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# Effect of liposomal resveratrol on sperm cell motility

**Stefan Thomassen**

*Supervisor: Purusotam Basnet professor II, Department of Clinical Medicine*

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## Preface

The purpose of this thesis was to investigate the *in vitro* effects of resveratrol on the sperm cell motility and viability. In order to do this, resveratrol was encapsulated in liposomal formulations in collaboration with the Department of Pharmacy, UiT-The Arctic University of Norway. Semen samples were obtained from the IVF clinic, University Hospital of Northern Norway (UNN). Current work was approved by regional ethic committee (REK), REK-2014/032 REK Nord.

I have always found that reproductive medicine, especially infertility treatment is quite interesting. On the second year of medical school, I chose to focus on IVF treatment in my elective period. I was at the IVF clinic, UNN, in January 2015. I found the work there amazingly interesting, the choice of topic for the 5th year thesis therefore was focused on the sperm cell. I would like to thank my supervisor, Prof. Purusotam Basnet, for offering me this interesting thesis work which started in autumn 2017. Thank you for the support, help and for sharing your knowledge with me on this journey! Thank you for using several weeks teaching me about laboratory work and about fertility! We used two weeks in August 2017 and one week in March 2018 together on the laboratory in order for me to obtain the laboratory skills needed in this thesis. Thank you for arranging everything and making this thesis possible!

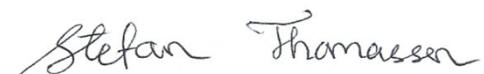
I also express my gratitude to the Prof. Natasa Skalko-Basnet, Department of Pharmacy, The Arctic University of Norway (UiT), for allowing me to prepare the liposomal formulation of resveratrol for this thesis by your established method. Resveratrol incorporated in liposomes and liposomes without any additional substance (empty liposomes) were prepared. I did not personally prepare the resveratrol formulation of the liposomes used in this thesis, due to the time limitation. However, I observed some of the experiments preparing curcumin liposomal formulation at the Department of Pharmacy, UiT, for two days in April. I would therefore like to thank PhD student Selenia Ternullo and master student Laura Schulte, for spending time with me in order for me to understand with such techniques.

I would also like to thank the IVF clinic, UNN for providing semen samples for this project. I express my gratitude to the two bioengineers, Sylvi Johansen and Sissel A. Hansen for recruiting patients and providing the semen samples!

The process of working with this thesis has given me greater insight and understanding of the importance of scientific laboratory work! Before I started working with this thesis, I had minimal of experience and knowledge about how research works and the process of finding new knowledge plays out. Working with this project, I feel that my laboratory skills are significantly improved. My ability to find and evaluate literature critically as well as analysis of our findings are also enhanced. The learning curve has been steep. Even though it has been many intensive weeks in the lab, many hours of microscopy from the beginning of April to the middle of May (sometimes even weekends and holidays), I will be forever grateful for everything I have learned during this amazing journey! I have a gotten a much deeper scientific insight and understanding of how research works and its importance in the medical sciences!

Tromsø, June 2018

Stefan Thomassen

A handwritten signature in black ink that reads "Stefan Thomassen". The signature is written in a cursive style with a large, sweeping initial 'S'.

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## Summary

**Background:** Resveratrol is found to have antioxidative properties and is reported as a protective pharmacological agent against several diseases. It is however a lipophilic and unstable substance because of its photosensitivity. In order to enhance the drug stability, solubility and to deliver the drug in the targeted area, it can be incorporated in a nano-sized vesicle of liposome formulations. The aim of this study was to identify whether the antioxidants such as resveratrol liposomal formulation has some *in vitro* effects on the motility and viability in human spermatozoa. Well known antioxidants such as vitamin C and vitamin E, were also included for comparison.

**Materials and methods:** The liposomal formulation of resveratrol was prepared in collaboration with the Department of Pharmacy. Four drug samples, resveratrol-loaded liposomes, empty liposomes, vitamin C and vitamin E were used at three concentrations (1, 10, and 100  $\mu\text{g}/\text{mL}$ ). Seventeen semen samples obtained from the IVF clinic, University Hospital of Northern Norway (UNN) were studied. After purification of the semen samples, sperm cells were treated with specified drug concentration in a 96 well plate. The motility of the sperm cells was observed with the microscope after 24 and 48 hours incubation at 5%  $\text{CO}_2$  and 37 °C. The current work was approved by regional ethic committee (REK), REK-2014/032 REK Nord.

**Results:** Among more than 90% motile spermatozoa immediately after purification decreased to 35.5% after 24 hours under the normal *in vitro* conditions. Sperm cells containing antioxidants were found to be significantly less motile and significantly more immotile as compared to controls. No significant differences were seen between empty liposomes and control groups. After 48 hours of incubation, in the control group, 9.3% of spermatozoa were motile. Sperm cells containing the drug samples (empty liposomes, resveratrol, vitamin C and vitamin E) were significantly less motile and significantly more immotile especially at the highest concentration (100  $\mu\text{g}/\text{mL}$ ) as compared to the control.

**Conclusion:** The direct antioxidant treatment on sperm cells did not improve the motility or viability of spermatozoa *in vitro*. Although we did not study the use of antioxidants for the IVF outcome, the use of such antioxidant substances in the semen sample purification media should be avoided in the IVF procedure.

## 1. Introduction

Infertility is a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse in a couple with reproductive age (1). Infertility affects approximately 15% of couple worldwide (2). Male factors contribute 20-30%, while female factors are responsible for 50% of causes of infertility. The remaining 20-30% are due to the combination of both male and female factors (3, 4). Male factor infertility affects approximately 7% of the general male population. For men, semen quality has been considered as one of the main factors in infertility (4).

### 1.1. Current trends on semen quality

Semen quality has been considered as one of the major parameters in men's reproductive health. Generally, semen quality is examined by observing the seminal fluid volume, seminal fluid viscosity, sperm cell concentration or total sperm cell counts in the ejaculation, morphology and motility of sperm cells (5). According to WHO guidelines, for the semen quality to be the normal, there should be more than 1.5 mL seminal fluid volume, more than 15 million sperm cells/mL or more than 39 million sperm cells/ejaculate, more than 40% motile spermatozoa and more than 4% spermatozoa with normal morphology (5).

Several studies have shown that the semen quality among men has been declining worldwide. Carlsen et al. published a meta-analysis in 1992, comparing semen quality data published from all six continents in the time period of 50 years (from 1938 to 1991). The results indicated a significant decline in seminal fluid volume and sperm cells concentration. Total sperm cell count during the past 50 years has been decreased to half (6).

There are also regional differences in semen quality in men among the Scandinavian countries. Danish and Norwegian men have shown to have significantly poorer semen counts compared to Finish men (7-9). The study on the 20 year period (1993-2012) archival data of 5739 north Norwegian men showed that the semen quality of men among couples seeking infertility treatment has been found to be progressively declining (10).



## 1.2. Cause of declining semen quality

It is difficult to point out the only one cause for declining semen quality. There are reports suggesting that some factors such as increasing heat around testicles, working in the area of high intensity of radio frequency, keeping mobile phone in the pocket close to testicles and using laptops close to testicles, are among the physical causes of declining semen quality (11-14). In addition, pollution and unhealthy food habits are also considered for the causes of declining semen quality (15, 16). One of the widely discussed research topic pointing as the causes of declining semen quality is the oxidative stress (3).

## 1.3. Oxidative stress

Oxidative stress is caused by the uncontrolled and overproduction of reactive oxygen species (ROS). Reactive oxygen species (ROS), also called oxygen free radicals, contain one or more unpaired electrons and therefore they are highly reactive oxidizing agents. They are formed as by-products during enzymatic reactions with oxygen during normal cellular metabolism (3, 17). Like all living cells under aerobic conditions, spermatozoa also face oxygen ( $O_2$ ) and therefore produce ROS during their normal metabolism (18). Free radicals “steal” electrons from surrounding structures, thereby causing disruption in neighboring structures as well as making other molecules to become subsequent free radicals (19). Examples of highly reactive ROS are the superoxide anion radical ( $O_2^-$ ), the hydroxyl radical (OH), the peroxy radical (ROO) and a subclass of free radicals derived from nitrogen (e.g. nitric oxide (NO), peroxynitrate ( $ONOO^-$ )) (17, 20). Normally there is balance between oxidants and antioxidant systems to protect human cells against potential damage by ROS. When the production of ROS overwhelms the biological antioxidant defenses, a condition called oxidative stress occurs. This can lead to partly or fully cellular physiology destruction (21).

Sperm cells contain only minimal amount of enzymes and antioxidant systems because of their significant lack of cytoplasm, the majority of antioxidants are contained in the seminal fluid (22). This includes enzymes such as superoxide dismutase (SOD) and catalase as well as non-enzymatic substances like ascorbic acid (vitamin C),  $\alpha$ -tocopherol (vitamin E), vitamin A (retinoid), urate, albumin among others (23). Leucocytes and immature spermatozoa have been shown to be the two main sources of ROS in man’s ejaculate (23, 24). However, a variety

of other factors enhance the oxidative stress, such as cigarette smoking, toxins, life style factors or conditions such as varicocele (3, 21).

Oxidative stress can damage variety of the components and functions of the sperm cell. It can cause severe damages to plasma membrane and DNA, and also induce apoptosis (3). The plasma membrane of spermatozoa is particularly vulnerable, because of its high content of polyunsaturated fatty acids, and hence double bonds (25). This can affect semen parameters such as concentration, motility, morphology and therefore fertilization (3, 23). Although excessive concentrations of ROS cause sperm pathology, low and controlled (physiological levels) are believed to be important in important processes in normal sperm physiology and fertilization (3).

#### 1.4. Antioxidants and semen quality

Oxidative stress has become an important target in the therapy of men's reproductive health. Modification of lifestyle behaviors such as smoking, diet, alcohol use, obesity or psychological stress have been proposed together with the antioxidant supplement. Antioxidants are helpful in reducing the ROS activity (21, 22). Dietary antioxidants are obtained through oral intake of food (e.g. fruits, nuts, vegetable) or supplements (e.g. supplemental vitamins, minerals) (26). The role of antioxidants and their significance to semen quality, have also been investigated and debated in several studies. Therefore, antioxidants supplement, diet and lifestyle modifications have been considered to be the important factors to improve the semen quality (22, 27). One of the small but interesting antioxidant molecules, resveratrol among other substances might be useful for therapeutic application however it has not been studied so far in my knowledge.

#### 1.5. Resveratrol

Resveratrol, also known as 3,5,4-trihydroxystilbene and is characterized as a polyphenol. It is a compound that occurs naturally in some plants, including grapes, blueberry, groundnuts and pines. Red wine is one of the widely consumed dietary sources of resveratrol. It is produced by plants in response to pathogenic infection. And it is believed to exhibit fungicidal, antimicrobial and antiviral activities (28). Resveratrol is found to have antioxidative properties and protective against ROS (29). It also can prevent and slow progression of several chronic diseases,

including cancer, cardiovascular diseases and neurodegenerative diseases among others because of its antioxidant properties (30). Chemical structure of resveratrol together with other well known antioxidants such as vitamin C and vitamin E are shown in fig 1.

Although, resveratrol is a potential multi-targeted antioxidant, it is a less stable lipophilic substance. Therefore, to obtain its optimal therapeutic effects, a novel and nano-formulation is necessary. For the current targeted aims, preparation and development of a liposomal formulation is necessary for the improvement of stability and solubility of resveratrol (28).

## 1.6. Liposomes

Liposomes are small nano-size vesicles with spherical shape consisting of one or more concentric lipid bilayers enclosing a discrete aqueous space. They are usually prepared from phospholipids (31). Phospholipids can generally form closed structures when they are hydrated in water, because of their amphipatic nature, that is, having both hydrophilic and hydrophobic structures. When lipids are exposed to water, they arrange themselves exposing their polar (hydrophilic) head groups toward the water phase, while the apolar (hydrophobic) part stick together in the bilayer – thus forming closed concentric bimolecular lipid leaflets separated by aqueous compartments (32). The vesicle consists of simple lipid bilayers that resemble biological membranes, in the form of a spherical shell (33) as shown in Fig 2.

Liposomal formulations can be characterized by properties such as particle size, charge, number of lamella, lipid composition as well as surface modification with polymers and ligands (31). The choice of bilayer components determines the rigidity or fluidity and the charge of the bilayer. Lipids composed of unsaturated phosphatidylcholine gives much more permeable and less stable bilayers, compared to saturated phospholipids that form rigid and less permeable barrier. The size of liposomes range very small (25 nm) to large (over 1000 nm) vesicles and they may also have one or bilayer membranes. Based on the number of bilayer, they are classified into multilamellar or unilamellar vesicles (32). Therefore, the type of lipid can control the liposome function in the drug delivery system.

Because of their entrapping ability, liposomes are considered as drug-carrying structures of vesicles (33). The unique structure of liposomes, with both hydrophilic and hydrophobic parts, makes it possible to entrap different types of drugs. Hydrophilic drugs can be entrapped in the

aqueous interior. Lipophilic drugs can be incorporated in the hydrophobic core of the phospholipid bilayer as shown in fig. 2. Liposomal drug formulations mainly enhance the drug stability and solubility. Moreover, it can deliver the drug in the targeted area and time (31, 32).

## 2. Aim of the thesis

The direct effect of resveratrol on the sperm cell motility and viability has not been reported so far. In IVF clinics, semen sample has to be taken out of the body and exposed to the atmospheric oxygen during the process of purification. The *in vitro* oxidative stress might have effects on the sperm cell functions during IVF treatment. Therefore, it is important to know whether antioxidants such as resveratrol might have effect on sperm cell motility and viability. The outcome of IVF treatment can be improved by using supplement of resveratrol in the medium or oral treatment to the patient for the improvement of semen quality if it is showing pharmacological effects.

In order to achieve the goal, it is aimed for a simple and preliminary screening test. Liposomal formulation of resveratrol is prepared and standardized. Semen samples from men were obtained from IVF clinic and purified by the standard methods. Different concentrations of resveratrol together with the liposomes were treated with the defined amounts of sperm cells and its effects on motility and viability were observed directly under microscope by the standard methods. Well known antioxidants such as vitamin C and vitamin E were also taken for comparisons.

### 3. Materials and methods

#### 3.1. Chemicals / media

Following chemicals and media were used in the experiment: Methylene blue hydrate (Sigma-Aldrich, Steinheim, Germany); Quinn's Advantage™ Fertilization (HTF) (SAGE In-Vitro Fertilization); Human serum albumin (Inc. a Cooper Surgical Company, Trumbull, USA); Quinn's ® Sperm Washing Medium (SAGE In-Vitro Fertilization, Inc., Trumbull, USA); Spermgrad lower layer and Spermgrad upper layer (Vitrolife, Sweden). Lipoid S100 Phosphatidylcholine (> 94% purity) from soybean, Lipoid GmbH, Ludwigshafen, Germany. Resveratrol (≥99 %, powder), Sigma-Aldrich Chemie GmbH, Steinheim, Germany

#### 3.2. Materials and equipment

Following materials and equipments were used: Eppendorf tubes; Heraeus Sepatech; Neubauer improved, Tiefe Depth Profondeur 0,100mm (Superior, Marienfeld, Germany); Falcon 96-Well Cell Tissue Culture Plates (Corning Incorporated, Corning, New York, USA); Falcon serological pipet 5 mL; Finnpiquette: 0.2-2 µL, 2-20 µL, 20-200 µL, 100-1000 µL (Termo Fisher Scientific, Waltham, Massachusetts, USA); Büchi Rotavapor R-124 with Büchi Vac C-500 Vacuum Pump System, Büch Vacuum Controller B-721 and Büchi Water bath B-480 (Büchi Laboratechnik, Flaxil, Switzerland); Eppendorf Centrifuge; Forma Scientific, CO<sub>2</sub> water jacked incubator; Megafuge 1.0, NICOMP Submicron Particle Sizer Model 370, Santa Barbara, California, USA; Zeiss Standard Binocular Microscope, Carl Zeiss, Oberkochen, Germany; Zetasizer Nanno-ZS, Malvern Instruments Ltd., Malvern, UK

#### 3.3. Collection of semen sample

Semen samples were collected from men who came for their routine treatment in the IVF clinic, University Hospital of Northern Norway (UNN). The current work was approved by regional ethic committee (REK), regulations were strictly followed (REK-2014/032 REK Nord). All patients were informed of the purpose of donating their semen samples and signed a consent form (appendix 1).

Semen samples were collected from the IVF clinic at University Hospital of Northern Norway (UNN). Samples were delivered to the clinic within one hour after ejaculation. The patients were instructed to be sexually abstinent for minimum 2-5 days before delivering the sample.



All patients received these guidelines before delivering the sample (appendix 2). Semen quality was assessed by the IVF lab initially using a standard protocol. The following semen parameters were assessed:

- Seminal fluid volume (mL)
- Sperm cell concentration (millions sperm cells/mL)
- Total motility (%)
- Motility grade (1-4)
- Viscosity grade (1-3)

All semen samples were analyzed in the IVF lab as routine clinical test. Only samples having normal semen parameters according to the WHO-criteria (5) were used in this thesis:

- Seminal fluid volume: more than 1.5 mL
- Total sperm cells number: more than 39 millions sperm cells per ejaculate
- Sperm cells concentration: more than 15 millions sperm cells per mL
- Total motility: more than 40%
- Progressive motility: more than 32%

A total of 17 semen samples from different men were studied in this thesis.

### 3.4. Preparation of semen samples

The seminal fluid contains many debris, non-germ cells, dead spermatozoa, among others in addition to normal spermatozoa. It is important to separate spermatozoa from seminal fluid within one hour after ejaculation as products from non-sperm cells can potentially damage the spermatozoa. Purification of semen sample is done by the standard clinical practice in IVF clinic (5).

The two most common techniques used to yield a final preparation containing a high percentage of morphologically normal and motile cells are: 1) the density gradient centrifugation method or 2) swim-up method (5). Both methods of purifications were used in our experiments.

### 3.4.1. Density gradient centrifugation method

This method was carried out according to a protocol by the IVF clinic. The semen sample were collected in a falcon specimen container and delivered to the IVF clinic within 30 minutes after ejaculation. At the lab the container was stirred using a Stuart tiling-machine, 20-25 tilts per minute for 10 minutes in order to liquefy the samples. Thereafter the sample volume, the motility-grade of the spermatozoa, viscosity and total number of spermatozoa were estimated. Standardized WHO-criteria was used as references (5).

For the purification of semen sample, two different gradients (spermgrad lower layer and spermgrad upper layer) were used. Upper layer (1.5 mL) was pipetted on top of lower layer (1.5 mL) into a Nunc-tube (10 mL). In order to have two distinct layers, this had to be done slowly and carefully. Semen sample (1.5 mL) was pipetted on top of these two layers carefully, in order to keep the layers separated. The tube was centrifuged at 500 x g for 20 minutes.

After centrifugation the two top layers were removed. Most of the healthy sperm cells were collected at the bottom. The sperm cells (approx. 1 mL) containing layer on the bottom of the Nunc-tube was mixed with pre warmed Quinn's sperm washing medium (5 mL). The tube was centrifuged at 300 x g for 10 minutes. After centrifugation, the supernatant was removed, leaving the pellet of sperm cells in the bottom of Nunc-tube. The same washing-procedure was repeated once more with washing medium (5 mL) thereafter centrifugation at 300 x g for 10 minutes supernatant was carefully removed. After the second washing procedure, a Nunc-tube with a pellet of sperm cells was left. Then Quinn's Advantage Fertilization medium containing human tubal fluid (0.5 - 1 mL) was added and mixed carefully. After this procedure, motility-grade of spermatozoa, total number, concentration was assed once more. The prepared sample was kept into a CO<sub>2</sub>-incubator at 37 °C until clinical procedures were performed. After the clinical use, the remaining sample was used in the experiment.

### 3.4.2. Direct swim up method

This method is based on the facts that spermatozoa can be selected by their ability to swim out of seminal plasma into the culture medium (5).

The semen sample was transferred into a 10 mL centrifuging tube and diluted with 5 mL Quinn's sperm washing media, and thereafter centrifuged at 700 x g for 10 minutes. After centrifugation, spermatozoa and other cells were collected at the bottom of the tube formed in a pellet with overlying supernatant containing semen plasma. Supernatant was removed as far down to the pellet as possible by pipetting. Thereafter Quinn's sperm washing media (5 mL) was added to the centrifuging tube once more, and centrifuged at 700 x g for 5 min. The supernatant was once more removed. Thereafter the 1 mL of Quinn's Advantage Fertilization (HTF) media was added to the pellet. This was done as gently as possible, in order to prevent mixing of the pellet. The tube was put in a CO<sub>2</sub> incubator for one hour. After one hour there was formation of a motile spermatozoa containing turbid cloudy area in the middle of the layer, some millimeters above the pellet. This cloudy portion, containing high concentration of spermatozoa, was transferred into another test tube. Immotile sperm cells and other cells such as leucocytes were left as pellet. Motile sperms were used in the experiments.

### 3.5. Preparation of liposomes

Lipid (SPC-100) was weighed (200 mg) and transferred into a round bottom flask (100 mL), thereafter 10 mg of resveratrol was added into it. Methanol (approx. 20 mL) was added into round bottom flask in order to dissolve lipid and resveratrol together. The flask was stirred by hand, until all the substances were dissolved.

In order for the methanol to evaporate, a rotatory evaporator (rotavapor), Büchi Rotavapor R-124 with Büchi Vac V-500 Vacuum Pump System, Büchi Vacuum Controller B-721 and Büchi Water Bath B-480, were used. The flask rotated for one hour with optimally adjusted pressure and temperature. All methanol was evaporated during one hour leaving behind a very thin and uniform film of lipid layer together with the resveratrol on the walls of the flask. In order to hydrate this lipid layer, a buffer (10 mL) was added to this mixture. It was shaken by hand in order to form vesicles. Thus formed liposomes were stored in the refrigerator for a minimum 24 hours before further preparation. Both resveratrol incorporated in liposomes and liposomes only (empty liposomes) were prepared by this method.

### 3.5.1. Extrusion of liposomes and size reduction

We were expecting that a uniform liposome size of approximate 100 nm would be up taken by the sperm cells. Size reduction was carried out by the extrusion method. Both resveratrol liposomes and empty liposomes were stored for at least 12 hours in the refrigerator. Before extrusion the liposomes were kept on the bench for one hour to bring at room temperature. For the extrusion, 800 nm filter pore size of Nuclepore® polycarbonate membranes (Whatman International Ltd., UK) were used. By using membranes pores size of 400 nm and 200 nm the liposomes size was further reduced. Subsequently the liposomal dispersion (approx. 10 mL) was further extruded using the membrane sizes 100 nm. Extrusion through membrane was repeated five times through each membrane in order to obtain a homogenous size distribution. After finishing the extrusion, the liposomes were stored in the refrigerator at 4 °C until their use.

### 3.5.2. Characterization of liposomes

The sample was prepared in a laminar airflow bench to avoid dust contamination. NICOMP Submicron Particle Sizer Model 370 with a Helium-Neon diode laser operating at 632.8 nm (NICOMP, USA) was used to measure the particle size. Glass test tubes were filled with distilled water and then sonicated in an ultrasonic bath for 15 min. At first the sonicated tubes were rinsed three times with filtered distilled water (Bulk Acrodisc® syringe filter with a 0.2 µm Supor® Membrane, PALL Life Sciences, Fribourg, Switzerland) and then the dispersion was diluted to an intensity of 250-350 kHz (34). The measurement settings were adjusted to current conditions such as temperature, the resulting viscosity of water and the incident angle was set to 90°. Each sample was measured 3 times for 10 min. The results are presented as intensity weighted distributions. Resveratrol entrapment efficiency was more than 80%.

### 3.6. Preparation of drug samples

In this study, the following drug samples were used: empty liposomes (liposomes without resveratrol), resveratrol incorporated in liposomes, vitamin C, and vitamin E (soluble form). All four drug samples were diluted to three different concentrations with Quinn's advantage fertilization medium in order to form 100 µg/mL (A), 10 µg/mL (B) and 1 µg/mL (C) as the

final concentrations to sperm cells treatment. Sperm cells containing Quinn's advantage fertilization medium only was used as control.

Vitamin C and vitamin E were prepared to an initial stock solution concentration of 10 mg/mL, while resveratrol incorporated in liposomes and liposomes only had an initial concentration of lipid as 20 mg/mL. In order to get concentration A (100  $\mu\text{g/mL}$ ), the vitamin C and E were 10 times diluted with Quinn's advantage fertilization media by mixing stock solution of vitamin C or vitamin E (50  $\mu\text{L}$ ) with 450  $\mu\text{L}$  of Quinn's advantage fertilization media, then mixed thoroughly by vortex shaking for approximately 5 seconds. Resveratrol incorporated in liposomes and liposome only was diluted 20 times i.e. of empty liposomes or resveratrol incorporated in liposomes (25  $\mu\text{L}$ ) was diluted with 475  $\mu\text{L}$  of Quinn's advantage fertilization media, respectively in order to obtain concentration A.

In order to get concentration B (final concentration as 10  $\mu\text{g/mL}$  to the sperm cells), sample A was diluted 10 times. For this, of sample A (50  $\mu\text{L}$ ) was diluted to 500  $\mu\text{L}$  with Quinn's advantage fertilization media, from each drug sample. By the similar way, in order to get sample C (final concentration 1  $\mu\text{g/mL}$  to the sperm cells), sample B was diluted 10 times (50  $\mu\text{L}$  of sample B + 450  $\mu\text{L}$  of Quinn's advantage fertilization media). Resveratrol-lipid ratio in the final concentration was 0.8:20.

The samples were stored in the fridge when not in use. However, they were kept at room temperature for minimum one hour before using the samples for the experiments. Each set of drug samples were used for five semen samples, thereafter a set of fresh drugs samples were prepared from the stock solutions.

### 3.7. Preparation of sperm cells

Cell counting is one of the important steps for this experiment. Cell counting is followed as described in WHO manual (5). In brief, the purified semen samples were diluted with methylene blue solution as 10 or 20 times depending on the initially estimated number of spermatozoa in the sample. Semen samples containing more than 20 million/mL were diluted 20 times by mixing 50  $\mu\text{L}$  of purified semen sample with 950  $\mu\text{L}$  of methylene blue. Samples containing less than 20 million/mL sperm cells concentration were diluted 10 times by mixing 50  $\mu\text{L}$  of semen sample with 450  $\mu\text{L}$  of methylene blue. Semen samples were thoroughly



mixed and left on the bench for 10 minutes to stain properly. Thereafter, it was transferred to a improved Neubauer haemocytometer with covering slit, on both sides of the haemocytometer. This was kept into a humid chamber for 10 minutes, in order for the spermatozoa to sediment and be properly visualized under the light microscope.

There were 25 big squares in the middle part of improved Neubauer haemocytometer, each of these had 16 smaller squares. Depending on the number of spermatozoa in one big square, we determined how many big squares that were needed to count.

- <10 spermatozoa in one big square, all 25 big squares were counted
- 10-40 spermatozoa in one big square, 10 big squares were counted
- >40 spermatozoa in one big square, 5 big squares were counted

In average, to calculate the sperm cell concentration approximately 200 cells were counted. Counting was done on both sides of the hemocytometer, in order to check if the number were close. The sperm cells counted in 25 large square was taken as number of sperm cells per 100 nL. Based on the dilution factor, the concentration in the original purified sample were calculated by multiplying by the dilution factor.

### 3.8. Dilution of purified semen samples

Every semen sample was diluted to having a sperm cell concentration of 250 000 cells per mL with the fertilization media in order to have approximately 100 spermatozoa per vision filed under the microscope with 200 times expansion. This number was based on laboratory exercises and preparations before starting the thesis work. For our experiments, it was aimed at having 3 mL of diluted purified semen sample with the concentration of 250 000 cells per mL in order to have enough volume ( $90 \mu\text{L} * 26 = 2340 \mu\text{L}$ ).

Diluted purified semen sample ( $90 \mu\text{L}$  each) was transferred into each of the middle portion 26 wells in the Falcon 96-well plate, as shown in the figure 3. In five rows, which are as shown in the figure for control, liposomes only, resveratrol in liposomes, vitamin C and vitamin E. In each well,  $10 \mu\text{L}$  of each additive media only or drug containing samples were transferred.

### 3.9. Application of drug sample to Sperm cells

After the drug samples and semen sample were prepared, as mentioned earlier four different drug samples, with three concentrations (A, B, C), each with 10  $\mu\text{L}$  in volume, were added into 90  $\mu\text{L}$  of semen samples. For control group, 10  $\mu\text{L}$  media only was added into the semen sample. Therefore, we had the following samples prepare the following combinations:

- Sperm cell + media only = control
- Sperm cell + liposomes only (A, B and C) = sample 1
- Sperm cell + resveratrol incorporated in liposomes (A, B and C) = sample 2
- Sperm cell + vitamin C (A, B and C) (positive control) = sample 3
- Sperm cell + vitamin E (A, B and C) (positive control) = sample 4

Each set of experiment was duplicated, in order to minimize the risk of experimental errors. Therefore, a total of 26 wells were used as shown in figure 3.

Each time we aimed of using the same amount and concentration of sperm cells, in order to get uniform results. Each of the plates were incubated at 37 °C and 5% CO<sub>2</sub> for 24 hours before motility and viability was assessed under the microscope.

### 3.10. Observation of sperm cell viability and motility

The plates were examined for sperm cell motility and viability after approximately 24 and 48 hours of drug treatment and incubation. The spermatozoa motility and viability was assessed under the light microscope according to guidelines in the WHO lab manual for examining and processing human semen (5). The motility of spermatozoa can be divided into three different categories:

- Progressive motility (PR): Spermatozoa moves actively, either linearly or in a large circle, regardless of speed.
- Non-progressive motility (NP): All patterns of motility with an absence of progression, e.g. swimming in small circles, the tail force hardly displacing the head, or when a tail beat can be observed.
- Immotility (IM): No movement.

All 26 wells were counted in microscope after 24 hours and 48 hours of incubation. The spermatozoa were observed under an inverted microscope. They were examined with an objective 20 x. When looking into the lens, there was a marked rectangular area in the eyepiece field area, as shown in fig. 4. Only spermatozoa inside this area, were counted. Approximately a total between 60 and 120 sperm cells in each field area were counted, with the number of progressive, non-progressive and immotile cells assessed. Two randomly chosen regions were counted in each well. Regions close to the rim of the wells were excluded. In total of 17 semen samples from different patients were studied.

### 3.11. Statistical methods

The results from microscopic observations were transferred into Microsoft Excel, version 2018 (Microsoft Corporation, Redmount, Washington, USA). The data were categorized according type of sample used, time of observation (24 or 48 hours) and classified as motile or immotile. The statistics were calculated using Excel. The means of motile and immotile as well as percentage, was calculated for each patient sample. When all samples were calculated the mean of the percentages from each category for each patient sample were calculated. Each data was the average of four point observations. The results are expressed as mean  $\pm$  SD (standard deviation) and n =17.

For the statistically difference a two-tailed student t-test was performed to compare the difference between controls and the respective samples.

## 4. Results

### 4.1. Baseline data

The data in this thesis were collected between 21<sup>st</sup> March to 16<sup>th</sup> May in 2018. Semen samples were obtained from the IVF clinic, UNN. All patients had signed a consent form in advance. Only semen samples having normal semen quality according to the WHO criteria were included (5).

In total, the results of 17 semen samples were included in this thesis. Ten samples were purified by the IVF clinic using the gradient separation method, while 7 samples were purified in the IKM-lab by using the swim-up method. Table 1 shows the mean age and general semen characteristics as well as standard deviation of the patients included in this thesis. The youngest patient was 29 years and the oldest 42 years.

In this thesis we tested *in vitro* how motility and viability of sperm cells were affected by the direct treatment resveratrol, vitamin C and vitamin E at 100  $\mu\text{g}/\text{mL}$  (A), 10  $\mu\text{g}/\text{mL}$  (B) and 1  $\mu\text{g}/\text{mL}$  (C) concentrations. Resveratrol, a poorly soluble in water, is an unstable substance because of its photosensitivity (28). Resveratrol was therefore encapsulated in liposomes. Empty liposomes were also used as an additional substance to see whether lipid itself has some effects. The liposomal formulations were prepared in collaboration with the Department of Pharmacy, UiT. Therefore, there were four different samples such as empty liposomes, resveratrol encapsulated liposomes, vitamin C and vitamin E at three different concentrations; 100  $\mu\text{g}/\text{mL}$  (A), 10  $\mu\text{g}/\text{mL}$  (B) and 1  $\mu\text{g}/\text{mL}$  (C). Media only treated group was used as control. For all dilution of drug samples and semen samples the same Quinn's advantage fertilization media was used.

#### 4.2. Effects of empty liposomes, resveratrol, vitamin C and vitamin E on sperm cell motility and viability

Generally, over 90% sperm cells are motile after purification in a normal sample. We observed the motility of spermatozoa after 24 hours. Among the controls, only 35.5% of the spermatozoa were found to be motile. The effects of empty liposomes, resveratrol liposomes, vitamin C and vitamin E on sperm cell motility after 24 hours treatment as comparing to control, are shown in Table 2. There is no significant difference in motility with the addition of empty liposomes compared to controls, however motility is slightly decreased.

Spermatozoa exposed to antioxidants are significantly less motile than normal spermatozoa at all concentrations. Samples with encapsulated resveratrol are significantly less motile compared to control at all concentrations, the motility decreases as the concentrations gets higher. Both vitamin E, vitamin C and resveratrol makes the spermatozoa significantly less motile at 100  $\mu\text{g}/\text{mL}$ , however vitamin C is found to most effective to reduce the sperm cell motility as only 10.2% of spermatozoa are motile, compared to 25.7% with resveratrol and 26.3% with vitamin E, respectively.

Generally, less than 10% of normal sperm cells are found immotile after immediate purification. We observed that the percentage of immotile sperm cells had increased up to 64.5% in *in vitro* condition after 24 hours. The results in Table 3 shows the effects of empty liposomes, resveratrol, vitamin C and vitamin E on sperm cell immotility after 24 hours of treatment at 37°C and 5% CO<sub>2</sub>. There is no significant difference in sperm cell immotility between controls and empty liposomes groups. Semen samples containing the antioxidants had significantly increased the percentage of immotile sperm cell after 24 hours compared to controls. 89.8% of spermatozoa exposed to vitamin C at 100  $\mu\text{g}/\text{mL}$  were significantly more immotile compared to control, a very high percentage comparing both resveratrol and vitamin E (74.3% and 73.7% respectively).

It is observed that 9.3% of sperm cells were found motile under *in vitro* condition after 48 hours. Table 4 shows the effects of empty liposomes, resveratrol, vitamin C and vitamin E at various concentrations on sperm cell motility after 48 hours of treatment. Sperm cells containing empty liposomes at 100  $\mu\text{g}/\text{mL}$  are significantly less motile as compared to controls, however no differences are seen at 1  $\mu\text{g}/\text{mL}$  and 10  $\mu\text{g}/\text{mL}$ . Sperm cells on treating with resveratrol at 100  $\mu\text{g}/\text{mL}$  are found significantly less motile compared to controls but no



significantly differences are seen at 1 and 10  $\mu\text{g}/\text{mL}$ . Sperm cells on treating with vitamin C and vitamin E are significantly less motile at 100  $\mu\text{g}/\text{mL}$  and 1  $\mu\text{g}/\text{mL}$  compared to normal, however no difference is seen at 10  $\mu\text{g}/\text{mL}$ .

The percentage of immotile sperm cell increased up to 90.7% at *in vitro* condition after 48 hours. The results in Table 5 shows the effects of empty liposomes, resveratrol, vitamin C and vitamin E on sperm cell immotility after 48 hours of incubation at 37°C and 5% CO<sub>2</sub>. Sperm cells containing empty liposomes at 100  $\mu\text{g}/\text{mL}$  are significantly more immotile as compared to controls, no difference is seen at 1 and 10  $\mu\text{g}/\text{mL}$ . Sperm cells with resveratrol at 100  $\mu\text{g}/\text{mL}$  significantly increased immotile cells compared to controls, no difference is seen at 1 and 10  $\mu\text{g}/\text{mL}$ . Sperm cells on treating with vitamin C and vitamin E significantly increased the percentage of immotile sperm cell at 100  $\mu\text{g}/\text{mL}$  and 1  $\mu\text{g}/\text{mL}$  compared to normal, however no difference is seen at 10  $\mu\text{g}/\text{mL}$ .

## 5. Discussion

We investigated the effects of various concentrations of liposomal formulations of resveratrol as well as vitamin C and vitamin E on sperm cell motility and viability after 24 hours and 48 hours of treatment. Three different concentrations (1, 10, 100  $\mu\text{g}/\text{mL}$ ) were used in the preliminary screening. The spermatozoa were counted under the microscope as progressively motile (PR), non-progressive (NP) and immotile (IM) sperm cells. However, in order to make simple screening results the percentage of progressively motile (PR) and non-progressive motile (NP) sperm cell are expressed to total percentage of motile sperm cell. Indirectly, motile sperm cells are considered as viable sperm cells and immotile sperm cells are non-viable sperm cells. No studies have reported so far on the potential influence of resveratrol on sperm cell motility and viability *in vitro*, therefore resveratrol was chosen for this study. We also selected well known antioxidants such as vitamin C and vitamin E for comparison.

The current research project was designed as a simple screening tool to observe a direct *in vitro* effect of antioxidants like resveratrol, vitamin C and vitamin E on the sperm cell motility and viability. In the IVF clinic, sperm cells and egg cells are needed to culture in media, which include several supplements. It is not clear whether all supplements included in the different kinds of media are needed. Their consequences and effects on *in vitro* fertilization and embryo development still are not understood.

Resveratrol, vitamin C and vitamin E are well established antioxidants. The structures of these compounds are shown in figure 1. Vitamin C is a hydrophilic and vitamin E is a lipophilic antioxidant, and they have well defined bioavailability. Resveratrol however is lipophilic and poorly soluble in water. It is also a photosensitive and an unstable compound. Therefore a way of making it more soluble for reaching the drug target and increase the stability, a liposomes formulation was designed (28). A special nanoparticle-based formulation using liposomes is a way to deliver therapeutic agents. Liposomes have been extensively studied for their controlled and targeted drug delivery and are used in the treatment of various diseases (35).

As expected, the motility of spermatozoa decreased after 24 hours of incubation, 35.5% of spermatozoa were found to be motile. The antioxidants such as resveratrol, vitamin C and vitamin E did not improve neither the semen motility or viability. After 24 hours of incubation, quite the reverse effect was seen. All semen samples containing the antioxidative substances (resveratrol, vitamin C and vitamin E), were significantly less motile and viable at all concentrations. No difference was seen between the empty liposomes and controls. 64.5% of the spermatozoa were immotile after 24 hours, the immotility in semen samples containing antioxidants, was significantly higher compared to normal. These effects were seen especially in vitamin C. At the higher concentrations, only 26.9 and 10.2% of spermatozoa containing vitamin C were motile at 10 and 100  $\mu\text{g}/\text{mL}$  respectively (73.1 and 89.8% immotility).

No improvement on motility was seen after 48 hours either, the differences were also less significant at this time. Both empty liposomes and the antioxidants caused a significant decrease in motility and viability, and therefore immotility, at the highest concentration, 100  $\mu\text{g}/\text{mL}$  respectively. Vitamin C and vitamin E, decreased motility and viability, and immotility at the lowest concentration, 1  $\mu\text{g}/\text{mL}$ , after 48 hours respectively.

As mentioned, there are no reports on sperm cell motility and viability concerning resveratrol *in vitro*. However, the effect of resveratrol on cryopreservation has been studied. Marcia et. al. found resveratrol to be able to avoid oxidative damages to sperm in the cryopreservation process. Resveratrol was found to prevent damage to the lipid membrane of spermatozoa. However, it was not able to prevent loss of motility, which is observed after cryopreservation (36). In this thesis, resveratrol was not able to improve neither motility nor viability *in vitro*.

In our experiment, the application of antioxidants *in vitro*, caused significant decrease in both motility and viability in all semen samples at all concentrations after 24 hours. The differences were less prominent after 48 hours. Despite this, there are a number of reports showing dietary supplements and antioxidants can improve the semen quality. Mendiola *et. al.* found that men with normal semen parameters had significantly higher intake of carbohydrates, fiber, folate, vitamin C and lycopene compared to patients with normal semen parameters. The intake of protein and total fat was also found to be lower in men with normal semen quality (37). Eskenazi *et. al.* found positive associations between intake vitamin C, vitamin E and beta carotene concerning semen parameters. Men with high intake of these antioxidants had better sperm concentration, more progressively motile sperm compared to men with lower intake (38).

Keskes-ammar *et. al.* examined the therapeutic effect of increased antioxidant intake on semen parameters in a prospective study. 54 men were randomized to treatment with vitamin E-selenium supplementation or vitamin B, lasting 3 months. Semen parameters were analyzed, as well as measurement of MDA-levels (lipid peroxidation marker) and levels of vitamins in serum. They found that patients receiving vitamin E combined with selenium had significantly decreased lipid peroxidation and improved semen motility after 3 months of supplementation. Patients receiving vitamin B didn't have any effect (39). Wirletner *et. al.* investigated the influence of antioxidant on semen quality. Men were divided into two groups based on their semen quality that is, having oligoastenoteratozoospermia (OAT) or non-OAT (normal semen quality). Both groups received an antioxidant supplementation for two months (containing vitamin C, vitamin E, folic acid, zinc, selenium, N-acetyl-L-cysteine, L-carnitine, Citrulline, Glutathione red., Lycopene and Coenzyme Q10). They observed significantly more motile spermatozoa and the number of immotile cells had decreased significantly in the OAT-group. There was also significantly higher number of total sperm, and the percentage of morphologically normal sperm cells was also increased this group. Non-OAT patients did not have significant changes in motility, total sperm cell number however (40).

The results obtained by using antioxidants on in clinical trials as discussed above, cannot be directly compared to the results of ours. In contrast to the reports described above, we did not examine the dietary intake of antioxidants in this thesis, nor factors such as lifestyle, smoking, exercise or others. We examined the effect of *direct* application of antioxidants in spermatozoa *in vitro*. Based on the results we got, we cannot see any improvement in motility

or viability with the use of antioxidants. Wirletner *et. al.* also noted that antioxidants did not improve the semen quality on the normal patient (non-OAT) (40). We were also using only normal semen samples. As already mentioned, sperm cells on treating with antioxidants were less viable and motile, compared to controls. It appears that direct application of antioxidants has a toxic effect on sperm cells, especially effects of vitamin C. After 24 hours, only 10.2% of spermatozoa were motile at 100  $\mu\text{g}/\text{mL}$  of vitamin C treatment, as compared to 35.5% of normal sperm cells (thus 89.8% immotility with vitamin C vs. 64.5% in controls). After 48 hours of incubation, only 0.4% of spermatozoa were motile at 100  $\mu\text{g}/$  of vitamin C treatment, compared to 9.3% in controls (thus 99.6% immotility with vitamin C vs. 90.7% in controls).

All semen samples were obtained from the IVF clinic. We aimed to include as many patients as possible in this thesis, however, only 17 patients could be eligible for the study during stated period of time. Ten of the semen samples were purified by the density gradient separation method, while the other 7 were purified in by the swim-up method. We are aware, that the samples should have been purified the same and uniform way. Generally, both methods are used in order to separate spermatozoa from seminal plasma and are well accepted in clinics. The swim-up method is often used in semen samples that are considered to be largely normal, whereas the gradient separation method is preferred in cases of severe oligozoospermia, teratozoospermia or asthenozoospermia. The two methods are somewhat different in the efficiency of getting the total number of motile spermatozoa, yielding morphologically normal spermatozoa and in producing different levels of contamination. Despite the differences in terms of efficiency reported, both methods are used to separate the spermatozoa from seminal plasma and yield the best spermatozoa from samples (5). The results in the two methods were not compared at this thesis. The use of two methods therefore constitutes a limitation. In addition, it is only a preliminary screening test and the effects of antioxidants were compared with control of their own in each time.

## 6. Conclusion

The antioxidants used in this thesis, did not improve the semen motility and viability. Rather the reverse effects were seen after 24 hours of treatment, as all spermatozoa containing antioxidative substances, at all concentrations, were less motile and viable. Lipid, phosphatidylcholine used to prepared liposomes, did not significantly change the sperm cell viability and motility as compared to controls. Vitamin C is found to be highly toxic comparing to resveratrol and vitamin E.

It appears that the direct antioxidants application on sperm cells do not have any beneficial effects concerning motility and viability of spermatozoa *in vitro*. Therefore, using antioxidants on the media for the purification or culture of sperm cell or human embryo should be debated. This thesis is only a simple and preliminary screening tool. In order to clarify further, more semen samples should be examined, and more extensive investigations are needed. More researches on antioxidants are necessary for their possible use for reproductive health.

## 7. References

1. Zegers-Hochschild F, Adamson GD, de Mouzon J, Ishihara O, Mansour R, Nygren K, et al. The international committee for monitoring assisted reproductive technology (ICMART) and the world health organization (WHO) revised glossary on ART terminology, 2009. *Human reproduction*. 2009;24(11):2683-7.
2. Agarwal A, Mulgund A, Hamada A, Chyatte MR. A unique view on male infertility around the globe. *Reproductive Biology and Endocrinology*. 2015;13(1):37.
3. Agarwal A, Virk G, Ong C, du Plessis SS. Effect of oxidative stress on male reproduction. *The world journal of men's health*. 2014;32(1):1-17.
4. Forti G, Krausz C. Evaluation and treatment of the infertile couple. *The Journal of Clinical Endocrinology & Metabolism*. 1998;83(12):4177-88.
5. Organization WH. WHO laboratory manual for the examination and processing of human semen. 2010.
6. Carlsen E, Giwercman A, Keiding N, Skakkebaek NE. Evidence for decreasing quality of semen during past 50 years. *Bmj*. 1992;305(6854):609-13.
7. Jørgensen N, Andersen A-G, Eustache F, Irvine DS, Suominen J, Petersen JH, et al. Regional differences in semen quality in Europe. *Human Reproduction*. 2001;16(5):1012-9.
8. Jørgensen N, Carlsen E, Nermoen I, Punab M, Suominen J, Andersen A-G, et al. East–West gradient in semen quality in the Nordic–Baltic area: a study of men from the general population in Denmark, Norway, Estonia and Finland. *Human Reproduction*. 2002;17(8):2199-208.
9. Jørgensen N, Askund C, Carlsen E, Skakkebaek NE. Coordinated European investigations of semen quality: results from studies of Scandinavian young men is a matter of concern. *International journal of andrology*. 2006;29(1):54-61.
10. Basnet P, Hansen SA, Olaussen IK, Hentemann MA, Acharya G. Changes in the semen quality among 5739 men seeking infertility treatment in Northern Norway over past 20 years (1993–2012). *Journal of Reproductive Biotechnology and Fertility*. 2016;5:2058915816633539.
11. Agarwal A, Deepinder F, Sharma RK, Ranga G, Li J. Effect of cell phone usage on semen analysis in men attending infertility clinic: an observational study. *Fertility and sterility*. 2008;89(1):124-8.
12. Avendano C, Mata A, Sarmiento CAS, Doncel GF. Use of laptop computers connected to internet through Wi-Fi decreases human sperm motility and increases sperm DNA fragmentation. *Fertility and Sterility*. 2012;97(1):39-45. e2.
13. Jung A, Schuppe HC. Influence of genital heat stress on semen quality in humans. *Andrologia*. 2007;39(6):203-15.
14. Møllerlækken OJ, Moen BE. Is fertility reduced among men exposed to radiofrequency fields in the Norwegian Navy? *Bioelectromagnetics*. 2008;29(5):345-52.
15. Kumar S, Murarka S, Mishra V, Gautam A. Environmental & lifestyle factors in deterioration of male reproductive health. *The Indian journal of medical research*. 2014;140(Suppl 1):S29.
16. Povey A, Stocks S. Epidemiology and trends in male subfertility. *Human fertility*. 2010;13(4):182-8.
17. Sikka SC. Relative impact of oxidative stress on male reproductive function. *Current medicinal chemistry*. 2001;8(7):851-62.
18. de Lamirande E, Jiang H, Zini A, Kodama H, Gagnon C. Reactive oxygen species and sperm physiology. *Reviews of reproduction*. 1997;2(1):48-54.

19. Du Plessis SS, Agarwal A, Halabi J, Tvrda E. Contemporary evidence on the physiological role of reactive oxygen species in human sperm function. *Journal of Assisted Reproduction and Genetics*. 2015;32(4):509-20.
20. Lanzafame FM, La Vignera S, Vicari E, Calogero AE. Oxidative stress and medical antioxidant treatment in male infertility. *Reproductive biomedicine online*. 2009;19(5):638-59.
21. Tremellen K. Oxidative stress and male infertility—a clinical perspective. *Human reproduction update*. 2008;14(3):243-58.
22. Kefer JC, Agarwal A, Sabanegh E. Role of antioxidants in the treatment of male infertility. *International journal of Urology*. 2009;16(5):449-57.
23. Agarwal A, Makker K, Sharma R. Clinical relevance of oxidative stress in male factor infertility: an update. *Am J Reprod Immunol*. 2008;59(1):2-11.
24. Griveau J, Lannou D. Reactive oxygen species and human spermatozoa: physiology and pathology. *International journal of andrology*. 1997;20(2):61-9.
25. Henkel RR. Leukocytes and oxidative stress: dilemma for sperm function and male fertility. *Asian journal of andrology*. 2011;13(1):43.
26. Ko EY, Sabanegh ES, Agarwal A. Male infertility testing: reactive oxygen species and antioxidant capacity. *Fertility and sterility*. 2014;102(6):1518-27.
27. Sharma RK, Agarwal A. Role of reactive oxygen species in male infertility. *Urology*. 1996;48(6):835-50.
28. Jøraholmen MW, Škalko-Basnet N, Acharya G, Basnet P. Resveratrol-loaded liposomes for topical treatment of the vaginal inflammation and infections. *European Journal of Pharmaceutical Sciences*. 2015;79:112-21.
29. Soleas GJ, Diamandis EP, Goldberg DM. Resveratrol: a molecule whose time has come? And gone? *Clinical biochemistry*. 1997;30(2):91-113.
30. Baur JA, Sinclair DA. Therapeutic potential of resveratrol: the in vivo evidence. *Nature reviews Drug discovery*. 2006;5(6):493.
31. Sercombe L, Veerati T, Moheimani F, Wu SY, Sood AK, Hua S. Advances and challenges of liposome assisted drug delivery. *Frontiers in pharmacology*. 2015;6:286.
32. Akbarzadeh A, Rezaei-Sadabady R, Davaran S, Joo SW, Zarghami N, Hanifehpour Y, et al. Liposome: classification, preparation, and applications. *Nanoscale research letters*. 2013;8(1):102.
33. Vemuri S, Rhodes C. Preparation and characterization of liposomes as therapeutic delivery systems: a review. *Pharmaceutica Acta Helvetiae*. 1995;70(2):95-111.
34. Ingebrigtsen L, Brandl M. Determination of the size distribution of liposomes by SEC fractionation, and PCS analysis and enzymatic assay of lipid content. *AAPS PharmSciTech*. 2002;3(2):9-15.
35. Lamichhane N, Udayakumar TS, D'Souza WD, Simone II CB, Raghavan SR, Polf J, et al. Liposomes: Clinical Applications and Potential for Image-Guided Drug Delivery. *Molecules*. 2018;23(2):288.
36. Garcez ME, dos Santos Branco C, Lara LV, Pasqualotto FF, Salvador M. Effects of resveratrol supplementation on cryopreservation medium of human semen. *Fertility and sterility*. 2010;94(6):2118-21.
37. Mendiola J, Torres-Cantero AM, Vioque J, Moreno-Grau JM, Ten J, Roca M, et al. A low intake of antioxidant nutrients is associated with poor semen quality in patients attending fertility clinics. *Fertility and sterility*. 2010;93(4):1128-33.
38. Eskenazi B, Kidd S, Marks A, Slotter E, Block G, Wyrobek A. Antioxidant intake is associated with semen quality in healthy men. *Human Reproduction*. 2005;20(4):1006-12.



39. Keskes-Ammar L, Feki-Chakroun N, Rebai T, Sahnoun Z, Ghozzi H, Hammami S, et al. Sperm oxidative stress and the effect of an oral vitamin E and selenium supplement on semen quality in infertile men. *Archives of andrology*. 2003;49(2):83-94.
40. Wirleitner B, Vanderzwalmen P, Stecher A, Spitzer D, Schuff M, Schwerda D, et al. Dietary supplementation of antioxidants improves semen quality of IVF patients in terms of motility, sperm count, and nuclear vacuolization. *Int J Vitam Nutr Res*. 2012;82(6):391-8.

## 8. Tables

**Table 1:** Baseline data of age and semen parameter.

Age and semen parameters	Mean and standard deviation
Age (years)	35.6 ± 6.6
Semen volume (mL)	3.1 ± 1.0
Viscosity grade	2.4 ± 0.7
Sperm cell concentration (millions/mL)	52.5 ± 19.6
Motility (%)	59.1 ± 10.4
Motility grade	2.6 ± 0.5

Results are expressed as mean ± SD (n = 17).

**Table 2:** Effects of empty liposomes, resveratrol-liposomes, vitamin C and vitamin E on *in vitro* sperm cells motility after 24 hours at 37°C and 5% CO<sub>2</sub>.

	Media /drug concentration	Total motile (PR + NP) %
Control	Media only	35.5 ± 11.0
Empty liposomes	1 µg/mL (C)	31.7 ± 7.8
	10 µg/mL (B)	33.5 ± 7.3
	100 µg/mL (A)	33.7 ± 8.4
Resveratrol	1 µg/mL (C)	31.0 ± 7.2 *
	10 µg/mL (B)	29.0 ± 11.4 **
	100 µg/mL (A)	25.7 ± 11.8 **
Vitamin C	1 µg/mL (C)	29.2 ± 8.0 **
	10 µg/mL (B)	26.9 ± 8.0 **
	100 µg/mL (A)	10.2 ± 8.7 **
Vitamin E	1 µg/mL (C)	26.7 ± 11.6 *
	10 µg/mL (B)	28.1 ± 9.1 *
	100 µg/mL (A)	26.3 ± 8.1 **

Results are expressed as mean ± SD (n = 17). \*P-value < 0.05, \*\*P-value < 0.01

**Table 3:** Effects of empty liposomes, resveratrol, vitamin C and vitamin E on *in vitro* sperm cells immotility after 24 hours in incubator at 37°C and 5% CO<sub>2</sub>.

	<b>Media /drug concentration</b>	<b>Immotile %</b>
Control	Media only	64.5 ± 18.8
Empty liposomes	1 µg/mL (C)	68.3 ± 20.5
	10 µg/mL (B)	66.5 ± 20.6
	100 µg/mL (A)	66.3 ± 21.5
Resveratrol	1 µg/mL (C)	69.0 ± 20.6 *
	10 µg/mL (B)	71.0 ± 26.6 **
	100 µg/mL (A)	74.3 ± 18.2 **
Vitamin C	1 µg/mL (C)	70.8 ± 16.3 **
	10 µg/mL (B)	73.1 ± 21.4 **
	100 µg/mL (A)	89.8 ± 17.8 **
Vitamin E	1 µg/mL (C)	73.3 ± 15.6 *
	10 µg/mL (B)	71.9 ± 18.6 *
	100 µg/mL (A)	73.7 ± 18.8 **

Results are expressed as mean ± SD (n = 17). \*P-value < 0.05, \*\*P-value < 0.01

**Table 4:** Effects of empty liposomes, resveratrol, vitamin C and vitamin E on *in vitro* sperm cells motility after 48 hours in incubator at 37°C and 5% CO<sub>2</sub>

	<b>Media /drug concentration</b>	<b>Total motile (PR + NP) %</b>
Control	Media only	9.3 ± 7.4
Empty liposomes	1 µg/mL (C)	7.9 ± 5.2
	10 µg/mL (B)	9.4 ± 7.0
	100 µg/mL (A)	5.2 ± 6.1 *
Resveratrol	1 µg/mL (C)	7.2 ± 4.7
	10 µg/mL (B)	6.3 ± 5.5
	100 µg/mL (A)	3.3 ± 4.0 **
Vitamin C	1 µg/mL (C)	6.5 ± 4.4 *
	10 µg/mL (B)	7.2 ± 4.6
	100 µg/mL (A)	0.4 ± 0.9 **
Vitamin E	1 µg/mL (C)	5.8 ± 4.4 *
	10 µg/mL (B)	8.1 ± 5.9
	100 µg/mL (A)	5.2 ± 4.0 *

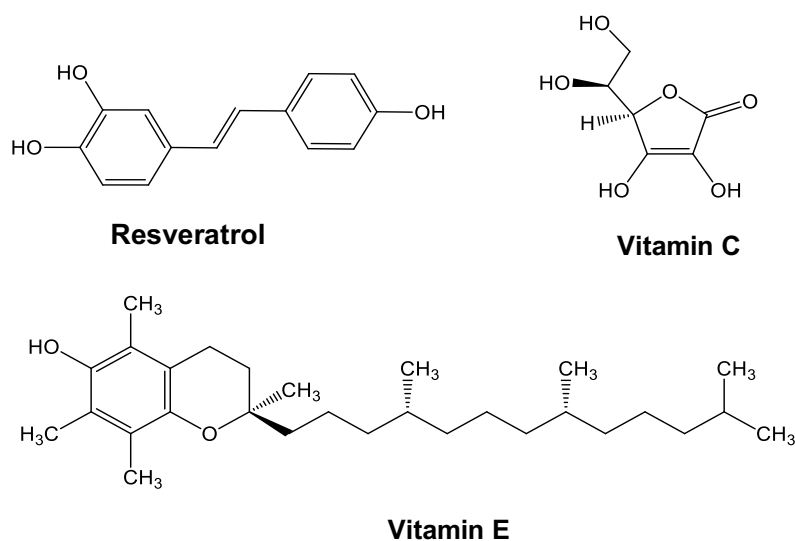
Results are expressed as mean ± SD (n = 17). \*P-value < 0,05, \*\*P-value < 0,01

**Table 5:** Effects of empty liposomes, resveratrol, vitamin C and vitamin E on *in vitro* sperm cells immotility after 48 hours in incubator at 37°C and 5% CO<sub>2</sub>.

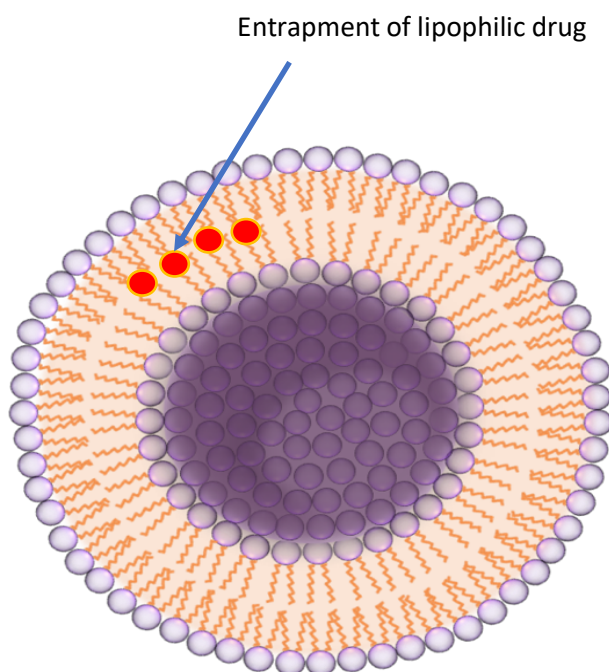
	<b>Media /drug concentration</b>	<b>Immotile %</b>
Control	Media only	90.7 ± 17.5
Empty liposomes	1 µg/mL (C)	92.1 ± 16.3
	10 µg/mL (B)	90.6 ± 21.9
	100 µg/mL (A)	94.8 ± 23.0 *
Resveratrol	1 µg/mL (C)	92.8 ± 19.4
	10 µg/mL (B)	93.7 ± 23.2
	100 µg/mL (A)	96.7 ± 19.0 **
Vitamin C	1 µg/mL (C)	93.5 ± 17.0 *
	10 µg/mL (B)	92.8 ± 17.0
	100 µg/mL (A)	99.6 ± 16.2 **
Vitamin E	1 µg/mL (C)	94.2 ± 18.8 *
	10 µg/mL (B)	91.9 ± 16.9
	100 µg/mL (A)	94.8 ± 10,3 *

Results are expressed as mean ± SD (n = 17). \*P-value < 0,05, \*\*P-value < 0,01

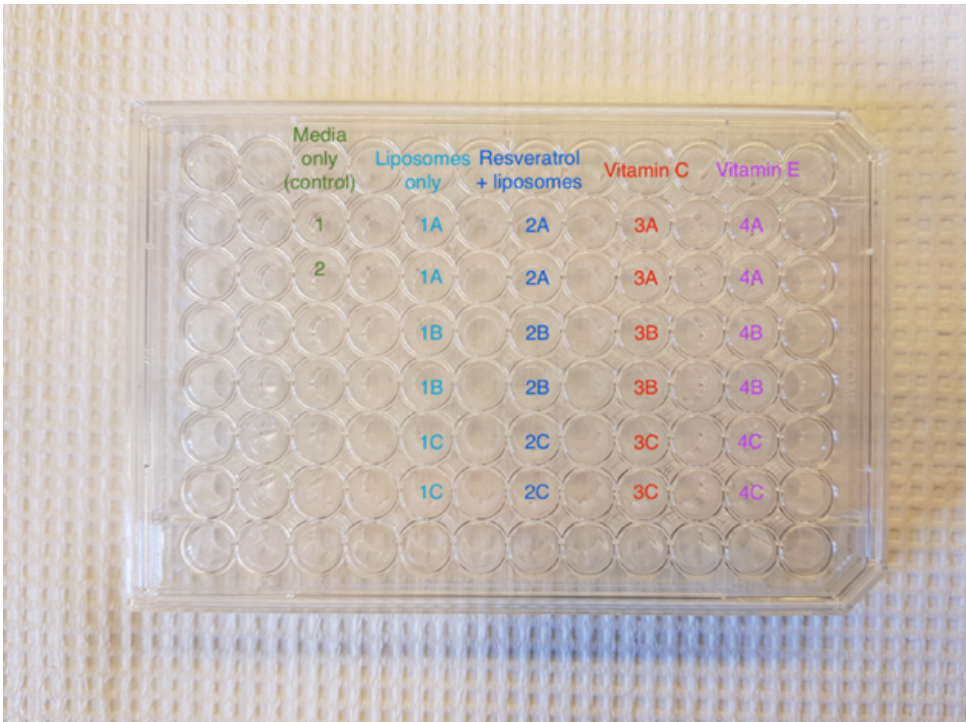
## 9. Figures



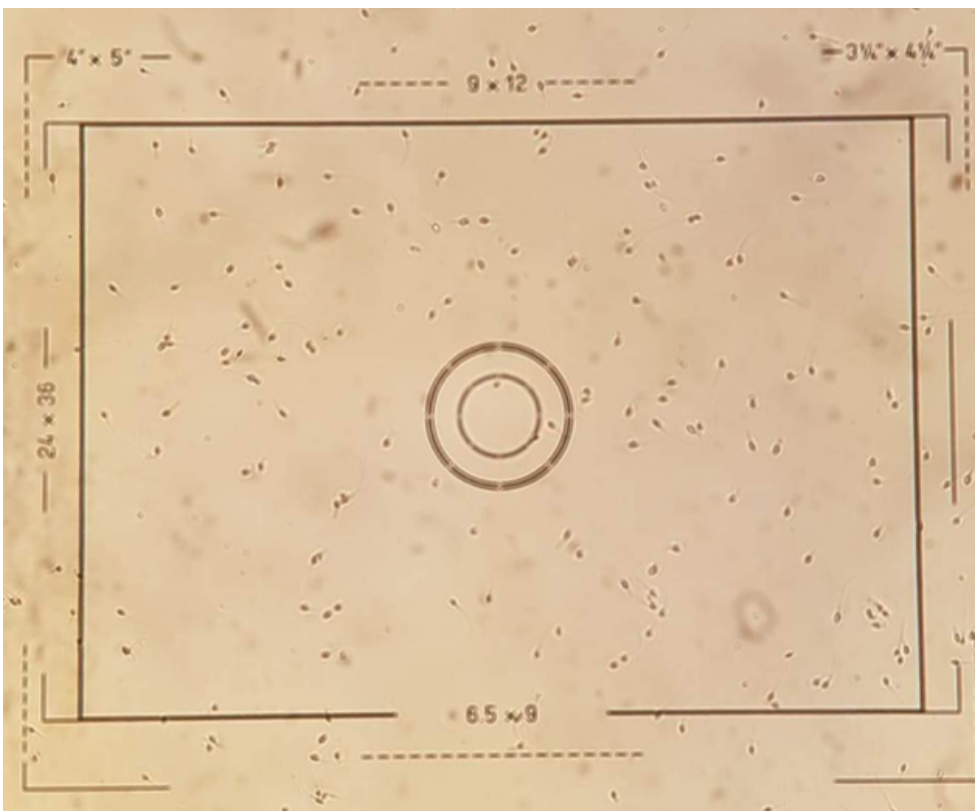
**Figure 1:** Chemical structures of antioxidants (resveratrol, vitamin C and vitamin E) used in the experiment (28).



**Figure 2:** The schematic representation liposomes, and entrapment of lipophilic drug. The unstable and less soluble substance like resveratrol in the liposomal formulation can show its optimal pharmacological effects. Therefore resveratrol liposomal formulation was chosen in the current project. (This figure is taken from PhD Thesis of May Wenche Jøraholmen with her permission and small modifications are made.)



**Figure 3:** Sample application into the plate. Photo: Stefan Thomassen



**Figure 4:** Observation field seen in the microscope where sperm cell viability and motility were observed. Photo: Stefan Thomassen

## 10. Grade evaluations

Reference: Keskes-Ammar L, Feki-Chakroun N, Rebai T, Sahnoun Z, Ghozzi H, Hammami S, et al. Sperm oxidative stress and the effect of an oral vitamin E and selenium supplement on semen quality in infertile men. Archives of Andrology. 2003;49(2):83-94.			Grade
			Level
			Recommendation
			2b
			B
Aim	Materials and methods	Results	Discussion and comments
<p>To evaluate the effects of vitamin E and selenium supplementation on lipid peroxidation and on sperm parameters.</p> <p><b>Conclusion</b></p> <p>Oral treatment of unselected infertile men by vitamin E-selenium over period of 3 months significantly improved sperm cell motility and caused significant decrease in the lipid peroxidation (MDA concentration) in sperm cells.</p>	<p>Study design: RCT study.</p> <p>Study population: Targeted to 78 infertile men who came to clinic for the fertility treatment. 54 of them were recruited for open randomized control test over a period of 10 month. Patients were randomly allocated to one of the two groups: Oral dose vitamin E and selenium (28 men) Oral dose vitamin B (26 men) Composite vitamin B was used as control instead of placebo and was chosen on the basis of its usual prescription in some cases of infertility. Three months of treatment was chosen to allow for a full spermatogenesis cycle. At the end of the experiment, only 20 patients completed trial with vitamin E-selenium (N = 12) and vitamin B (N = 8).</p> <p>Recruitment criteria: Not clear</p> <p>Laboratory analysis: Semen parameters such as, volume, concentration, motility, viability and morphology were determined on freshly ejaculated semen obtained after minimum 3 days of sexual abstinence. Oxidative stress were evaluated by by measuring lipid peroxidation markers, malondialdehyde (MDA) levels and body antioxidative status were preformed by measurement of vitamin E and cholesterol levels.</p> <p>Statistical analysis:</p> <ul style="list-style-type: none"> <li>• Chi-square test and the student t-test to compare statistical significance.</li> <li>• Regression equations and correlation factors were calculated using the least-squares method.</li> <li>• P-values &lt;0.05 were considered as significant.</li> </ul>	<p>There were no statistically significant between all parameters of the two initially treated groups of patients (vitamin E-selenium and vitamin B) at the point of entry in the trial,</p> <p>MDA-concentration:</p> <ul style="list-style-type: none"> <li>• In the initial treated group (N: 54), MDA-concentrations in sperm pellet suspensions were much less than that in seminal plasma. There was wide variability in semen MDA-concentration.</li> <li>• A positive correlation between MDA-concentrations in seminal plasma and in sperm pellet was found.</li> <li>• Motility and viability were highly and inversely correlated with semen MDA-level in the initial treated group of patients (N: 54).</li> <li>• MDA-levels were significantly higher in patients having semen anomalies compared with normospermic patients.</li> <li>• No correlation was seen between MDA-concentration and vitamin E/cholesterol ratio.</li> </ul> <p>Treatment of patients with vitamin E-selenium produced a significant increase in serum vitamin E levels and a significant decrease in semen MDA-concentrations. No significant effect on semen MDA-levels and serum vitamin E concentrations were seen after vitamin B supplementation.</p> <p>Treatment with vitamin E-selenium induced improvement of sperm cell motility and concentration. There was an inverse significant correlation between semen MDA-concentration (in seminal plasma and in sperm pellet) and sperm motility and viability in patients treated with vitamin E-selenium.</p>	<p><b>Was the objective of the study described clearly?</b> Yes.</p> <p><b>Were the samples allocated to the defined groups with randomized procedure?</b> Yes, with random numbers which was withheld from researchers and patients.</p> <p><b>Were all participants accounted for at the end of the study?</b> No, 64% patients dropped out.</p> <p><b>Were the participants/study-person blinded in terms of group identification?</b> Yes.</p> <p><b>Were the groups equal at the start of the study?</b> Yes, from what they measured.</p> <p><b>Were the groups treated equally?</b> Yes, the oral drug administration in both groups.</p> <p><b>What are the results?</b> Vitamin E-selenium treatment significantly lowered MDA-levels and improved sperm motility</p> <p><b>Can the results be transferred/used in clinical practice?</b> Based on the having one study, we cannot state this clearly. However, to give vitamin E-selenium is a simple and practically possible – as well as measurement of MDA-levels.</p> <p><b>Were all the outcome validations assessed?</b> Yes.</p> <p><b>Are the benefits worth the harm/costs?</b> Unclear from the evidence as is stands today. Too little evidence.</p> <p><b>Strengths</b></p> <ul style="list-style-type: none"> <li>• Only infertile men were included</li> <li>• The treatments in the trial were randomized, both to patients and researchers and sufficiently described.</li> <li>• Long enough follow-up time.</li> <li>• Refers to other studies, showing similar results.</li> <li>• The groups were comparable in terms of semen parameters and other parameters measured.</li> <li>• Exposures and and outcomes was measures the same way.</li> </ul> <p><b>Limitations</b></p> <ul style="list-style-type: none"> <li>• The trial was open.</li> <li>• Diagnosed infertility was not a criteria for attending the study.</li> <li>• Few sample size.</li> <li>• Many patients dropped out of treatment.</li> <li>• Diagnosed infertility was not a selection criteria. Appears as all patients admitted for semen analysis in the particular clinic were recruited, inclusion/exclusion criteria are poorly described/not existing.</li> <li>• Drug compliance not assessed.</li> <li>• The person who evaluated the results (endpoints) was not blinded to the group identification.</li> </ul> <p><b>Does the results have biological explanations?</b> Yes</p> <p><b>Does the author refer to other literature that supports or contradicts the results?</b> Yes</p> <p>Low quality randomized trial.</p>
<b>Country</b>	Tunisia		
<b>Year of data sampling</b>	Not reported		



<b>Reference:</b> Wirleitner B, Vanderzwalmen P, Stecher A, Spitzer D, Schuff M, Schwerda D, et al. Dietary supplementation of antioxidants improves semen quality of IVF patients in terms of motility, sperm count, and nuclear vacuolization. International Journal of Vitamin and Nutrition Research. 2012;82(6):391-8.			<b>Grade</b>
			<b>Level</b>
			<b>2b</b>
			<b>Recommendation</b>
			<b>B</b>
<b>Aim</b>	<b>Materials and methods</b>	<b>Results</b>	<b>Discussion and comments</b>
Investigate the influence of oral antioxidative supplementation on sperm quality of <i>in vitro</i> fertilization (IVF) patients.	<p>Study design: Non-randomized experimental study (OAT and non-OAT were assigned antioxidative supplement treatment, semen quality quality before and after treatment).</p> <p>Study population: The study population were IVF 147 patients. These patients were divided into two groups: OAT group (39 men) and Non-OAT group (108 men) based on the semen quality according to WHO criteria.</p> <p>Recruitment criteria: They consented to an antioxidative supplementation. Had no or were not planning to have testicular sperm extraction (TESE). Did not exhibit indication of azoospermia. Did not have genetic reasons for an oligoasthenoteratozoospermia (OAT) syndrome. Had no chemotherapy or exposure to other noxious agents in the past and did not have any other medication with known influence on sperm quality.</p> <p>Treatment: After the first semen analysis all patients were treated with oral Fertilocit, an antioxidant supplement containing vitamin C, vitamin E, folic acid, zinc, selenium, N-acetyl-L-cysteine, L-carnitine, citrulline, glutathione red., lycopene and Coenzyme Q10 for 2 months.</p> <p>Laboratory analysis: Semen parameters such as, volume, concentration, motility, viability and morphology were determined on freshly ejaculated semen according to WHO guidelines. For motile sperm organelle morphology examination (MSOME) spermatozoa were classified as grade I: normal shape, size, and no vacuoles or only small vacuole(s) (&lt; 4 % of the sperm's head), Grade II: spermatozoa with normal shape and size, but with one or more vacuoles (&gt; 4 % of the sperm's head) and Grade III: abnormal shape and/or size with or without vacuoles, after purification of sperm cells by gradient method. Semen parameter data before Fertilocit treatment and after treatment were compared</p> <p>Statistical analysis: Statistical significance differences between groups (sample volume, total motile sperm count and MSOME criteria) non-parametric Wilcoxon test was applied.</p>	<p>OAT group (39 men) revealed lower sperm motility, sperm concentration, smaller ejaculation volume and lower percentage of MSOME Grade I spermatozoa before treatment comparing to non-OAT group (108 men).</p> <p>The second semen analysis was performed 2 to 12 months after an antioxidative supplementation. There was significantly more progressive motile sperm in the OAT-group. The total sperm number of the OAT-group was significantly increased, but not in the non-OAT group. OAT-group had significantly increased non-linear motility and the decreased percentage of immotile sperm. In the non-OAT group there was no significant increase of progressive motility or in total motility. The percentage of Grade I spermatozoa according to MSOME criteria was significantly higher after antioxidative supplementation in the OAT-group.</p> <p>In Summary: After the antioxidative therapy there was significant improvement in semen parameter. The percentage of class I spermatozoa according to MSOME criteria was significantly higher after antioxidative supplementation treatment.</p>	<p><b>Are the groups recruited from the same population/section of the population?</b> Yes.</p> <p><b>Were the exposed individual representative from the defined population/section of the population?</b> Yes, they were typical IVF-attenders.</p> <p><b>Were the groups comparable in terms of background factors?</b> Yes.</p> <p><b>Was the study prospective?</b> Yes, semen samples were collected and analysed after antioxidants treatment.</p> <p><b>Was expositions and outcome measured the same way and reliable?</b> Outcome was measured by semen analysis of all patients. The time of antioxidant consumption seems however to vary greatly and intake of drug/compliance was not controlled by blood tests e.g. So maybe this is not reliable.</p> <p><b>Sufficient number of persons followed up?</b> Yes, 147 men were included.</p> <p><b>Was the follow-up time long enough to detect positive/negative outcomes?</b> Yes.</p> <p><b>Are important confounders considered in the study design?</b> Yes.</p> <p><b>Is the person who evaluated the results (endpoints) blinded group identification?</b> No.</p> <p><b>Strengths</b></p> <ul style="list-style-type: none"> <li>• Clear recruitment criteria and group separation</li> <li>• Relatively a good number of patient included.</li> <li>• Defined inclusion criteria.</li> </ul> <p><b>Limitations</b></p> <ul style="list-style-type: none"> <li>• Great variation of the length of supplement consumption (2-12 months) and the time of second semen analysis. Standardized duration lacking.</li> <li>• Antioxidant therapy was composed of many different substances, which one had effects?</li> <li>• Higher number of patients are in non-OAT group vs. OAT group.</li> </ul> <p><b>Does the results have biological explanations?</b> Yes</p> <p><b>Does the author refer to other literature that supports or contradicts the results?</b> Yes</p>
<b>Conclusion</b>			
Antioxidants dietary supplements improved in semen quality, especially for oligoasthenoteratozoospermia (OAT) patients. An antioxidative and micronutrient supplementation has remarkable benefit for IVF patients having restricted sperm parameters, in particular.			
<b>Country</b>			
Austria.			
<b>Year of data sampling</b>			
January 2008 – July 2011.			

<b>Reference:</b> Mendiola J, Torres-Cantero AM, Vioque J, Moreno-Grau JM, Ten J, Roca M, et al. A low intake of antioxidant nutrients is associated with poor semen quality in patients attending fertility clinics. <i>Fertility and Sterility</i> . 2010;93(4):1128-33.			<b>Grade</b>	
			<b>Level</b>	<b>4</b>
			<b>Recommendation</b>	<b>C</b>
<b>Aim</b>	<b>Materials and methods</b>	<b>Results</b>	<b>Discussion and comments</b>	
To compare specific nutrient intake between normospermic and oligoasthenoteratospermic patients attending infertility clinics in two Mediterranean provinces of Spain.	Study design: Case-control study  Study population: A total of 61 men were participated in the study from three infertility centers were classified into two groups on the basis of seminal quality as: Case (n = 30): men with severe or moderate oligozoospermia and severe teratozoospermia and Control (n = 31): normospermic patients.	<ul style="list-style-type: none"> <li>Distribution of age, BMI and hormone levels were similar between case and control subjects.</li> <li>Control subjects presented significantly higher intakes of carbohydrates, fiber, folate, vitamin C and lycopene and lower intakes of proteins and total fat in logistic regression. Control subjects had also been exposed less frequent to occupational toxics than case subjects.</li> </ul>	<b>Were the case-control groups recruited from comparable populations?</b> Yes. <b>Are the groups comparable in terms of important backgrounds factors?</b> Yes. <b>Are the casus groups condition sufficient described? Is the diagnose validated?</b> Yes, the cases were diagnosed with oligozoosperma and teratozoospermia before participation. <b>Is the control groups healthy?</b> Yes, normozospermic patients. <b>Are important confounders considered in the design/analysis?</b> Yes. Age, tobacco smoking, occupational factors e.g. were adjusted for. <b>Is the exposure for danger/harm/ intervention measured and graded equally in the groups?</b> Yes. Semen analysis, interviews, questionnaires. <b>Was the one who measured the exposure blinded in terms of casus/control?</b> Yes. <b>Was the response rate sufficient in both groups?</b> Yes. <b>Strengths</b> <ul style="list-style-type: none"> <li>Defined cases and controls.</li> <li>Comparable groups.</li> <li>Equal examination.</li> <li>Blinded researcher.</li> <li>Significantly differences in terms of diet and occupational exposure between cases and controls.</li> </ul> <b>Weakness</b> <ul style="list-style-type: none"> <li>Small sample number</li> <li>Other confounders could be included; medications? Alcohol? Exercise?</li> </ul>	
<b>Conclusion</b>	Laboratory analysis: Semen parameters such as, volume, concentration, motility, viability and morphology were determined on freshly ejaculated semen obtained after minimum 3 days of sexual abstinence following Krugers strict criteria. Subjects provided at least two semen samples.			
A low intake of antioxidant nutrients was associated with a poor semen quality in a Mediterranean population of Spanish men.				
<b>Country</b>	Self reported questionnaires on dietary intake: Study participants were interviewed by the same trained fieldworker, who was blinded regarding case-control patient status. Structured questionnaires to collect information			
Spain	Questionnaire to collect information regarding occupational history. A semi-quantitative food frequency questionnaire of 93 food items was used to assess usual dietary intake.			
<b>Year of data sampling</b>	Statistics: SPSS was used. Results presented as percentage and mean with standard deviation (SD). Student T-test was used for means comparison of continuous variables, X <sup>2</sup> -tests were used for categoric Variables. Statistical significance was set less than 0.05 p values and all test were two-tailed.			
2005-2007				

<b>Reference:</b> Eskenazi B, Kidd S, Marks A, Slotter E, Block G, Wyrobek A. Antioxidant intake is associated with semen quality in healthy men. Human Reproduction. 2005;20(4):1006-12.		<b>Grade</b>																																																																																																																																																																															
		<b>Level</b> 4																																																																																																																																																																															
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<b>Aim</b>	<b>Materials and methods</b>	<b>Results</b>																																																																																																																																																																															
To determine whether dietary and supplement intake of specific micronutrients (zinc and folate) and antioxidants (vitamin C, E and β-carotene) are associated with semen quality.	Study design: Observational study (cross-sectional)  Study population: The study population consisted of 97 healthy male volunteers. Male volunteers were recruited from advertisements, e-mail list servers, posters and newsletters. Fifteen men from each decade from 20 to 60 years of age were enrolled, and additionally 25 men from 60 to 80 years of age were screened over telephone.	After controlling for covariates, a high intake of antioxidants was associated with better semen quality, but in almost all cases there was no clear dose relationship in that moderate intake groups had the poorest semen quality.  For example, positive associations were observed between vitamin C intake and sperm number as reflected in the higher mean count, concentration and TPMS; between vitamin E intake and progressive motility and TPMD; and between β-carotene intake and sperm concentration and progressive motility. Folate and zinc intake were not associated with improved semen quality.																																																																																																																																																																															
<b>Conclusion</b>	Exclusion criteria: Cigarette smokers, the last 6 months. Current fertility or reproductive problems Had a vasectomy History of prostate cancer or undescended testicles Received chemotherapy or radiation for cancer Previous semen analysis with zero count Having a fever >101 °F in the prior 3 months Twenty men were excluded based on the above criteria.																																																																																																																																																																																
In a convenience sample of healthy non-smoking men from a non-clinical setting, higher antioxidant intake was associated with higher sperm cell number and motility. Antioxidant intake may attenuate the impact of age on sperm motility.	Laboratory analysis: Semen parameters such as semen volume, sperm cell concentration, total sperm count, motility, progressive motility and total progressively motile sperm count (TPMS) were determined on freshly ejaculated semen obtained after minimum 3 days of sexual abstinence following WHO strict criteria. Subjects provided at least two semen samples.																																																																																																																																																																																
<b>Country</b>	Self reported dietary assessment: Men were mailed a questionnaire and semen collection container with instructions. Questionnaire asked about medical and reproductive history, socio-demographic characteristics, occupation, lifestyle, and habits and characteristics. Daily intake of nutrients were estimated by self-administered modified block food frequency questionnaire (FFQ).																																																																																																																																																																																
<b>Year of data sampling</b>	Statistical analysis: Multivariate regression analysis with covariate selection based on the literature																																																																																																																																																																																
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1997-1998																																																																																																																																																																																	
		<p><b>Table III. Adjusted means (95% CI) for semen quality parameters by level of micronutrient or antioxidant intake: AGES Study, California, 1997-1998</b></p> <table border="1"> <thead> <tr> <th></th> <th>Folate</th> <th>Zinc</th> <th>Vitamin C</th> <th>Vitamin E</th> <th>β-Carotene</th> <th>Antioxidant composite</th> </tr> </thead> <tbody> <tr> <td><b>Semen volume (ml)<sup>a</sup></b></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> </tr> <tr> <td>Low</td> <td>2.8 (2.3-3.3)</td> <td>2.5 (2.0-3.1)</td> <td>2.9 (2.4-3.4)</td> <td>2.7 (2.2-3.2)</td> <td>2.5 (2.0-3.0)</td> <td>2.8 (2.3-3.3)</td> </tr> <tr> <td>Moderate</td> <td>2.9 (2.5-3.3)</td> <td>3.0 (2.6-3.5)</td> <td>2.6 (2.2-3.1)</td> <td>2.8 (2.4-3.2)</td> <td>3.0 (2.6-3.4)</td> <td>2.7 (2.4-3.1)</td> </tr> <tr> <td>High</td> <td>2.5 (1.7-2.9)</td> <td>2.2 (1.7-2.9)<sup>b</sup></td> <td>2.5 (2.0-3.1)</td> <td>2.5 (2.0-3.1)</td> <td>2.5 (2.0-3.0)</td> <td>2.4 (1.8-3.0)</td> </tr> <tr> <td><b>Sperm concentration (10<sup>6</sup>/ml)<sup>a</sup></b></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> </tr> <tr> <td>Low</td> <td>136 (88-194)</td> <td>113 (69-169)</td> <td>133 (87-189)</td> <td>107 (66-158)</td> <td>102 (61-152)</td> <td>107 (64-160)</td> </tr> <tr> <td>Moderate</td> <td>116 (81-158)</td> <td>120 (85-162)</td> <td>95 (65-131)</td> <td>116 (82-156)</td> <td>108 (76-146)</td> <td>112 (81-148)</td> </tr> <tr> <td>High</td> <td>120 (75-175)</td> <td>137 (87-197)</td> <td>171 (117-225)<sup>b</sup></td> <td>160 (106-226)</td> <td>177 (123-242)<sup>b,c</sup></td> <td>191 (126-270)<sup>b,c</sup></td> </tr> <tr> <td><b>Total sperm count (10<sup>7</sup>)<sup>a</sup></b></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> </tr> <tr> <td>Low</td> <td>301 (149-506)</td> <td>242 (120-405)</td> <td>337 (197-514)</td> <td>222 (104-384)</td> <td>240 (119-402)</td> <td>288 (114-407)</td> </tr> <tr> <td>Moderate</td> <td>205 (103-338)</td> <td>282 (174-416)</td> <td>199 (113-310)</td> <td>224 (118-364)</td> <td>267 (164-395)</td> <td>280 (167-338)</td> </tr> <tr> <td>High</td> <td>231 (110-398)</td> <td>289 (153-470)</td> <td>385 (231-578)<sup>b</sup></td> <td>275 (129-475)</td> <td>345 (196-535)</td> <td>330 (159-563)</td> </tr> <tr> <td><b>Sperm motility (%)<sup>a</sup></b></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> </tr> <tr> <td>Low</td> <td>30 (20-39)</td> <td>34 (26-42)</td> <td>32 (24-40)</td> <td>32 (23-40)</td> <td>31 (23-40)</td> <td>30 (22-39)</td> </tr> <tr> <td>Moderate</td> <td>33 (25-40)</td> <td>34 (27-40)</td> <td>32 (26-38)</td> <td>27 (19-34)</td> <td>33 (27-39)</td> <td>28 (21-35)</td> </tr> <tr> <td>High</td> <td>27 (19-35)</td> <td>32 (24-40)</td> <td>36 (29-44)</td> <td>35 (26-44)</td> <td>37 (29-45)</td> <td>35 (26-47)<sup>b</sup></td> </tr> <tr> <td><b>Progressive sperm motility (%)<sup>a</sup></b></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> </tr> <tr> <td>Low</td> <td>14 (8-21)</td> <td>15 (9-22)</td> <td>16 (10-22)</td> <td>18 (12-24)</td> <td>14 (9-20)</td> <td>13 (8-20)</td> </tr> <tr> <td>Moderate</td> <td>14 (9-21)</td> <td>16 (11-21)</td> <td>14 (10-20)</td> <td>14 (10-18)</td> <td>13 (9-18)</td> <td>11 (7-15)</td> </tr> <tr> <td>High</td> <td>11 (6-17)</td> <td>15 (9-23)</td> <td>17 (11-25)</td> <td>17 (11-25)</td> <td>22 (15-30)<sup>b,c</sup></td> <td>22 (14-32)<sup>b,c</sup></td> </tr> <tr> <td><b>Total progressively motile sperm (TPMS) (10<sup>6</sup>/ml)<sup>a</sup></b></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> </tr> <tr> <td>Low</td> <td>51 (21-94)</td> <td>53 (25-93)</td> <td>50 (24-87)</td> <td>53 (26-89)</td> <td>43 (17-80)</td> <td>49 (23-84)</td> </tr> <tr> <td>Moderate</td> <td>29 (11-56)</td> <td>38 (19-65)</td> <td>31 (13-56)</td> <td>51 (14-86)</td> <td>41 (21-68)</td> <td>28 (11-51)</td> </tr> <tr> <td>High</td> <td>32 (10-66)</td> <td>49 (20-90)</td> <td>68 (35-110)<sup>b</sup></td> <td>74 (38-122)<sup>b</sup></td> <td>43 (30-108)</td> <td>85 (44-139)<sup>b</sup></td> </tr> </tbody> </table> <p><sup>a</sup>All volume models were adjusted for age, abstinence and smoking. <sup>b</sup>All concentration models were adjusted for age and abstinence. Concentration was square-root transformed for analysis and back-transformed for presentation in the table. <sup>c</sup>All models of count were adjusted for age, abstinence, smoking and season. In addition, models for vitamin C and β-carotene were adjusted for body mass index (BMI), and models for folate, vitamin E and antioxidant composite were adjusted for BMI and history of urinary tract infection (UTI). Count was square-root transformed for analysis and back-transformed for presentation in the table. <sup>d</sup>Models for motility were adjusted for age, abstinence, time to sample analysis and season. In addition, models for folate, vitamin E and antioxidant composite were adjusted for UTI. <sup>e</sup>Models for progressive sperm motility were adjusted for age, abstinence and time to sample analysis. In addition, models for zinc, vitamin C and β-carotene were adjusted for smoking, and models for folate and antioxidant composite were adjusted for smoking and UTI. Progressive motility was square-root transformed for analysis and back-transformed for presentation in the table. <sup>f</sup>Models for TPMS were adjusted for age, abstinence and time to sample analysis. In addition, the model for zinc was adjusted for smoking and season; models for vitamin C, vitamin E and β-carotene were adjusted for smoking, season and BMI; the model antioxidant composite was adjusted for smoking, BMI and UTI, and the model for folate was adjusted for smoking, season, BMI and UTI. TPMS was square-root transformed for analysis and back-transformed for presentation in the table. <sup>g</sup>ANCOVA P &lt; 0.10. <sup>h</sup>ANCOVA P &lt; 0.05. <sup>i</sup>ANCOVA P &lt; 0.01. <sup>j</sup>Adjusted test for trend P &lt; 0.10. <sup>k</sup>Adjusted test for trend P &lt; 0.05.</p>		Folate	Zinc	Vitamin C	Vitamin E	β-Carotene	Antioxidant composite	<b>Semen volume (ml)<sup>a</sup></b>							Low	2.8 (2.3-3.3)	2.5 (2.0-3.1)	2.9 (2.4-3.4)	2.7 (2.2-3.2)	2.5 (2.0-3.0)	2.8 (2.3-3.3)	Moderate	2.9 (2.5-3.3)	3.0 (2.6-3.5)	2.6 (2.2-3.1)	2.8 (2.4-3.2)	3.0 (2.6-3.4)	2.7 (2.4-3.1)	High	2.5 (1.7-2.9)	2.2 (1.7-2.9) <sup>b</sup>	2.5 (2.0-3.1)	2.5 (2.0-3.1)	2.5 (2.0-3.0)	2.4 (1.8-3.0)	<b>Sperm concentration (10<sup>6</sup>/ml)<sup>a</sup></b>							Low	136 (88-194)	113 (69-169)	133 (87-189)	107 (66-158)	102 (61-152)	107 (64-160)	Moderate	116 (81-158)	120 (85-162)	95 (65-131)	116 (82-156)	108 (76-146)	112 (81-148)	High	120 (75-175)	137 (87-197)	171 (117-225) <sup>b</sup>	160 (106-226)	177 (123-242) <sup>b,c</sup>	191 (126-270) <sup>b,c</sup>	<b>Total sperm count (10<sup>7</sup>)<sup>a</sup></b>							Low	301 (149-506)	242 (120-405)	337 (197-514)	222 (104-384)	240 (119-402)	288 (114-407)	Moderate	205 (103-338)	282 (174-416)	199 (113-310)	224 (118-364)	267 (164-395)	280 (167-338)	High	231 (110-398)	289 (153-470)	385 (231-578) <sup>b</sup>	275 (129-475)	345 (196-535)	330 (159-563)	<b>Sperm motility (%)<sup>a</sup></b>							Low	30 (20-39)	34 (26-42)	32 (24-40)	32 (23-40)	31 (23-40)	30 (22-39)	Moderate	33 (25-40)	34 (27-40)	32 (26-38)	27 (19-34)	33 (27-39)	28 (21-35)	High	27 (19-35)	32 (24-40)	36 (29-44)	35 (26-44)	37 (29-45)	35 (26-47) <sup>b</sup>	<b>Progressive sperm motility (%)<sup>a</sup></b>							Low	14 (8-21)	15 (9-22)	16 (10-22)	18 (12-24)	14 (9-20)	13 (8-20)	Moderate	14 (9-21)	16 (11-21)	14 (10-20)	14 (10-18)	13 (9-18)	11 (7-15)	High	11 (6-17)	15 (9-23)	17 (11-25)	17 (11-25)	22 (15-30) <sup>b,c</sup>	22 (14-32) <sup>b,c</sup>	<b>Total progressively motile sperm (TPMS) (10<sup>6</sup>/ml)<sup>a</sup></b>							Low	51 (21-94)	53 (25-93)	50 (24-87)	53 (26-89)	43 (17-80)	49 (23-84)	Moderate	29 (11-56)	38 (19-65)	31 (13-56)	51 (14-86)	41 (21-68)	28 (11-51)	High	32 (10-66)	49 (20-90)	68 (35-110) <sup>b</sup>	74 (38-122) <sup>b</sup>	43 (30-108)	85 (44-139) <sup>b</sup>
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		<p><b>Discussion and comments</b></p> <p><b>Is the study population clearly defined?</b> Yes. <b>Is the study representative for the general population?</b> Yes. <b>Is it described how non-responders differ in characteristics from responders?</b> No, this is not studied. This can be a selection bias issue. <b>Is the answer % high enough?</b> Not given. <b>Was the data sampling standardized?</b> Yes. <b>Are objective criteria used for evaluating the outcome measures?</b> Yes, semen quality was evaluated in all patients. <b>Is the data analysis relevant?</b> Yes, they used adjusted regression analysis.</p> <p><b>Strengths</b></p> <ul style="list-style-type: none"> <li>• Many participants</li> <li>• Wide distribution of age</li> <li>• General population represented, clear exclusion criteria</li> <li>• Published in high-impact journal</li> </ul> <p><b>Weaknesses</b></p> <ul style="list-style-type: none"> <li>• Unclear whether analysis of semen was blinded concerning results from questionnaire and interview.</li> <li>• Simplified investigation of dietary and supplement habits.</li> <li>• Many potential confounders; physical activity, smoking the past, somatic diseases etc.</li> </ul>																																																																																																																																																																															

Reference: Garcez ME, ds Santos Branco C, Lara LV, Pasqualotto FF, Salvador M. Effects of resveratrol supplementation on cryopreservation medium of human semen Fertility and Sterility. 2010;94(6):2118-21			Grade
			Level 4
			Recommendation C
Aim	Materials and methods	Results	Discussion /comments
<p>To analyse oxidative stress markers and seminal standard parameters after using resveratrol (0.1, 1.0 and 10.0 mM), an important antioxidant, in the cryopreservation of human semen.</p>	<p>Study design: Observational study (case-control <i>in vitro</i> perspective study)</p> <p>Study population: Subjects: The study group included infertile (20 men) and fertile (10 men) subjects. Infertile men were diagnosed after at least two successive semen analysis with a partner having no problem. Fertile men were proven fertility either pregnant partner of delivered a child within the previous 2 years.</p> <p>Exclusion criteria: Presence of azoospermia, severe oligozoospermia or leukocytospermia, use of medications/antioxidants, exposure to chemotherapy or radiation or varicoceleotomy.</p> <p>Laboratory analysis: Semen parameters such as semen volume, sperm cell concentration, total sperm count, motility, progressive motility were determined on freshly ejaculated semen obtained after minimum 3 days of sexual abstinence following WHO criteria and assessment of morphology by using Krugers criteria.</p> <p>Oxidative stress markers Levels of thiobarbituric acid-reactive species (TBARS), superoxide dismutase (SOD) and catalase (CAT) were assessed,</p> <p>Cryopreservation with resveratrol Semen samples were separated into four aliquots; without resveratrol and with 0.1, 1.0 and 10.0mM of resveratrol. Thereafter cryopreserved with standard procedure.. Post-thawing analysis was after performed 72 hours. Samples were removed from liquid nitrogen, thawed in room temperature for 5 minutes and incubated at 37 °C for 5-10 minutes. Thereafter washed with medium and then evaluated for oxidative and seminal parameters.</p> <p>Statistical analysis: SPSS, statistical significance <math>P &gt; 0,05</math>. Mann-Whitney-U.test, Wilcoxon's test, Spearmans test.</p>	<p>Infertile men presented with lower sperm concentration, motility and morphology when compared with fertile men. There was also significant higher average of sperm with cryoplasmic droplets among infertile when compared with the control group. Infertile men had lower SOD and CAT activities and higher levels of lipid damages in prefreezing samples compared to fertile men. Cryopreservation induced decrease in SOD activity and increase in CAT activity and TBARS levels in infertile men.</p> <p>Fertile men had no alteration in SOD activity after cryopreservation, however increase in CAT activity in post-thawing samples. Increase in lipid damages after cryopreservation.</p> <p>Addition of resveratrol (0.1, 1.0 and 10.0 mM) was able to prevent lipid damages induced by cryopreservation in both fertile and infertile men. The effect was not dose-dependent.</p> <p>In fertile men, the addition of resveratrol did not change SOD and CAT activities. In infertile, an increase in SOD activity was observed.</p> <p>The cryopreservation process was not able to change sperm concentration or motility. A decrease in sperm motility was observed in both fertile and infertile men.</p>	<p><b>Were the case-control groups recruited from comparable populations?</b> Yes, samples were collected from patients in one clinic. Fertile and infertile male were recruited.</p> <p><b>Are the groups comparable in terms of important background factors?</b> Yes.</p> <p><b>Are the casus groups condition sufficiently described? Is the diagnose validated?</b> Yes, they are sufficiently described. Infertile men were diagnosed after at least two semen analysis.</p> <p><b>Is the control groups healthy?</b> Yes.</p> <p><b>Are important confounders considered in the design/analysis?</b> Yes, defined exclusion/inclusion criteria helps reducing the risk of confounders.</p> <p><b>Is the exposure for danger/harm/ intervention measured and graded equally in the groups?</b> Yes, the same laboratory techniques were used.</p> <p><b>Was the one who measured the exposure blinded in terms of casus/control?</b> No.</p> <p><b>Was the responsrate sufficient in both groups?</b> Yes.</p> <p><b>Strengths</b></p> <ul style="list-style-type: none"> <li>• Defined inclusion/exclusion criteria</li> <li>• Standardized methods used</li> <li>• Long enough follow-up time to detect outcomes.</li> </ul> <p><b>Limitations</b></p> <ul style="list-style-type: none"> <li>• Small sample</li> <li>• Unequal distribution of patients in the two groups</li> </ul>
<b>Conclusion</b>			
Resveratrol avoids oxidative damages induced by the cryopreservation of human semen, but it not able to restore the decrease in sperm motility.			
<b>Country</b>			
Brazil			
<b>Year of data sampling</b>			
2010			

## Appendix 1: Consent form

### **Samtykkeskjema for bruk av sædprøve til andre formål enn pasientbehandling**

**Forskningsstudien:** Oksidativt stress og sæd kvalitet

**Avdeling:** Kvinnehelse og perinatologi forskjæring gruppe i Universitetet i Tromsø

**Ansvarlig person/Prosjektleder:** Professor Purusotam Basnet

#### **Formål med bruk av prøven:**

Denne forskningsstudien blir gjennomført av Med. Stud/PhD under veiledning av Prof. Purusotam Basnet for å undersøke sammenhenger mellom sædkvalitet og oksidativt stress.

Bevegelsen av sædceller vil være studier under ulike oksidative stress og antioksidanter, og endringer i mitokondriet vil bli observert.

#### **Konfidensialitet**

Alle prøver og data vil bli anonymisert nor vi mottar prøven. Det er derfor ikke i ettertid ikke mulig å spore giver eller knytte resultater opp mot person.

Forsøksdeltakere vil ikke bli utsatt for noe risiko. Prøvene vil bare bli brukt til dette formålet, ikke i noe annet formål.

Signatur prosjektleder

Dato:

Signatur pasient

Dato:

## Appendix 2: Rettledning for levering av sædprøve ved Fertilitetspoliklinikken UNN



UNIVERSITETSSYKEHUSET NORD-NORGE  
DAVVI-NOROGGA UNIVERSITEHTABUOHCCVEI ESSU

Kirurgi-, kreft- og kvinnehelseklinikken, Fertilitetspoliklinikk (IVF)

HELSE  NORD

### Rettledning for levering av sædprøve ved Fertilitetspoliklinikken UNN Tromsø

Du har fått tildelt time for sædundersøkelse, og i den forbindelse må du levere en sædprøve ved Fertilitetspoliklinikken. Les nøye gjennom informasjonen nedenfor.

#### Det er ikke mulighet for prøvetaking på vår avdeling.

- Prøven må tas hjemme eller på et toalett på UNN før ankomst Fertilitetspoliklinikken og leveres innen 1 time.
- Prøven må ikke utsettes for temperatursvingninger. For høy/lav temperatur kan påvirke resultatet. For å unngå dette bør den oppbevares så nært inntil kroppen som mulig.
- Det bør være **minst 2** og **ikke mer enn 5** dager siden siste sædavgang. Avvik fra dette kan påvirke analyseresultatet.
- Prøven må samles direkte i den utleverte prøveboksen. **Kondom må ikke brukes.** Dersom noe kommer utenfor boksen skal det oppgis "ikke fullstendig prøve" i opplysningene nedenfor.

Pasienten kan få resultatet hos lege ved Fertilitetspoliklinikken samme dag.

Prøvesvar sendes elektronisk til fastlege og henvisende lege. Navn og fødselsdato på partner blir oppgitt i besvarelsen.

Denne egenerklæringen fylles ut og leveres sammen med sædprøven:

Fastlege: \_\_\_\_\_

Henvisende lege: \_\_\_\_\_

Henvisende leges adresse: \_\_\_\_\_

Ditt navn og fødselsnummer: \_\_\_\_\_

Adresse: \_\_\_\_\_

Partners navn og fødselsnummer: \_\_\_\_\_

Tidspunkt for prøvetaking (dato, klokkeslett): \_\_\_\_\_

Prøven er fullstendig  ikke fullstendig

Har du tidligere utført sædanalyse? \_\_\_\_\_

Hvis ja, når/hvor: \_\_\_\_\_

Pasienter som skal til kontroll etter sterilisering behøver ikke oppgi navn på partner.

Postadresse:  
UNN HF  
Fertilitetspoliklinikk  
Postboks 24  
9038 Tromsø

Besøksadresse:  
Fertilitetspoliklinikk (IVF)  
C4  
UNN  
9038 Tromsø

Telefon:  
77 62 64 81

Kontonr:  
4700.04.02008

Org.nr.:  
MVA 983 974 899 NO

Internett:  
[www.unn.no](http://www.unn.no)