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In vitro **effects of alcohol and oxidative stress on sperm cell motility and viability**

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Acknowledgements

This master thesis focuses on the effect of alcohol and oxidative stress on sperm motility and viability. The research for this thesis was conducted at Department of Clinical Medicine, The Faculty of Health Sciences, UiT The Arctic University of Norway, Tromsø in cooperation with the IVF lab., University Hospital of North Norway (UNN), Tromsø. Semen sample collection and analysis was initiated in January 2020 and completed in March 2020.

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Abstract

Background: The deteriorating semen quality is one of the increasing problems of male reproductive health worldwide and the exact cause of this has not been fully understood yet. Many stress factors may affect semen quality such as sperm cell motility and viability and thus impair the fertilization outcome. Among the different stress factors, alcohol can inhibit steroidogenesis, suppresses LH release from the pituitary gland, decrease the levels of testosterone, induce apoptosis of sperm cells and reduce the anti-oxidant defence systems. However, it is not clear so far that the chronic and low dose effects of alcohol and oxidative stress on the fate of sperm cell motility and viability. A method of identification of alcohol or oxidative stress affected sperm cells by using artificial intelligence technique could be the useful tool for the treatment of assisted reproductive technology (ART). Such technology has not been available for the clinical application so far. The main objective of the study is to observe the effects of various concentrations of alcohol and hydrogen peroxide on the motility and viability of sperm cells at different time periods.

Material and methods: In this study, we collected and evaluated semen samples from 13 men who came for the *in vitro* fertilization (IVF) treatment attending the IVF clinic at the University hospital in North Norway (UNN), Tromsø. Three samples were studied with high concentrations of ethanol (10%, 5%, 2%, and 1%) and hydrogen peroxide, (100 mM, 10 mM, 1 mM and 0.1 mM) and the motility and viability were observed within 2 h. Ten samples were studied with lower concentrations of ethanol (1.0%, 0.5%, 0.2%, and 0.1%) and hydrogen peroxide (100 μM, 50 μM, and 10 μM) and their direct effects on the sperm cell motility and viability were observed by using microscope after 24 hours.

Results: A significantly reduced motility and viability were observed at the higher concentrations of ethanol and hydrogen peroxide within a short time period. Sperm cells treated with 10% ethanol showed reduced motility within 15 min. and all cells remained immotile within 30 min. The total motility of the sperm cells reduced to 80% by 1% ethanol in 2 h. Higher concentrations of hydrogen peroxide concentration were found to be highly toxic to sperm cells. Lower concentrations of ethanol (1.0%, 0.5%, 0.2%, and 0.1%) rather enhanced progressive and non-progressive motilities in 24 h comparing to the corresponding control groups. Sperm cells treated with 100 μM, 50 μM, and 10 μM of hydrogen peroxide reduced progressive and non-progressive motility comparing to the corresponding control groups.

Conclusion: It is a preliminary study for the preparation and adjustment of experimental conditions to understand the direct effects of alcohol and oxidative stress on sperm cell motility and viability. Low levels of alcohol concentration will not affect the sperm cell motility and viability while oxidative stress with hydrogen peroxide pathway could affect the sperm cell motility and viability. Although it is a manually observed results from microscope, this will be highly useful information after correlated with the quantitative phase microscopy and artificial intelligence techniques and possible clinical application in the future.

Introduction

The infertility rate in the world may be as high as 15%, particularly in industrialized nations (1). Infertility occurred in couple contributed by male factors is approximately 30% of cases, female contributes 30% and almost 40% infertility cases are by both or unknown (2). Male infertility is mainly due to the degrading semen quality. A progressive deterioration of semen quality occurring in most of the Western countries has been taken as a serious concern in male reproductive health (3, 4). A study reports 32% decline in sperm concentration in European men over the past 50 years (5). Similar results are also reported from Scandinavian countries. Furthermore, regional studies in Scandinavia indicated that the Danish and Norwegian men, have significantly poorer semen count than the Finish men (6, 7).

In an archival data study, among men seeking infertility treatment in the Northern part of Norway revealed a gradually reduction in seminal fluid volume, sperm concentration and total sperm count for the past 20 years (8). This study showed that the proportion of hypospermic, azoospermic and oligozoospermic men had increased by 24.6%, 109.5% and 9.5%, respectively, comparing to the first decade (1992-2002) to the last decade (2003-2012) of the study period (8). The gradual decline in semen quality has raised concerns about the effects of a variety of substances that could be suspected to be responsible for this deterioration (8) Increased alcohol consumption could be one the factors assumed to influence sperm cell morphology and motility (9, 10), however it is difficult to pin point to only one factor.

Although modern techniques such as assisted reproductive technologies (ART) especially development of intracytoplasmic sperm injection (ICSI) techniques have provided a partial solution for the treatment of infertility in case of reduced sperm cell counts and motility, infertility cases due to the semen quality are continuously increasing. The birth of the first “test tube baby” Louise Brown in 1978 made a substantial breakthrough in ART and since then more than 7 million children have been born with the ART application. The success of assisted reproduction would not have been possible without the advances in the laboratory identification, manipulation and proper preparation of spermatozoa. There is always further space for its development.

Many physical factors are known to undesirably infer male infertility, these include; infections, varicocele, cryptorchidism, lesions causing obstruction, trauma and tumours (11). However, in

many cases, the suboptimal semen quality is of idiopathic origin, with no clear explanation for impaired quality. The decline in semen quality in recent decades, has raised concern about the effect of variety of factors that could be responsible for this deterioration (5).

Research indicates that a number of unknown lifestyle factors, such as excessive consumption, high cholesterol intake, cigarette smoking, malnutrition and physical inactivity might disturb sperm parameters (12). Reactive oxygen species (ROS), such as hydrogen peroxide, are products of normal cell metabolism (13, 14) is another important factor. However, exogenous factors such as smoking, environmental toxins and alcohol, promote the formation of ROS, thereby increasing the oxidative stress load on cells (13). When the level of ROS exceeds the cell defence mechanism against these substances, ROS might cause damage to essential parts of the cell, such as lipoproteins, DNA and RNA (13, 15). Among various stress factors, especially oxidative stress followed by denaturation of sperm cells have been pointed as one of the major causes for unsuccessful fertilization and embryo development (13).

Alcohol has a dual role on male reproductive function by affecting both testosterone production and spermatogenesis in the hypothalamic and pituitary axis (HPA) as illustrated in Figure1. This role of alcohol is probably a dose-dependent, where heavy drinkers are more likely to have a poor testicular function than moderate consumers (9). In additional, alcohol consumption might have direct effect on sperm morphology and function (16).

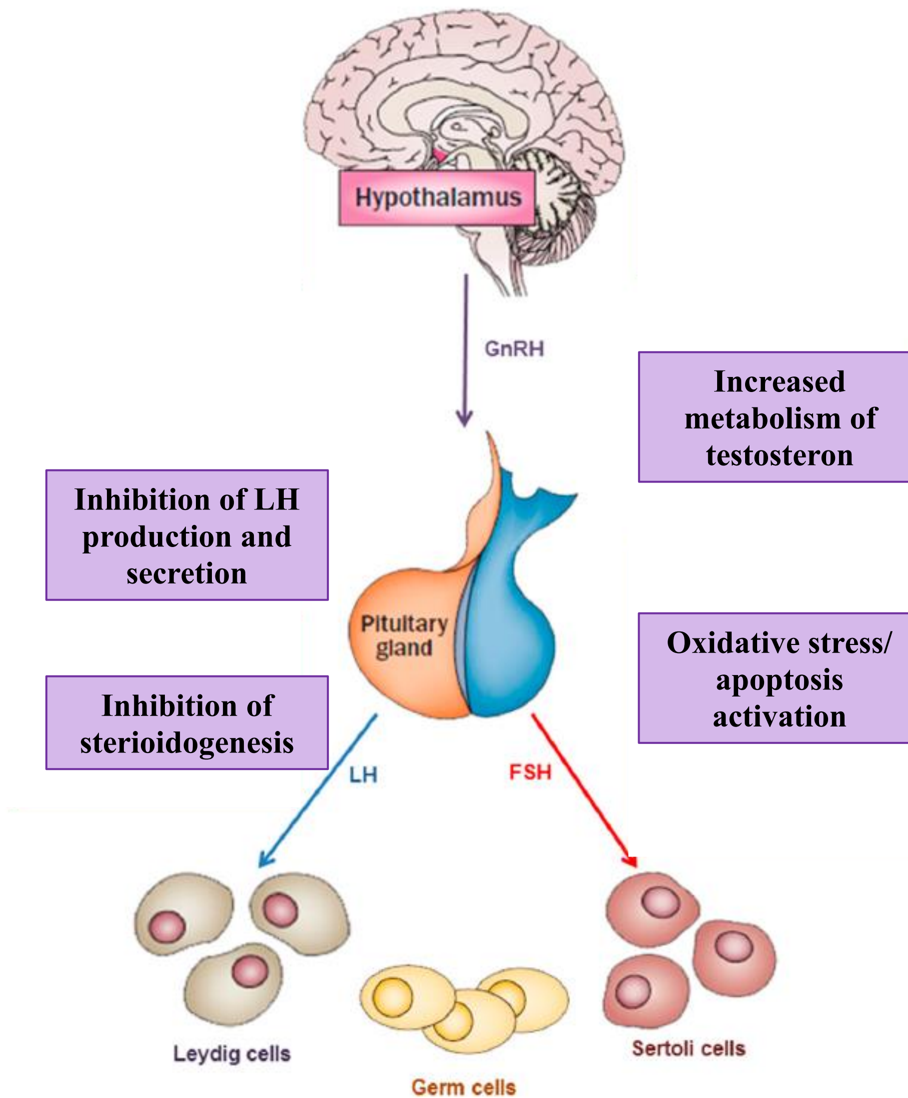

Figure 1 The detrimental effects of alcohol on testosterone levels and sperm production. Abbreviations: FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone.

However, it is also reported as an apparent protective role of moderate to social alcohol consumption on sperm quality. This effect can possible be explained by the antioxidant effect of some alcoholic beverages, for example red wine (17). It is also found in a metanalysis study that alcohol consumption did not significantly affected the fertility outcome (10). Therefore, in order to confirm the effect of alcohol consumption on male fertility needs further study.

Aim of the study

It is a preliminary screening procedure to understand the direct effects of alcohol and oxidative stress on the motility and viability on sperm cells. Therefore, the sperm cells are directly exposed to the externally induced oxidative stress by taking hydrogen peroxide and ethanol with various concentrations. The motility and vitalities were investigated by the use of microscope at different time intervals. The specific aim of the study was to find out the experimental conditions of appropriate time and concentrations affecting the motility and viability of sperm cells which can be applied in the future study to identify the viable sperm cells using artificial intelligence with quantitative phase microscopy.

Materials and methods

Semen collection

Semen samples were obtained from 13 men attending infertility treatment at the University Hospital of North Norway (UNN), Tromsø. Only the normal quality of semen samples (seminal fluid volume: 1.5 mL or higher, sperm cell concentration: 15 x 10^6/mL or higher, Total count: 39 x 10^6/mL or higher, motility percentage: 50% or more) as per WHO guidelines were used in the experiments. Baseline data of the semen quality is presented in Table 1. Semen samples were collected in the IVF clinic, UNN as per clinical protocol and the remaining samples after using in IVF treatment were used in the experiments. The men were instructed to abstain ejaculation for 2-5 days before delivering semen sample. Semen samples were collected by masturbation into a sterile Falcon specimen container (Corning Incorporated, Life Sciences, Tewksbury, MA, USA). Collected semen samples were advised to be kept at temperature close to body temperature and to be delivered to the IVF lab within 30-60 min.

All ethical guidelines were strictly followed. All patients signed consent forms permitting the use of their semen samples for this study (Appendix 1). The subjects were also received guidelines for optimal deliverance of high-quality sperm (Appendix 2). The current work was approved by the Regional Ethic Committee (REK) with REK number 2014/932 (Appendix 3).

Sperm cell preparation

In all cases semen samples were purified by gradient separation method at IVF Clinic, UNN (Appendix 4). Semen samples were allowed to liquefy using a Stuart tiling-machine with 25 tilts per minute for 10 min at room temperature. Semen analysis of sample volume, motility-grade of the spermatozoa, viscosity and total number of spermatozoa count were evaluated according to standard WHO criteria (18). The results of the semen quality is presented in **Table 1**.

Purification of semen samples were carried out by two-step discontinuous Percoll gradient centrifugation (Appendix 4). Briefly, sperm separation was performed by two-layer Percoll using the SpermGrad Lower Layer and SpermGrad Upper Layer purchased from Vitrolife (Västra Frölunda, Sweden). Both lower and upper layers of each gradient with 1.5 ml volume were cautiously pipetted into a 10 ml tube (Nunc, Roskilde, Danmark). The semen sample (1.5 ml) were carefully placed on top of the two Percoll layers and centrifuged at 500 x *g* for 20 min. The supernatant layer with seminal fluid was removed. The resulting bottom layer was diluted

in 5 ml pre-warmed (37 °C) Quinn's sperm washing medium (SAGE-In vitro Fertilization, Trumbull, CT, USA) and centrifuged at 300 *x g* for 10 min. The supernatant was removed and pallet of sperm cells was washed with 5 ml washing medium by homogenizing and centrifugation at 300 *x g* for 10 min. The resulting sperm cell pellet was homogenized in 0.5-1.0 ml pre-warmed (37 °C) Quinn's Advantage Fertilization medium (SAGE-In vitro Fertilization, Denmark).

Following purification of semen sample, the sample volume, motility-grade of the spermatozoa, and total number of spermatozoa were assessed once more for the purified sample. All samples were of over 90% progressive motile sperm cells after purification. The purified sperm cell samples were kept in an incubator at 37 °C with 5 % CO_2 until further use.

Dilution of purified semen samples

After receiving purified sperm cell samples from the IVF lab., the samples were evaluated for sperm cell counting and motilities once again in laboratory of Women´s Health and perinatology, UiT-The Arctic University of Norway, Tromsø according to WHO guidelines. Makler counting chamber is used to count and evaluate motility. The sperm cells samples were diluted in Quinn's Advantage Fertilization medium (SAGE-In vitro Fertilization, Denmark) to the sperm cell concentration of 250 000 cells/ml. Previous in-house laboratory experience has revealed that this will leave approximately 100 spermatozoa per vision field using the microscope with 200 times magnification in 96 well plate (19, 20).

For our experiments, it was aimed having approximately 3 ml of diluted purified semen samples with the concentration of 250,000 cells/ml in order to have enough volume for each experiment. The diluted sperm cell samples were transferred into 96 well plate containing each 90 µl cell suspension in the well.

Ethanol concentrations preparation

In this experiment, 99.9% absolute ethanol from Merck was used. Ethanol concentrations were diluted to four different concentrations with Quinn's advantage fertilization medium (SAGE-In vitro Fertilization) in order to form 1%, 0.5%, 0.2% and 0.1% as the final concentration exposure to the sperm cells. Alcohol concentrations were chosen to mimic serum equivalents of different levels of alcohol drinking. The high concentrations of ethanol (10%, 5%, 2% and 1%) on sperm cell motility and viability were also prepared to observe time course lethality up

to 2 hours. In all cases, the concentration of the prepared ethanol samples were 10 times higher than desired final concentration treated to the sperm cells.

Hydrogen peroxide concentrations preparation

In this experiment, 30% hydrogen peroxide (purchased from Sigma-Aldrich) was used. Hydrogen peroxide concentrations were diluted to three different concentrations with Quinn's advantage fertilization medium (SAGE-In vitro Fertilization) in order to form 100 μM 50 μM, 10 μM as the final concentrations. The high concentrations of hydrogen peroxide (1000 μM, 500 μM and 200 μM) also prepared by diluting with Quinn's advantage fertilization medium (SAGE-In vitro Fertilization) for the study of short time exposure and time course lethality. In all cases, the concentration of the prepared hydrogen peroxide samples were 10 times higher than desired final concentration treated to the sperm cells.

Preforming the in vitro motility and viability study

The purified and diluted sperm cell sample (90 µl) was transferred into each well of the Falcon 96-Well Cell Tissue Culture Plates (Corning Inc.). The different alcohol concentrations, hydrogen peroxide or medium only each 10 µl were transferred to the same well.

All experiments were performed in duplicates in order to minimize experimental errors. The sperm counts were aimed to have the same concentration of sperm cells to maximise the chance of receiving uniform results. The 96-Well Cell Tissue Culture Plates were incubated for 24 h at 37 °C with 5% CO_2 before motility and viability was assessed.

Sperm motility and viability assessment

The spermatozoa motility and viability were assessed using an inverted light microscope according to guidelines of the WHO laboratory manual for examining and processing human semen (18).

The motilities of spermatozoa were 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.

- **Immobility (IM)**: No movement.

Immotile sperm cells were considered as non-viable sperm cells. Sperm cells with progressive and nonprogressive motilities were considered as viable sperm cells.

All wells were manually counted using a microscope with an objective of 20 x . A defined rectangular microscopic field is fixed as in the Figure 2. The sperm cells seen the 96-Well Cell Tissue Culture Plates in the microscopic field have a rectangular area (Figure 2) and only spermatozoa inside this area were counted. Approximately between 60 and 120 sperm cells in each field were counted, with the number of progressive, non-progressive and immotile cells assessed. Sperm cells were evaluated in two randomly chosen regions in each well.

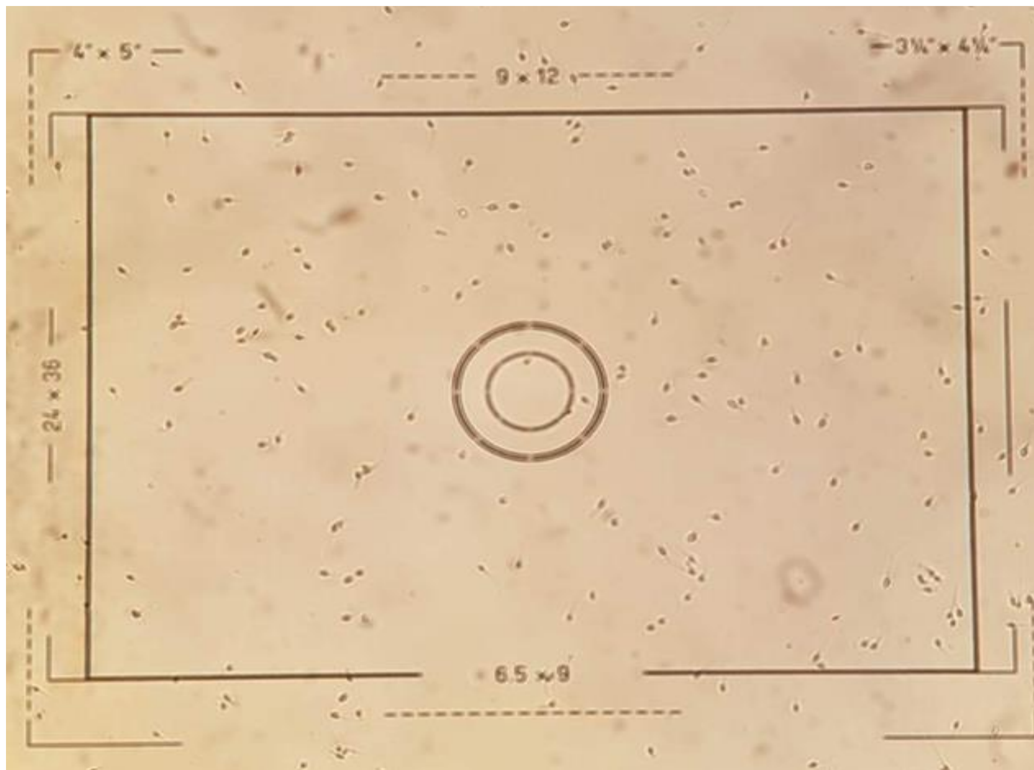

Figure 2. The observation field seen in the microscope where sperm cell viability and motility were counted. Photo by Stefan Thomassen.
Regions close to the rim of the wells were excluded.

Statistical methods

Semen samples from 10 men were used for the lower concentrations of ethanol or hydrogen peroxide and semen samples from 3 men were used to observe the effect of higher concentrations of ethanol or hydrogen peroxide. For mean value of four observations for each sample were expressed in percentage progressively motile (PR), non-progressively motile (NP) and immotile (IM) sperm cells. Results are expressed as mean percentage value of ten men or three men for each concentration of ethanol or hydrogen peroxide. The value is compared to control group (treated only media) to compare. The statistical significance $P< 0.05$ was considered as significant calculating two tailed and two paired student t-test from the excel program.

Results

The preliminary semen analysis was carried out in IVF Clinic, UNN, Tromsø. The base line data of 13 men with their mean age and clinical semen parameters evaluated in IVF clinic are presented in **Table 1.** To evaluate the semen parameters, the clinical protocols was followed as guided by the WHO guidelines (18). Only the men having good quality of semen parameter were selected for the study after their approval for research. Results in the **Table 1** are expressed as mean and standard deviation. The average age of the men was 35.6 years. Men´s age ranged from 29 years to 43 years. The average seminal fluid volume was 3.2 ml which was ranged from 1.8 ml to 4.9 ml.

Table 1: Base line data of men´s age and semen parameters.

Parameter	Mean (SD)
Age (years)	35.4 (6.5)
Seminal fluid volume (ml)	3.3 (1.0)
Viscosity (n)	2.4 (0.7)
Sperm cell concentration (millions)	56.4 (19.3)
Total count (millions)	186.1 (77.4)
Motility (%)	58.5 (9.4)
Motility grade (n)	3. 1 (0.5)

Results are shown as mean (SD: standard deviation) of 13 men. Viscosity and motility grade are expressed in number as per WHO guidelines.

The assessment of semen parameters such as seminal fluid volumes, viscosity, sperm cell concentration, total count in ejaculation, motility percentage, morphology were evaluated and graded. After assessment, each semen sample was purified by density gradient centrifugation separation method as described in the experiment section and appendix 4. A part of the purified sperm cells were used in the IVF clinic and the remaining sample was used in the experiment.

After transferring sample to research group lab., the sperm cell concentration and motility were evaluated by using the Makler Counting Chamber. All samples were of more than 90% sperm cell motilities with various sperm cell concentrations. 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. Motile sperm cells are considered as viable sperm cells and immotile sperm cells are nonviable sperm cells.

A. Effects of higher concentration of Alcohol and Hydrogen peroxide

In order to find out appropriate concentration, first a small number of samples were tested with very high concentrations of ethanol or hydrogen peroxide and sperm cell motility and viability were evaluated within a short time period.

For the high concentration of alcohol, 10%, 5%, 2% and 1% of ethanol were directly exposed to the sperm cells in the medium containing 96 well plate and sperm cells motilities were evaluated up to 2 hours. Results are shown in **Table 2**.

Table 2: Effects of higher concentration of ethanol on total motilities of sperm cells at various time periods.

	Total motility in percentage			
Concentration of Ethanol	15 min	30 min	60 min	120 min
10 %	23.3 (2.9)	0 (0)	0 (0)	0 (0)
5 %	38.3 (10.4)	15.0 (5.0)	2.3 (2.5)	0 (0)
2 %	76.7 (5.8)	71.7 (10.4)	56.7 (5.8)	53.3 (11.5)
1 %	90.0 (5.0)	90.0 (0)	86.7 (5.8)	80.0 (10.0)

Results are expressed as percentage mean (SD), n = 3. Mean percentage motilities for controls (untreated groups) were 90 % or more up to 120 min. Total motility shows the progressive and non-progressive together.

Sperm cells exposed with 10% ethanol showed reduced motility within 15 min. and all cells remained immotile in 30 min. By the treatment of 5% ethanol, number of motile sperm cells gradually reduced and in 120 min all cells remained immotile. In the similar way, 2% ethanol changed almost half of the motile sperm cells to immotile. The effect of 1% ethanol on sperm cell motility observed in 2 h. slightly decreased, however 80 % of the total cells were motile. Therefore, it is decided to use less that 1% of alcohol to see the effect in 24 h.

For the high concentration of hydrogen peroxide, four different concentrations such as 100 mM, 10 mM, 1 mM and 0.1 mM of hydrogen peroxide were directly exposed to the sperm cells in the medium containing 96 well plate and sperm cells motilities were evaluated up to 1 hour. Results are shown in **Table 3**.

Table 3: Effects of higher concentration of hydrogen peroxide on total motilities of sperm cells at various time periods

	Total motility in percentage			
Concentration of H_2O_2	1 min	5 min	30 min	60 min
100 mM	0 (0)	0 (0)	0 (0)	0 (0)
10 mM	10.7 (4.0)	0 (0)	0 (0)	0 (0)
1 mM	73.3 (5.8)	61.7 (2.9)	38.3 (2.9)	1.3 (0.6)
0.1 mM	85.0 (5.0)	80.0 (0)	70 (0)	63.3 (5.8)

Results are expressed as percentage mean (SD: standard deviation), n = 3. Mean percentage motilities for controls (untreated groups) were 90% or more up to 60 min. Total motility shows the progressive and non-progressive together.

All sperm cells which are treated with 100 mM concentration of hydrogen peroxide turned to immotile in 1 min. Those sperm cells which were exposed to 10 mM of hydrogen peroxide changed to immotile in 5 min. Almost 100% sperm cells changed to immotile in 1 hour by the treatment of 1 mM hydrogen peroxide. By the use of 0.1 mM (100 µM) hydrogen peroxide, sperm cells motility was gradually decreased within 1 h. Therefore, for the further experiments, hydrogen peroxide concentration used were less than 100 µM.

B. Effects of lower concentrations of Alcohol and Hydrogen peroxide

For the lower concentration of alcohol, 1.0%, 0.5%, 0.2% and 0.1% of ethanol are directly exposed to the sperm cells in the medium containing 96 well plate and sperm cells motilities were evaluated after 24 h. Results are shown in **Table 4**.

From **Table 4**, it is observed that the average progressive and non-progressive motilities of sperm cells were found to be higher in the alcohol (1.0%, 0.5%, 0.2% and 0.1%) treated groups after 24 h. than that of corresponding control groups. In the meantime, the non-viable cells i.e. immobility of sperm cells in the alcohol treated groups were found to less as comparing to control groups. The result show that alcohol concentrations at 1.0%, 0.5%, 0.2% and 0.1% is rather improving the motilities and viabilities of sperm cells.

Table 4: Effects of alcohol on motilities and viability of sperm cells after 24h.

Alcohol concentration	Motility	Mean percentage	SD	P-value
1.0 %	Progressive	21.4	13.4	0.1450
	Nonprogressive	21.7	8.4	**0.0444**
	Immotile	57.1	15.7	**0.0534**
0.5 %	Progressive	29.0	10.6	**0.0168**
	Nonprogressive	17.1	7.8	0.3606
	Immotile	54.2	12.6	**0.0388**
0.2 %	Progressive	27.7	12.6	**0.0173**
	Nonprogressive	16.1	8.4	0.4818
	Immotile	55.9	16.3	**0.0398**
0.1 %	Progressive	26.7	13.8	**0.0287**
	Nonprogressive	15.7	5.6	0.4523
	Immotile	57.6	15.1	0.0602
Control	Progressive	15.3	15.7	-
	Nonprogressive	15.9	8.5	-
	Immotile	68.9	19.6	-

Results are expressed as percentage mean (SD: standard deviation), n = 10. Mean percentage motilities for controls (untreated groups) were 100% and it is reduced after 24 h. incubation. P-values less than 0.05 is considered as statically significant comparing to control and shown in bold fonts.

Hydrogen peroxide is one major factor to induce oxidative stress in the body. Therefore to understand the effect of oxidative stress lower concentration of hydrogen peroxide was directly exposed to sperm cells. For the lower concentration of hydrogen peroxide, three different concentrations such as 100 µM, 50 µM, and 10 µM were directly exposed to the sperm cells in the medium containing 96 well plate and sperm cells motilities were evaluated after 24 h. Results are shown in **Table 5**.

From **Table 5**, it is observed that the average progressive and non-progressive motilities of sperm cells were found to be reduced in the 100 µM, 50 µM, and 10 µM of hydrogen peroxide treated groups comparing to that of corresponding control groups. In the meantime, the non-viable cells i.e. immobility of sperm cells in the hydrogen peroxide treated groups were found to be increased as comparing to control groups. The results show that even very low level of oxidative stress in the hydrogen peroxide pathway deteriorates the motilities and viabilities of sperm cells.

Table 5: Effects of hydrogen peroxide on motilities and viability of sperm cells after 24 h.

Hydrogen peroxide concentration	Motility	Mean Percentage	SD	P-value
100 µM	Progressive	6.3	5.6	**0.0392**
	Nonprogressive	11.5	9.8	0.0958
	Immotile	82.3	14.6	**0.0289**
50 µM	Progressive	6.3	5.9	**0.0398**
	Nonprogressive	13.1	12.7	0.2239
	Immotile	80.5	18.3	0.0582
10 µM	Progressive	9.6	9.2	0.1388
	Nonprogressive	11.4	12.1	0.1120
	Immotile	79.0	21.2	0.0949
Control	Progressive	15.3	15.7	-
	Nonprogressive	15.9	8.5	-
	Immotile	68.9	19.9	-

Results are expressed as percentage mean (SD: standard deviation), n = 10. Mean percentage motilities for controls (untreated groups) were 100% and it is reduced after 24 h. incubation. P-values less than 0.05 is considered as statically significant comparing to control and shown in bold fonts.

Discussion

In this study, I have investigated the direct effects of alcohol and hydrogen peroxide concentrations on sperm cell motility and viability *in vitro*. As mentioned in the introduction, alcohol has been identified as one of the possible causes of decreasing the quality of semen. However it is difficult to pinpoint the direct correlation of alcohol consumption and male infertility. Ingested alcohol is absorbed quickly into the systemic circulation (21). This in turn might affect the sperm cells at different in their development through a variety of mechanisms. Though alcohol seem to have a negative impact on sperm morphology and motility, the direct causative role of oxidative stress as a result of excess alcohol consumption on male infertility is somewhat unclear (22) (23). Alcohol does increase systemically oxidative stress, but the direct effect on sperm cell *in vivo* is still not completely understood (23).

Previous studies have also shown that the effect of alcohol on male infertility is dose-dependent (24). In this study, one can assume that the 1.25 % ethanol in the systemic circulation is equivalently 5 drinks, while the 2.5 % ethanol corresponds to more than 10 orally ingested drinks. A recent mate-analysis found that both semen volume and morphology were significantly impaired in daily drinkers (10). However, when comparing social drinkers to subjects who abstain from alcohol all together, no significant effect on semen volume and morphology were found.

Chronic alcohol intake was found to have a detrimental effect on both semen quality and the levels of male reproductive hormones (25). Conversely, a study comprising 8344 healthy male volunteers found that moderate alcohol intake was associated with higher testosterone levels but not with semen quality (26). Chronic ethanol administration has been shown to decrease testicular steroidogenic and antioxidant enzyme activities resulting in increased oxidative stress (27), which could disrupt testosterone synthesis and compromise fertility. A study on the male partners of couples facing primary infertility found that 72% of subjects whom were classified as heavy drinkers (with an alcohol consumption>80 g/day) were teratozoospermic. Among moderate drinkers (with an alcohol consumption 40-80 g/day), 63% were found to be teratozoospermic present in 63% and 72% of males who drank alcohol moderately (40–None of the heavy alcohol drinkers were normozoospermic and most were oligozoospermic (64%), which is suggestive of progressive testicular damage in relation to increasing daily alcohol intake (22). Similarly, another study found alcohol consumption rates to be significantly higher

in men with severe oligozoospermia and with non-obstructive azoospermia compared to fertile controls (28).
The objective of this study was to investigate the direct effect of alcohol on sperm viability and motility. However, the effect of alcohol on spermatogenesis must also be reviewed, as a possible factor that negatively affects sperm quality and thus the male infertility. The successful production of sperm cells relies on the presence of both Leydig cells and Sertoli cells, and proper stimulation from the hypothalamic-pituitary-testicular axis. Leydig cells are stimulated by luteinizing hormone (LH) to produce testosterone. Alcohol supress LH secretion form the pituitary, thus reducing testosterone production. Furthermore, alcohol also have a direct toxic effect on Leydig cells (29). Sertoli cells, which have an essential function in nurturing immature sperm cells, depend on stimulation from follicle-stimulation hormone (FSH). Spermatogenetic arrest and the syndrome called "Sertoli-only-cells" is more frequently in men with high alcohol consumption (30). A high amount of Sertoli cells will lead to an increase in the conversion of testosterone into estradiol. In fact, alcohol has been shown to directly induce the enzyme aromatase, which is responsible for the conversion of testosterone to estradiol (29, 31).

As previously mentioned, alcohol increase the formation of reactive oxygen species (ROS), which in turn disturb regular cell functions. Besides alcohol, several other factors such as pathogens, environmental physical and chemical factors including types of foods as well as mental stress produce ROS. Hydrogen peroxide produced in our body has the central role in ROS production and oxidative stress. Therefore, we selected two main factors such as alcohol and hydrogen peroxide for this study.

The effects of alcohol on sperm cell motility and viability are indeed biological plausible. The high level of polyunsaturated fatty acid in the head part of spermatozoa membrane, makes them particularly susceptible to damages caused by ROS. Lipid peroxidation of the cell membrane leads to loss of structural integrity, which in turn affect number of essential cell functions (32, 33). When the level of ROS exceeds the cells´ defence mechanism against these substances, ROS might cause damage to essential parts of the cells, such as lipoprotein, DNA and RNA (32, 33). Among stresses, especially oxidative stress followed by denaturation of sperm cells has been pointed out as one of the major causes for unsuccessful fertilization and embryo development.

In addition to interfering with the structural, as well as the functional integrity of cells, accumulation of ROS might also cause cell apoptosis (34). However, a recent study showed that alcohol had no significant effect on sperm concentration, though sperm morphology was significantly altered. This result might indicate that alcohol plays a minor role in reducing sperm cell potential through apoptosis. At the same time, some have reported an apparent protective role of moderate to social alcohol consumption on sperm quality. In fact, polyphenols such as xanthohuminol which is found in various alcoholic beverages, are known to have a cell protective effect (35, 36). Furthermore, the protective effect of moderate alcohol consumption, might be explained by the presence of antioxidants in beverages such as red wine (17). There are number of literatures on alcohol and its correlation on male infertility, because of controversial results, it is difficult to conclude so far. In addition, the concomitant effect of other substances, such as cigarettes, environmental toxins or other cellular stressor might pose an additive effects. Therefore further studies are necessary to clarify the effect of alcohol on male infertility.

Sperm cell motility and viability are directly related total infertility contributed by the male factor. Out of several factors, alcohol and oxidative stress have been pointed out the main factors for the reasons of male infertility. Alcohol is consumed as the social drinks and absorbed quickly and reach into the systemic circulations. The certain concentrations of alcohol depending on the quantity of alcoholic drinks is directly exposed to several other cells including mature sperm cells which might have certain direct effects on motility and viability on sperm cells.

In our results, by direct exposing the various alcohol concentrations, lower concentrations of alcohols did not reduce the progressive motility and viability rather it significantly increased the motility and viability comparing to controls up to 24 h of study period. Higher concentration of alcohol significantly reduced motility and viability. The effect of oxidative stress induced by various concentrations of hydrogen peroxide progressively and significantly reduced motility and viability during short period within 1 h or lower concentrations (100 µM, 50 µM or 10 µM exposed up to 24 h. Our results cannot be correlated with the clinical studies about the impact of alcohol consumption and oxidative stress on fertility. Moreover, they are not our objectives of this research work too. The scope of this work was to observe the changes on single sperm cell behaviour by treating with alcohol or oxidative stress factors for the development of experimental set up for the future experiments.

In most of the IVF clinics, it is a routine procedure that a live sperm cell is directly injected into the ovum in case of a few number of sperm cells or a low grade of sperm cells were found in men which could not brought the successful fertilization previous treatment cycle. Such procedure is called intracytoplasmic sperm injection (ICSI) where microneedle manipulator and microscope are used to handle each single egg or sperm cell. In this procedure, sperm cells motility and morphology observed under the bright field microscopy and is the only criteria for selecting a particular sperm cell during operation. Several factors such as oxidative stress, cryopreservation, heat, smoking and alcohol consumption, are negatively associated with the quality of sperm cell and fertilization potential due to the changing of subcellular structures and functions, are overlooked so far. Because of this, possibilities of further success rate in infertility treatment outcomes are limited. Bright field imaging contrast in an ordinary microscope is not able to distinguish tiniest morphological cell features that might have influence the fertilizing ability of sperm cell. In our research group, one group are trying to develop a technology which can give morphological image data as well as quantitative data together with the artificial intelligence, the minor changes in the cell in the sperm cells (20) could be identified. A partially spatially coherent digital holographic microscope (PSC-DHM) for quantitative phase imaging (QPI) in order to distinguish normal sperm cells from sperm cells under different stress conditions such as cryopreservation, exposure to hydrogen peroxide and ethanol could be distinguished. However, in this study, very high concentrations of alcohol and hydrogen peroxide were used and quantitative phase imaging data were obtained in 1 hour. For the preparation of long exposure time and lower concentrations, information are essential before investigation of clinically pathogenic samples. Such QPI information and artificial intelligence framework will be applicable for further improving ICSI procedure and the diagnostic efficiency for the classification of semen quality in regard to their fertilization potential and other biomedical applications in general (20). This study is one of the small steps for the future plan of developing ICSI tool to identify damaged sperm cells which can be avoided to be injected into the ovum.

Conclusion

It is a preliminary screening study for the preparation and adjustment of experimental conditions to understand the direct effects of alcohol and oxidative stress on sperm cell motility and viability. The results obtained in this study suggests that low levels of alcohol concentration will not affect the sperm cell motility and viability while oxidative stress with hydrogen peroxide pathway might affect the sperm cell motility and viability. In order to confirm such conclusion, we need further clinical study. Although it is a manually observed results under microscope, it will be highly useful in the clinical application specially during the selection of sperm cells in intracytoplasmic sperm injection (ICSI) procedure in ART treatment after correlating with the quantitative phase microscopy and artificial intelligence techniques.

Limitations and perspective

Alcohol might interfere with male reproductive function on multiple levels, and the possible biological basis for the detrimental effects of alcohol on male reproductive function seems apparent. This study was conducted with a limited sample size and focused on biological study, for the purpose of screening to set experimental conditions. Furthermore, the semen samples were collected form men who underwent fertility treatment. As a result of this sampling bias, the appliance to the general population have not been studied yet. In addition, the biological significance of these *in vitro* results cannot be correlated alcohol consumption in relation to male fertility at this stage, however it provides a science based hypothesis for the further study. Furthermoreour study is also affected partly by the Corona pandemic specially on semen sample collection.

Hopefully, modern artificial intelligence technique will be able to distinguish pathological sperm cells affected by alcohol or any other stress factors from normal sperm cells without any intervene which can lead new development in ICSI procedure in ART treatment. For this purpose, current study will be only a small experimental set up (20).

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Grade evaluations

Reference: Alcohol intake and semen variables: cross-sectional analysis of a prospective cohort study of men referring to an Italian Fertility Clinic	**GRADE**	
	Level	4
	Recommendation	C

Aim	**Materials and methods**	**Results**	**Discussion and comments**
Study the effect of alcohol intake to semen variables in men undergoing assisted reproductive techniques (ARTs) **Conclusion** Moderate alcohol intake appears positively associated to semen quality in males undergoing ARTs **Country** Italy **Year of data sampling** September 2014 to December 2016	Study design: Cross sectional analysis of a prospective cohort study Population: 323 male patients, mean age 39.3 years of subfertile couples referring to an Italian Infertility Unit and eligible for ARTs. Men were asked about their lifestyle including alcohol the last year before ART procedure. Age, risk factors for impaired male fertility, caffeine intake, smoking, leisure physical activity, days of abstinence and daily calories intake were accounted for in the analyses. Semen volume, sperm cell concentration, total sperm count and sperm motility were determined. Information on alcohol intake was collected as usual weekly consumption (1 unit = 125 ml wine or 330 ml beer or 30 ml spirits, all containing approximately 12.5 g of alcohol).	From the studied population 9.6 % were abstainers, while 30.0 % drank <1-3, 30.3 % drank 4-7 and 30.0 % drank more than 8 alcohol units per week. The results showed that for men drinking 4-7 units per week both semen volume and total sperm count were higher compared to the men drinking <1-3 units per week No association emerged with sperm cell motility. Concentration and total sperm count increased with higher level of intake in men without risk factors for impaired fertility and was significant both in those drinking 4-7 units and >8 units per week.	Is the study population clearly defined? Yes Is the answer % high enough? Yes Was the data sampling standarized? Yes Are objective criteria used for evaluating outcome measures? Yes, semen quality was evaluated in all patients Is the data analysis relevant? Yes Strengths: The relatively good sample size showing significant results as this is a single institution study. Men were interviewed in the same Institution by the same personnel and participation was practically complete. Accounted for potential biases, such as age, smoking, BMI, calories intake, days of abstinence, that have been included which are important parameters with semen quality Weaknesses: Important limitation is that the findings represent only to the male patients of infertile couples. The information regarding alcohol use was self-reported, thus some misclassification may have occurred, an underreporting or over reported should tend to reduce the estimated association between alcohol and semen parameters.

<table>
<tr><td rowspan="3">Reference: Changes in the semen quality among 5739 men seeking infertility treatment in Northern Norway over past 20 years (1993–2012)</td><td colspan="2">Grade</td></tr>
<tr><td>Level</td><td>4</td></tr>
<tr><td>Recommendation</td><td>C</td></tr>
</table>

<table>
<tr><th>Aim</th><th>Materials and methods</th><th>Results</th><th>Discussion and comments</th></tr>
<tr><td>Evaluate the temporal trends in semen quality of men who attended the fertility clinic of University Hospital of North Norway, Tromsø</td>
<td rowspan="7">Study population: The laboratory records and reports on semen analysis of 5739 men who attended the fertility clinic of the University Hospital of North Norway, Tromsø over the past 20 years (1993-2012) were studied.
Method of semen analysis: Semen analysis was performed as a routine procedure during the clinical investigation of subfertile/infertile couples. All men received both oral and written instructions and after 3-5 days of sexual abstinence produced and submit a fresh semen sample. Semen was analyzed using the conventional techniques adapted from the methods described in World Health Organization (WHO) laboratory manuals and the data were recorded according to hospital protocol. Semen volume was measured with pipetting, and sperm concentration and motility assessed by direct observation under a microscope.
Data analysis and statistics: The percentage of motility was graded on a scale of 0–4; were 0 for immotile and 3-4 for the spermatozoa that presented rapid progression along a linear track. Assessment of morphology was based on the criteria described in the WHO manuals. Between the first decade (1993-2002) period and the last decade (2003-2012) period the mean values and yearly mean values of the seminal fluid volume, sperm cell concentration and total sperm cell count per ejaculate were compared. The proportion of hypospermia, azoospermia, and oligozoospermia were also compared between first and last decades.
Hypospermia: the seminal fluid volume of less than 1 mL
Azoospermia: the absence of spermatozoa observed in whole ejaculate.
Oligozoospermia: the sperm cell concentration and total sperm cell count per ejaculate less than the reference values.</td>
<td rowspan="7">During the study period the seminal fluid volume, sperm concentration and total sperm count were found to have gradually decreasing.
It was observed 11,4% decrease in the mean seminal fluid volumes in the first decade (1993–2002) and the last decade (2003-2012).
The mean sperm cell concentrations decrease in the first decade (1993–2002) and the last decade (2003–2012) was found as 23.1%.
The total sperm count in the first decade was 166.0 millions sperms per ejaculate, which was decreased by 28.9% ($p < 0.001$) in the last decade.
The proportion of hyposermic, azoospermic, and oligozoospermic population has been found to be in gradually increasing order from 1993 to 2012. The percentage of hypospermic and azoospermic population was found to be sharply increased than oligozoospermic population.</td>
<td rowspan="7">Is the study population clearly defined? Yes
Is the study representative for the general population? No
Is it described how non-responders differ in characteristics from responders? No
Is the answer % high enough? Not given
Are objective criteria used for evaluation the outcome measure? Yes
Is the data analysis relevant? Yes

Strenghts:
- Long observational period, 20år
- study was performed in a self-selected population of men seeking fertility treatment
- All subjects included in the study resided in the Northern region of Norway during the time of semen analysis.
Weaknesses:
- study did not identify the possible cause(s) of the observed deterioration in the semen quality.
- We did not have information on the lifestyle factors on these men,
-environmental factors might be responsible for the observed deterioration of semen characteristics
- analysis does not distinguish indigenous population from immigrated one. Therefore, the possibility of changes in semen profile due to the demographic changes cannot be excluded.
- causes of semen quality deterioration and its effect on fertility potential remain to be elucidated</td></tr>
<tr><td>Conclusion</td></tr>
<tr><td>The semen quality of men among couples seeking fertility treatment is progressively declining.</td></tr>
<tr><td>Country</td></tr>
<tr><td>Norway</td></tr>
<tr><td>Year of data sampling</td></tr>
<tr><td>1993-2012</td></tr>
</table>

<table>
<tr><td rowspan="3">Reference: Semen quality and alcohol intake: a systematic review and meta-analysis,</td><td colspan="2">Grade</td></tr>
<tr><td>Level</td><td>4</td></tr>
<tr><td>Recommendation</td><td>C</td></tr>
</table>

<table>
<tr><th>Aim</th><th>Materials and methods</th><th>Results</th><th>Discussion and comments</th></tr>
<tr><td>To determine what kind of association is between alcohol intake and semen quality.</td>
<td rowspan="7">Identification of studies: Literature search of all observational studies published or in press as original articles in English, up to April 2016. Electronic databases MEDLINE and Embase were used. Reference lists of retrieved articles to search for other pertinent studies was reviewed. Two authors reviewed the papers and independently selected the articles eligible for the systematic review.
Inclusion criteria:
- observational studies reporting original data
- parameters of semen
-quality provided as means and standard deviation (SD) or standard error (SE) or as medians and interquartile range (IQR)
– full-length articles, published in English.
Quality of studies was independently evaluated by two reviewers using strobe criteria
Data collection for meta-analysis: Data were extracted independently by two investigators and discrepancies were resolved by discussion. For each study, the following information was collected in a standard form: first author's last name; year of publication; country of origin; number of subjects; mean age, if available; category of alcohol consumption, if available; mean and SD (or SE) or median and IQR; covariates adjusted for in the statistical analysis.
Statistical analysis: The data were transformed into mean and SD. The estimates of the average effect of alcohol on semen parameters and 95%CI were calculated by using both fixed effect and random effect models.</td>
<td rowspan="7">Out of 179 papers they found, a total of 15 articles were included in the meta-analysis
The rest was excluded for not meeting the criterias?????
All studies had cross-sectional design, quality was according to the STROBE criteria.
Some authors found no effects on semen parameters and some underlined a detrimental effect of alcohol. Main results showed that alcohol intake has a detrimental effect on semen volume (pooled estimate for no/low alcohol consumption 0.25 ml, 95% CI, 0.07 to 0.42) and normal morphology (1.87%, 95% CI, 0.86 to 2.88%). The difference was more marked when comparing occasional versus daily consumers, rather than never versus occasional, suggesting a moderate consumption did not adversely affect semen parameters.</td>
<td rowspan="7">Is the study population clearly defined: Not answered
Is the study reprensentative for the general population: Not answered.
Strenghts
All included study included had the same design
Inclusion criteria for the studies are clearly defined
Quality of the studies was according to STROBE criteria
Funnels plot and Eggers test of all the measures were performed to detect publications
Weaknesses:
Information was collected by questionnaire.
Alcohol use was classified in different ways
Many variables were not normally distributed and had to be transformed to be able to include them in meta-analysis.</td></tr>
<tr><td>Conclusion</td></tr>
<tr><td>Alcohol intake has a detrimental effect on semen volume and normal morphology</td></tr>
<tr><td>Country</td></tr>
<tr><td>The Italy, Milan</td></tr>
<tr><td>Year of data sampling</td></tr>
<tr><td>Literature search from 1966 to April 2016</td></tr>
</table>

<table>
<tr><td rowspan="3">Reference: 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.</td><td colspan="2">Grade</td></tr>
<tr><td>Level</td><td>4</td></tr>
<tr><td>Recomendation</td><td>C</td></tr>
</table>

<table>
<tr><td>Aim</td><td>Material and methods</td><td>Results</td><td>Dicussion and comments</td></tr>
<tr><td>Groups of young men from Norway, Denmark, Finland and Estonia were investigated to elucidate whether semen parameters and other related parameters follow a gradient between these countries, as does the gradient in incidence of testicular cancer.</td><td rowspan="7">In total 968 young men born in 1979, 1980 or 1981 from Norway, Denmark, Estonia and Finland were investigated when attended the compulsory medical examination.

Participant had received written information about the study.

All participants were instructed to abstain ejaculation for at least 48h before attendance at the clinic.

On the day of attendance at the clinic, each man returned a completed questionnaire, underwent a physical examination, and provided both blood and semen samples.

WHO guidelines were followed for the analysis of semen samples.

Possible confounders were evaluated and included in the statistical analysis when appropriate. Inter-laboratory differences in assessment of sperm concentrations were controlled by an external quality control programme and morphology assessment was centralized to one person.</td><td rowspan="7">Differences were detected in both the qualitative and quantitative serum parameters between young men in these four countries.
Increasing duration of abstinence had an increasing effect on semen volume, sperm concentration and total sperm count up to 96 h.
Finland and Estonia had higher sperm concentration. Men from Estonia were found to have the highest percentage of motile sperm followed by men from Denmark, Finland and Norway in order.
Men from Finland and Estonia had the highest frequency of morphologically normal sperm than the Norwegian and Danish men. The Finish and Estonian men had an adjusted median sperm concentration of 54 and 57 x 10(6)/ml respectively and the Norwegian and Danish men 41x10(6)/ml. The corresponding total sperm counts were 185, 174, 133 and 144 x 10(6). The frequency of normal sperm in men from Finland was 8,9%, Estonia 9,2%, Norway 6,9%and Denmark 6,4%. Within all four groups of men from a relationship between increasing level of inhibi-B and increasing sperm counts was observed. However, inhibin-B levels were not predictive of sperm count differences between countries.</td><td rowspan="7">Is the study population clearly defined? Yes
Is the study representative for the general population? Yes
Is the data analysis relevant? Yes
Is the answer % high enough? Yes
Are objective criteria used for evaluating the outcome measures? Yes, semen quality was evaluated in all pastients.
Is the data analysis relevant? Yes
Strenghts
All four groups of men were investigated according to the same protocol.
No seasonal variation was found in semen quality.
Participations had no prior knowledge of their own fertility potential.
The assessment of sperm morphologies performed in a random order by 1 person.
Differences in assessment of sperm were controlled by an external quality control program
Weakness
The participation rate was low.
The motility assessment was highly subjective</td></tr>
<tr><td>Conclusion</td></tr>
<tr><td>It appears that an east-west gradient exists in the Nordic-Baltic area with regard to semen parameters, this being in parallel with the incidences of testicular cancer.</td></tr>
<tr><td>Land</td></tr>
<tr><td>Denmark, Norway, Estonia and Finland</td></tr>
<tr><td>Year of data sampling</td></tr>
<tr><td>March 1997 to January 2000</td></tr>
</table>

Reference:	**Grade**	
Alcohol intake and cigarette smoking: Impact of two major lifestyle factors on male fertility	Level	4
	Recommendation	C

Aim	**Material and methods**	**Results**	**Discussion and comments**
To find out the specific impact of alcohol and smoking on semen quality. **Conclusion** Asthenozoospermia, the most common semen variable in our study, can be an early indicator of reduction in quality of semen. Alcohol abuse apparently targets sperm morphology and sperm production. Smoke-induced toxins primarily hamper sperm motility and seminal fluid quality. **Country** India **Year of data sampling** Jan 2000 to Des 2005	Population: Male partners of infertile couples seeking treatment for primary infertility. Selection criteria: Male partners of couple facing primary infertility impaired for more than one year and not using any contraceptive measures Male suffering from azoospermia, ex-smokers, and ex-alcoholics, history of prolonged medication, above 45 years of age, negative semen fructose test and males with history of injury to testes, varicocele, hydrocele and more were excluded from the study groups. Three study groups were formed each comprising of 100 males. Group A: strict non-alcohol users and non-smokers who acted as controls. Group B: alcohol users but non-smokers divided into three subgroups according to the average daily alcohol consumption, -mild 40g or less, moderate 40-80g and heavy>80g. Group C: smokers, who were strict non-alcohol-consumers, were sub-grouped based on number of cigarette smoked per day.- light smokers 1-20sig/d, moderate smokers 21-40 and heavy smokers >41 sig/day. Semen collection: semen samples were collected after strict abstinence of 4 to 6 days. Only one sample per patient was included into the study. All semen samples were analyzed as per recommended guidelines according to WHO manuals and compared with 100 strict non-alcoholic and non-smoker males for presence of asthenozoospermia, oligospermia and teratozoospermia. Data was analyzed by F-test using Microsoft Office Excel 2003.	Group A: comprised of strict non-smokers and non-alcoholic. Normozoospermia was present in 37 cases and asthenozoospermia was the most commom anomaly of semen compared to oligo and teratozoospermia. Group B: Only 12% showed normozoospermia of which 9 of cases were mild alcoholics. Normozoospermia was not present in cases of heavy alcoholics. Teratozoospermia and oligozoospermia was seen in much higher number of cases among alcoholics in comparison to controls. Overall teratozoospermia dominated the semen variables and was present in 72% heavy alcoholics and 63% moderat alcoholics Similarly oligozoospermia was present in as high as 64% of heavy alcoholics.Thus heavy alcoholics showed a very high percentage of defects of sperm count, motility as well as morphology. Group C: Only 6 samples had semen parameters consistent with normozoospermia. Asthenozoospermia was dominant semen variable in smokers. Comparing to controls asthenozoospermia was seen only in 9% of cases, 39% in light smokers. By contrast, heavy smokers and moderate smokers had more samples with astheno-oligo and teratozoospermia than light smokers. Alcohol appeared to contribute mostly towards developmental defects of sperm morphology and sperm production. Cigarette smoke appears to contribute significantly towards impairment of sperm motility. Deterioration in semen quality apparated in direct proportion of the quantity of alcohol intake and cigarettes smoked.	Were the case-control groups recruited from comparable population? Yes Are the groups comparable in terms of important background factors? Yes Is the control group healthy? Yes Are important confounders considered in the design/analysis? Yes, defined exclusion/inclusion criteria help reducing the risk of confounders. Is the exposure for danger/harm/intervention measured and graded equally in the groups? Yes, the same laboratory techniques were used. Was the one who measured the exposure blinded in terms of casus/control? No Was the responsrate sufficient in all groups.? Yes. Strengths - Defined inclusion/exclusion criteria - Standardized methods used - Long enough follow up time to detect outcomes - Big sample - Equal distribution of patients in groups Weakness -unclear whether analysis of semen was blinded

Appendix 1. Consent form

Samtykkeskjema for bruk av sædprøve til andre formål enn pasientbehandling

Forskningsstudiet: Oksidativt stress og sæd kvalitet

Avdeling: Kvinnehelse og perinatologi forskningsgruppe ved Universitetet i Tromsø
Ansvarlig person/Prosjektleder: Professor Purusotam Basnet
Formål med bruk av prøven:
Denne forskningsstudiet blir gjennomført av Med. Stud 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 Signatur pasient

Appendix 2: Guidelines for deliverance of semen samples for IVF

UNIVERSITETSSYKEHUSET NORD-NORGE
DAVVI-NORGGA UNIVERSITEHTABUOHCCEVIESSU

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

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 prøvetakning 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

Appendix 3: REK approval

Region:	Saksbehandler:	Telefon:	Vår dato:	Vår referanse:
REK nord	Øyvind Strømseth	77620753	28.11.2014	2014/932/REK nord
			Deres dato:	Deres referanse:
			08.10.2014	

Vår referanse må oppgis ved alle henvendelser

Purusotam Basnet
Postboks 24

2014/932 Oxidative stress and semen quality

Forskningsansvarlig: Universitetet i Tromsø
Prosjektleder: Purusotam Basnet

Prosjektomtale
Sædkvalitet er en avgjørende variabel for vellykket reproduksjon, men blir stadig dårligere, uten kjent årsak. Rapporter om sædkvalitet hos menn fra forskjellige land blir publisert, men enda ikke fra Nord-Norge. Endringene i sædkvalitet påvirker fertilitet, som kan gi demografiske endringer. En av de mest diskuterte faktorene i den nyeste litteraturen, er rollen til oksidativt stress (OS). Det trengs derfor en systematisk studie for å overvåke og evaluere sækvaliteten hos menn i Nord-Norge. Mål: 1. Finne korrelasjon mellom OS-nivå og sædkvalitet 2. Å undersøke OS-nivå og sædkvalitet hos friske individer og individer som ønsker fertilitetsbehandling. 3. Å finde sammenhengen mellom OS og reproduksjonutfall i IVF–behandling. 4. Å måle sammenhengen mellom OS-nivå og sædkvalitet longitudinellt. 5. Å undersøke in vitro effekten av vanlige antioksidanter som vit C, vit E, polyfenoler, metallioner (Fe++/ Cu+) på å forbedre sædkvalitet ved sædlagring og -behandling i IVF-prosedyrer.

Vurdering
Søknaden ble behandlet av Regional komité for medisinsk og helsefaglig forskningsetikk (REK nord) i møte den 12.6.2014. Komiteen hadde merknader til søknaden og fattet utsettelsesvedtak hvor den videre behandling av søknaden vil bli foretatt på fullmakt av komiteens leder/nestleder og sekretær med mindre det reises spørsmål som må behandles av samlet komité. Vurderingen er gjort med hjemmel i helseforskningsloven (hfl.) § 10, jf. forskningsetikklovens § 4.

Prosjektleder har gitt tilfredsstillende tilbakemelding på komiteens merknader den 8.10.2014, vedlagt revidert protokoll, revidert samtykke mv.

Etter fullmakt er det fattet slikt

Vedtak

Med hjemmel i helseforskningsloven §§ 9, 10 og forskningsetikkloven § 4 godkjennes prosjektet.

Sluttmelding og søknad om prosjektendring
Prosjektleder skal sende sluttmelding til REK nord på eget skjema senest 30.04.2020, jf. hfl. § 12. Prosjektleder skal sende søknad om prosjektendring til REK nord dersom det skal gjøres vesentlige endringer i forhold til de opplysninger som er gitt i søknaden, jf. hfl. § 11.

Besøksadresse:
MH-bygget UiT Norges arktiske universitet 9037 Tromsø

Telefon: 77646140
E-post: rek-nord@asp.uit.no
Web: http://helseforskning.etikkom.no/

All post og e-post som inngår i saksbehandlingen, bes adressert til REK nord og ikke til enkelte personer

Kindly address all mail and e-mails to the Regional Ethics Committee, REK nord, not to individual staff

Klageadgang
Du kan klage på komiteens vedtak, jf. forvaltningsloven § 28 flg. Klagen sendes til REK nord. Klagefristen er tre uker fra du mottar dette brevet. Dersom vedtaket opprettholdes av REK nord, sendes klagen videre til Den nasjonale forskningsetiske komité for medisin og helsefag for endelig vurdering.

Med vennlig hilsen

May Britt Rossvoll
sekretariatsleder

Øyvind Strømseth
seniorrådgiver

Kopi til:purusotam.basnet@uit.no

Appendix 4: Sperm preparation by twostep discontinuous Percoll gradient centrifugation

Sperm preparation

Density gradient centrifugation method

Directions for supplementation of un-supplemented G5 Series™ media can be found in the G5 Series™ Manual on www.vitrolife.com. Once supplemented, the media should be used as the G5 Series™ PLUS media described below.

Day -1
the day before oocyte pick-up

G-IVF™ PLUS

Pre-equilibrate G-IVF™ PLUS at

37°C 6 % CO_2 overnight

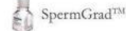
G-RINSE™

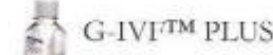
Pre-rinse all utensils, including tubes and dishes, with G-RINSE™.

Day 0

1. Assess the semen sample

2. Prepare gradient solutions


SpermGrad™


G-IVF™ PLUS

Mix SpermGrad™ with G-IVF™ PLUS in separate rinsed tubes to obtain 90 % and 45 % stock solutions. For 90 % stock solution, mix 9.0 mL SpermGrad™ with 1.0 mL G-IVF™ PLUS and for 45 % stock solution, mix 4.5 mL SpermGrad™ with 5.5 mL G-IVF™ PLUS.

3. Prepare gradients

Pipette 1.5 mL of the 90 % solution into the rinsed tube first and then slowly pipette 1.5 mL of the 45 % solution on top of it. Finally, 1.0 mL of the semen is layered on the top.

Make up 2-4 gradient tubes. Before use, allow the stock solutions to warm to ambient temperature.

4. Centrifuge the gradients at

300-600g 10-20 min

5. Wash I

Remove the two top layers. Transfer the pellets to new rinsed tubes and re-suspend with 5 mL equilibrated G-IVF™ PLUS and centrifuge again at

300-600g 10 min

6. Wash II

Aspirate and discard the supernatant. Transfer the pellets to new rinsed tubes and re-suspend with 5 mL equilibrated G-IVF™ PLUS and centrifuge again at

300-600g 10 min

7. Assess sperm preparation

Aspirate and discard the supernatants. Combine all pellets in a new rinsed tube and re-suspend in 0.5-1.0 mL of equilibrated G-IVF™ PLUS depending on sample quality.

Determine motility and concentration of spermatozoa in the washed sample.

8. Dilution

Dilute with equilibrated G-IVF™ PLUS to a final concentration of 75,000-200,000 motile sperms/mL.

9. Preparation of insemination dishes

Prepare rinsed insemination dishes with 0.5-1.0 mL of sperm solution and pre-equilibrate.

If oil overlay is used, droplets of at least 100 µL volume are recommended. Equilibrate the dishes at

37°C 6 % CO_2 >2 h

10. Insemination

Transfer the oocytes to the insemination dishes and leave at

37°C 6 % CO_2 overnight

Alternatively: Add equilibrated sperm suspension to equilibrated dishes with the oocytes already present.

Vitrolife

G5 Series