50% after thawing.

In this experiment, the frozen/thawed stallion semen samples were evaluated after 10 and 20 hours storage at 5 °C, then the samples were placed into 38 °C water bath for 1 hour and examined again. Samples stored in plastic straws at the concentration of 200 x 10^6 spermatozoa per ml were used for the experiment (using a modified INRA 82 extender as the freezing diluent [Vidament, M.- Yvon, J.M.- Couty, L- Arnaud, G.- Nguekam-Feugang, J.- Noue, P.- Cotton, S.- Le Tellier A.- Noel, F.- Palmer, E.- Magistrini, M. (2001) Advances in cryopreservation of stallion semen in modified INRA82. Anim Reprod Sci. 2001 68(3-4):201-18].) After thawing and quality control, the semen was divided into treatment groups. The first aliquot was stored without dilution. This was the first control sample (CON). A next part was diluted in 1:1 ratio with PBS phosphate buffered saline (PBS) containing 0.06% K<sub>2</sub>HPO<sub>4</sub> and 0.825% NaCl (CON2). This was the second control, to assess the effects within the treatment groups as real effects rather than an effect due to the dilution. Tahitian Noni® Juice /TNJ/ food supplement was neutralized in 1:4 dilution with 0.6% K<sub>2</sub>HPO<sub>4</sub> solution (pH 6.7). After sedimentation, the supernatant of the TNJ solution was added to the third sample in 1:1 ratio (N). Therefore, the TNJ was added in 10% ratio to the thawed semen. Smears were prepared from the samples at the time points mentioned above, and viability and acrosome integrity of the cells were evaluated by modified Kovacs-Foote live/dead and acrosome staining method (Kovacs, A.- Foote, R.H. Viability and acrosome staining of bull, boar and rabbit spermatozoa. Biotech. and Histochemistry. 1992; 67:19-124.; Kütvölgyi G.- Stefler J.- Kovács A.- Viability and acrosome staining of stallion spermatozoa by Chicago sky blue and Giemsa. Biotech. & Histochem. (2006) Biotechnic & Histochemistry. Vol. 81. (4-6) p.109 - 117.) 300 cells were counted in the microscopic assay and classified into 5 categories: 1. cells with completely intact membranes (intact). 2. cells with intact head and tail membranes and damaged acrosome, 3. sperm with intact head-, damaged tail membrane, 4. cells with intact tail-, damaged head membrane, 5. completely damaged (dead) cell. The statistical analyses were made by linear mixed ANOVA model with time and treatment as fixed factors and sample as random factor using the software R. Treatment "N" resulted in significantly more intact spermatozoa than either CON or CON2 after 20h storage (30.9% vs. 20.5%, p=0.0016 and 24.5%, p=0.036; respectively) and after the following heat stress for 1h at 38 °C (28.1% vs. 17.2%, p=0.02 and 19.1%, p=0.04; respectively). The noni supplement reduced the membrane damages of the frozen/thawed stallion sperm after chilled storage and during storage at 38 °C.

**Example 2**

*The use of Morinda citrifolia supplement in the sperm freezing protocol, treatment of diluted bull semen before equilibration and freezing*

Tahitian Noni® Juice (TNJ) food supplement was centrifuged in 10-ml centrifuge tubes at 600g for 5 minutes, then the supernatant devoid of filamentous pellet was removed and collected in a beaker. The pH of the resulting solution was 3.4. The solution was titrated with 1 M NaOH solution to pH 6.9. Bull semen after mounting were diluted with Bioxcell extender (IMV, L'Aigle, France) to 70-80 x 10^6 per ml concentration. 9 bulls were included in the
experiment. After dilution, the control group was placed into a 5 °C refrigerator for equilibration without treatment. In the case of the treated group, 2.5% from the noni fruit juice supernatant as neutralized above was added to the diluted semen at room temperature and placed into the 5 °C refrigerator. Equilibration time was 2 hours. The samples were frozen in 0.25-ml plastic straws in a programmed freezer, then placed into liquid nitrogen. The semen samples were thawed at 37 °C for 30 seconds, then the motility of the sperm was evaluated by CASA (computer assisted sperm analyzer)(MiniT Ub) system. The motility of the groups supplemented with noni was significantly (9%) higher than that of the controls (62% vs. 53%, P=O.01). The Morinda citrifolia supplementation of bull sperm before freezing resulted in higher ratio of motile, living cell number in the thawed semen.

3. Example 3
The use of Morinda citrifolia supplement in the spermfreezing protocol, treatment of diluted stallion semen before equilibration andfreezing
Tahitian Noni® Juice (TNJ) food supplement was centrifuged in 10-ml centrifuge tubes at 600g for 5 minutes, then the supernatant devoid of filamentous pellet was removed and collected in a beaker. The pH of the resulting solution was 3.4. The solution was titrated with 1 M NaOH solution to pH 6.9. Stallion semen after mounting was diluted with centrifugation diluent in 1:1, 1:2 ratio, depending on the stallion, and centrifuged at 800-100Og for 6-10 minutes. After the removal of the supernatant, the sperm pellet was diluted with modified INRA 82 freezing extender (Vidament, M.- Yvon, J.M.- Couty, L.- Arnaud, G.- Nguekam-Feugang, J.- Noue, P.- Cotron, S.- Le Tellier A.- Noel, F.- Palmer, E.- Magistrini, M. (2001) Advances in cryopreservation of stallion semen in modified INRA82. Anim Reprod Sci. 2001 68(3-4):201-18.) to 150 x 106 per ml concentration. 5 stallion were included in the experiment in 4 replicates. After dilution, the control group was placed into a 5 °C refrigerator for equilibration without treatment. In the case of the treated group, 5% from the noni fruit juice supernatant as neutralized above was added to the diluted semen at room temperature and placed into the 5 °C refrigerator. Equilibration time was 1 hour. The samples were frozen in 0.5-ml plastic straws, placed for 10 minutes 4 cm over the level of liquid nitrogen, then plunged into liquid nitrogen. The semen samples were thawed at 38°C for 30 seconds, then the motility of the sperms was evaluated by CASA (computer assisted sperm analyzer)(MiniT Ub) system. The motility of the groups supplemented with noni was 4.3% higher than that of the controls (47.5% vs. 43.2%). The Morinda citrifolia supplementation of stallion sperm before freezing resulted in higher ratio of motile, living cell number in the thawed semen.

The present invention is not limited to the use of the semen of the animal species disclosed in examples above. The scope of the invention includes every mammal and other animal species, whose sperm is used for artificial insemination or vitro fertilization methods. The scope of the invention includes, for example, the following species: human, horse, cattle, swine, dog, wild and Zoo vertebrate species. The present invention is not limited to the above disclosed centrifugation forces and times, the sedimentation of the filamentous components can be achieved by using different parameters. The present invention is not limited to the near neutral pHs disclosed in the above experiments, it may be varied based on the animal species and semen extender depending on the pH of the particular fresh or diluted sperm. The invention is not limited to the neutralization method disclosed above, the scope of protection includes any other method to achieve the near neutral pH usable in the field of spermatology from the strongly acidic pH of the Morinda citrifolia fruit juice (e.g. the use of 2M NaOH instead of IM NaOH for the titration). The present invention is not limited to the addition or dilution ratios described above for the semen, the optimal noni concentration may be different
based on the species and the cell concentration. The present invention is not limited to the
addition to diluted sperm, the supplementation may be performed by addition to the sperm
extender, then by the dilution of the sperm with the solution already comprising the noni
supplement.

By increasing the viability of spermatozoa after thawing, the insemination dose may be
decreased in those insemination protocols where several inseminations are performed, as well
as better result can be achieved with pre-ovulation insemination. The better quality frozen
sperm, the longer lifespan and consequently the better fertility of the sperm and the better
female pregnancy rate are industrially important factors for the breeders, as well as by
developing and preparing modified sperm diluents (comprising Morinda citrifolià), new
markets are opened for these products.
The advantage of the present invention is that the lifespan of the spermatozoa during storage,
after freezing/thawing, during heat stress is elongated due to the noni fruit, the ratio of live,
motile spermatozoa with intact-membranes is greater than that of the fresh, diluted,
frozen/thawed sperm under the same conditions without modification, therefore the
membrane damage, as well as the ratio of damaged spermatozoa decreases due to the noni
supplementation during freezing and storage.
Claims

1. Use of *Morinda citrifolia* fruit extract in the field of spermatology.

2. Method for improving the storability of spermatozoa, comprising centrifugation or sedimentation and neutralization of the fruit juice obtained from *Morinda citrifolia*, then the addition of the solution thus obtained being free from filamentous components to fresh, diluted or frozen/thawed semen in a predetermined ratio depending on the species and cell concentration.

3. The method according to claim 2, wherein the *Morinda citrifolia* fruit extract is added to the equine or bovine semen after dilution with freezing extender, and before equilibration and freezing.

4. The method according to claim 2, wherein the *Morinda citrifolia* fruit extract is added to the equine or bovine semen after freezing/thawing.

5. The method according to claim 2, wherein the *Morinda citrifolia* fruit extract is added to the fresh semen of an equine or bovine species before chilled storage.

6. The method according to claim 2, wherein the *Morinda citrifolia* fruit extract is added to the semen of a mammal or bird species after dilution with freezing extender, and before equilibration and freezing.

7. The method according to claim 2, wherein the *Morinda citrifolia* fruit extract is added to the semen of a mammal or bird species after freezing/thawing.

8. The method according to claim 2, wherein the *Morinda citrifolia* fruit extract is added to the fresh semen of a mammal or bird species before chilled storage.

9. The method according to claim 2, wherein the fibreless supernatant is separated by centrifugation of the fruit juice obtained from *Morinda citrifolia* fruit or mashed noni fruit, then the pH of the supernatant is neutralized (pH 6.5-7.4) and the noni extract is added to the fresh, diluted or frozen/thawed semen in 2-10% (v/v) depending on the species and cell concentration.

10. Use of the extract obtained as the supernatant of the solution obtained after the neutralization (1:4 dilution with 0.6% K₂HPO₄ solution (pH: 6.7)) and centrifugation or sedimentation of a noni fruit juice obtained from *Morinda citrifolia* in the field of spermatology, by adding said solution to the frozen/thawed stallion sperm in a ratio of 1:1, which thus comprises 10% noni fruit juice.

11. Use of the noni extract having a pH of 6.6 to 7.4 and devoid of filamentous components obtained after the centrifugation or sedimentation and neutralization of the fruit juice obtained from the mashed *Morinda citrifolia* fruit in the filed of spermatology, by adding said solution to fresh, diluted or frozen/thawed stallion or bull semen before the storage, equilibration or freezing, or after the freezing/thawing.

12. Use of the noni extract having a pH of 6.6 to 7.4 and devoid of filamentous components obtained after the centrifugation or sedimentation and neutralization of the fruit juice obtained
from the mashed *Morinda citrifolia* fruit in the filed of spermatology, by adding said solution to fresh, diluted or frozen/thawed semen of a male mammal or bird before the storage, equilibration or freezing, or after the freezing/thawing.

13. Use of the noni extract having a pH of 6.9 to 7.0 obtained after the neutralization of the supernatant obtained after the centrifugation of the fruit juice obtained from the *Morinda citrifolia* fruit before equilibration and freezing by adding it to stallion or bull semen in 1-5% (v/v).