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ANTI-MÜLLERIAN HORMONE AND CARNITINE CONCENTRATION IN THE SERUM OF HOLSTEIN COWS

ANTI-MÜLLERIAN HORMOONI JA KARNITIINI KONTSENTRATSIOON HOLSTEIN LEHMADE SEERUMIS

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ABSTRACT

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Ovum pick up (OPU) and *in vitro* fertilization has become a popular way of producing genetically superior cattle. The quality of oocytes has a big impact on the outcome of this procedure. Anti-Müllerian hormone (AMH) concentration in serum has been used as a marker for morphologically healthy follicles in ovaries. L-Carnitine has been proven to work as a marker for certain diseases in cattle up to four weeks before clinical signs of disease appear.

The aim of this study is to compare AMH L-carnitine concentration, body condition score, pregnancy status and oocyte quality, to determine the factors that affect the number of follicles punctured, cumulus-oocyte complexes (COCs) aspirated and the quality of them.

The OPU procedure was used to get oocytes from 24 Holstein cows, 9 non-pregnant (NP, 46-48 days postpartum) and 15 pregnant (P, 44-85 days). On the day of OPU blood samples were obtained and BCS was evaluated. AMH concentration was analyzed with ELISA and L-carnitine with mass spectrometry.

Results demonstrate that there is a positive correlation between the quality of the oocyte and the serum AMH concentration (r= 0.73, p=0.059). The pregnant animals had more morphologically healthy ovaries since they had a higher percentage of retrieved oocytes out of follicles aspirated (51.7%) compared to the non-pregnant animals (31.6%) and higher AMH concentration. In pregnant animals the AMH average is 0.28ng/ml and in nonpregnant animals 0.23ng/ml (p=0.255). L-Carnitine concentrations had a negative correlation (r=-0.35, p=0.091) with the follicle count.

Keywords: oocyte quality, ovum pick up, body condition score

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Transvaginaalne munasarjade punktsioon (OPU) ja in vitro viljastamine on muutunud populaarseks meetodiks veiste geneetilise materjali kiiremal paljundamisel. Munarakkude kvaliteedil on selle protseduuri lõpp-tulemustele suur mõju. Anti-Mülleri hormooni (AMH) kontsentratsiooni määramist vereseerumis on kasutatud morfoloogiliselt tervete folliikulite markerina munasarjades. On tõestatud, et L-karnitiin tõuseb veistel teatud haiguste markerina kuni neli nädalat enne kliiniliste tunnuste ilmnemist.

Selle uuringu eesmärgiks on võrrelda AMH, L-karnitiini kontsentratsiooni, kehakonditsiooni skoori, tiinuse järku ja munarakkude kvaliteeti, et määrata kindlaks tegurid, mis mõjutavad kogutud folliikulite arvu, munarakkude hulka (COC), ja nende kvaliteeti.

OPU protseduur teostati 24 Holsteini lehmal, kellest, 9 olid mittetiined ja 15 tiined. OPU päeval võeti vereproovid ja hinnati kehakonditsiooni. Vereseerumid eraldati ja säilitati kuni proovude uurimiseni. AMH kontsentratsiooni analüüsiti ELISA meetodil ja L-karnitiini mass-spektromeetriaga. Tulemused näitavad, et munaraku kvaliteedi ja seerumi AMH kontsentratsiooni vahel on positiivne seos ($r = 0,73$, $p = 0,059$). Tiinetel loomadel oli rohkem morfoloogiliselt terveid munasarju, sest neil oli suurem kätte saadud munarakkude protsent (51,7%), võrreldes mitte tiinete loomadega (31,7%) ja kõrgem AMH kontsentratsioon. Tiinetel loomadel on keskmine AMH 0,28 ng/ml ja mitte tiinetel loomadel 0,23ng/ml ($p = 0,255$). L-karnitiini kontsentratsioonil oli folliikulite arvuga negatiivne korrelatsioon ($r = -0,35$, $p = 0,091$)

Märksõnad: munaraku kvaliteet, transvaginaalne munasarjade punktsioon, kehakonditsiooni skoori

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ABBREVIATIONS

AETE Association of Embryo Technology in Europe

AFC Antral follicle count

BCS Body condition score

COC Cumulus oocyte complex

FSH Follicle stimulating hormone

GNRH Gonadotropin-releasing hormone

ICM Inner cell mass

IETS The International Embryos Transfer Society

IVF *In vitro* fertilization

LCFA Long-chain fatty acids

LOWESS Locally weighted scatterplot smoothing

MLC Meiotically less competent

MMC Meiotically more competent

NP Non-pregnant

OPU Ovum pick up

OPU-IVP Ovum pick up- *in vitro* embryo production

P Pregnant

PGF 2α Prostaglandin F2alpha

INTRODUCTION

This thesis focuses on the quality of the oocytes in donor cows for the ovum pick up and *in vitro* embryo production. It also looks at the cattle's body condition score, pregnancy status, the serum antimüllerian and L-carnitine concentrations.

Ovum pick up (OPU) is a method of production of *in vitro* embryos where follicular fluid and oocytes can be aspirated from ovaries under ultrasound control. OPU can be done in non-pregnant cows and also on pregnant animals up to 100 days from conception. The aspirated fluid is sent to the laboratory for recovery, evaluation, maturation, fertilization and cultivation of oocytes. The embryos are cultivated *in vitro* up to blastocyst stage for 7-8 days and are thereafter transferred to recipients or frozen for later use. The animals that take part in this study are part of an ovum pick up and *in vitro* embryo production (OPU-IVP) program.

There is a notable increase in the amount of OPU sessions in 2017 according to the Association of Embryo Technology in Europe (AETE) data collection report. According to this report the efficacy of OPU has increased from 1.6 embryos per session to 2.8 embryos per session in one year. While OPU has become more popular, the *in vivo* method amounts have stayed quite stable (Mikkola, 2017). OPU has some advantages compared to the *in vivo* embryo production methods and the superovulation of donor animals. OPU can be done weekly for months. In ideal circumstances the outcome of the whole process is 1-2 embryos from heifers and 3-4 from cows with OPU-IVP (Galli *et al.* 2005). There is no need for hormonal treatment so there are no side effects like infertility, ovarian cysts, milk drop, or trauma caused by hormonally influenced behavior.

The quality of the oocyte in this procedure is very important since in order to get good embryos, also good quality oocytes are needed. Oocytes can be evaluated in different ways like for example morphologically or functionally. Usually they are assessed by light microscope. Oocytes are divided into groups according to the COCs (Cumulus oocyte complexes). COCs with a complete and dense layer are considered to have a better outcome compared to COCs with a damaged and thin layer. (Goovaerts *et al*., 2010). This study looks at oocyte quality results by evaluating the COCs and morphology of the oocytes.

Antimüllerian hormone (AMH) is produced in female animals in the gonads by the granulosa cells that are produced by the growing ovarian follicles, the secondary follicles. The hormone has two functions it inhibits the premature growth of primary follicles and it reduces the response of follicle stimulating hormone (FSH) to preantral follicles, so they do not rupture prematurely. It is only released by growing, healthy follicles so it can be connected with the amount of morphically healthy follicles in cattle. Thus, in cattle the AMH concentration in the serum is indicative of the oocyte quality and quantity (Guerreiro *et al.,* 2014). The AMH serum concentrations in this study was analysed with ELISA.

Carnitine is an important part of the metabolism. It is an amino acid and takes part in the transporting of long-chain fatty acids from the cytoplasm to the mitochondria were the fatty acids are broken down. High carnitine concentrations have been linked with disease. Carnitine has been linked to be a potential marker for disease, levels of carnitine have shown to have increased up to four weeks before diseases like metritis, mastitis and laminitis occur. It is also linked to fatty liver disease in cattle (Hailemariam *et al.,* 2014). The serum samples in this study were analyzed with mass spectrometry for the L-carnitine concentration.

This study also classified the cattle according to the 5-point Fergusson-method. The Body condition score (BCS) of cattle has an impact on the fertility. It can be either direct for example high BCS has been linked with endometritis or poor quality of oocytes, or indirect via some disease like causing an increased risk for ketosis. According to studies if the BCS is too high there may be problems with reproduction in general (Olexikova *et al.*, 2017) and if BCS is too low there is also an increase in the risk of endometritis (Kadivar *et al.*, 2013). Thus, the risk for endometritis is increased in case of too high and too low BCS.

The hypothesis of this thesis was that there is a connection between oocyte quality, AMH and L-carnitine concentration in blood serum. This is evaluated with statistical analysis of the parameters chosen for the study.

1. LITERATURE REVIEW

1.1. Ovum pick-up and *in vitro* embryo production

The commercial production of *in vitro* embryos with ovum pick-up (OPU) has increased rapidly in the last 20 years within both dairy and beef cattle. The popularity of *in vitro* produced embryos is increasing while the popularity of *in vivo* produced embryos is staying at a constant level (Perry, 2016). The OPU-IVP technology is used to obtain more offspring from genetically superior females per year and to effectively use semen from expensive bulls or sex-sorted semen. It can also be used to get oocytes from calves and prepubertal heifers (Baruselli et al. 2016).

OPU can be done to the donors every 7 days or even twice a week (Viana et al. 2010). The procedure is done with a real-time B-mode ultrasound that has been equipped with a 7.5MHz micro-convex transducer and is connected to a follicular aspiration guide and a stainless-steel guide. The follicular fluid and oocytes are collected with a 18G hypodermic needle. All follicles over 5mm are punctured, and they are collected into the ovum pick-up media that is in a 50-mL conical tube via a silicon tubing system. The pressure is held using a vacuum pump that uses negative pressure between 50 and 80mmHg. Follicles should be aspirated one at a time. After aspirating the follicles, the system is washed with the OPU recovery medium to get all remains into the conical tube. (Cavalieri et al. 2018)

OPU can be carried out in donor animals for up to 100 days of pregnancy. After the collection of the oocytes they are sent to the laboratory for recovery, evaluation, maturation, fertilization and cultivation. After 7-8 days of *in vitro* cultivation, good quality blastocysts are selected for embryo transfer to recipients or vitrification. The recipients can be cows or heifers that are either synchronized into heat or have had spontaneous heat 8 days earlier. The other option is to freeze the embryos for later use. Frozen embryos have similar pregnancy rates as the ones produced *in vivo*, in case the quality of them is good before vitrification (Phillips *et al*., 2016). The average pregnancy rate for frozen embryos is approximately 50%. Fresh embryos have a higher pregnancy rate. It is between 50-60%

depending on the quality of the embryo (IETS, 2016). The results differ slightly according to source and whether looking at ideal circumstance results or farm-level results.

Sometimes IVP is done with synchronization prior to OPU with progesterone, gonadotropin-releasing hormone (GNRH), follicle stimulating hormone (FSH) or a combination. There is a large difference in the response of the cattle to the progesterone treatment. There is also variety of response to the other treatment protocols within cows, despite the fact that the protocols that have been enhanced over the years by developing different kinds of protocols for the donor cattle (Cavalieri et al., 2018). Different protocols for synchronization are for example two prostaglandin F2alpha (PGF 2α) injections in 11-day intervals (Getz *et al*., 2011). Another example of a synchronization protocol is inserting a progesterone-releasing intravaginal device and giving an injection of GnRH. 10 days later an injection of PGF 2α is given and two days later the intravaginal device is removed. On day 21 OPU is performed (Astiz *et al*., 2012). Some protocols give an injection of FSH on day 4 or 5 after insertion of the prostaglandin intravaginal device (Vieira *et al*., 2014).

The amount of good quality, transferrable embryos has not changed a lot during the years. This is because there is a lot of variation between individual oocyte donor cows. Some farms get a great result with OPU-IVP procedure and others don't. The results vary from none to 37 transferrable embryos per donor animal (Monniaux *et al*., 2009). Influencing factors on the success of OPU and IVP are health of the donor animal, for example body condition score, negative energy balance, metritis and mastitis (Boni R., 2012). The farm management also has an effect, for example the feed quality, rotten silage with toxins or too limited space per animal. Despite this, the reason why there are such large differences between the individual animals is still not fully understood.

1.2. Evaluation of oocytes

The fertilization rate of *in vitro* fertilization (IVF) in cattle is ideally estimated to be as high as 90%, but the calving rate ideally upon IVF is only 55%. The most dangerous time for the embryo is between the ages 8 to 16 days. In this time 70-80% of embryonic losses happen (Mossa *et al*., 2015). In order to get a healthy embryo, a healthy, good quality oocyte is needed. The quality of an oocyte is dependent on many different factors. These factors

comprise of health of animal, environmental effects, age and season (Boni, 2012). There are also different kind of ways to evaluate the quality of the oocyte. Some evaluations look at the ovary, the follicle, the cumulus cell complex or the oocyte. It can also be a combination of the above (Boni, 2012).

The oocytes can vary from each other according to the estrus cycle, age of the cells, hormonal status of the donor cow, the components of the follicular fluid, diameter of the follicle, atresia grade of the follicle and the morphology of the ovary and oocyte (Boni, 2012). All these factors have an effect on the outcome of *in vitro* production of embryos.

Oocyte quality can be assessed by looking at the functionality of it. The good quality oocyte is able to resume meiosis, produce the cleavage at fertilization, develop to blastocyst stage, bring pregnancy to term and result with a healthy calf (Boni, 2012). When using *in vitro* fertilization, the oocyte needs to be evaluated in an earlier stage. This is why different morphological features of the oocyte are evaluated after ovum pick up.

The estrus cycle does not have much impact on the morphology of individual oocytes, but the blastocyst production is highest in oocytes obtained in early luteal phase ovaries. The number and size of follicles in ovaries has also been studied. According to literature the lowest amount of cumulus oocyte complexes (COCs) was found in the ovaries that had the least follicles combined with the smallest follicles. Cows with small follicles also had the lowest number of blastocysts after fertilization when looking at developmental competence of the oocyte. (Boni, 2012)

Commonly light microscope evaluation of COCs are used to evaluate the quality of oocytes and embryos. It is a useful way to increase the blastocyst rate. Unfortunately, the cattle oocyte has dark ooplasm that limits the evaluation compared to humans that have a light ooplasm (Nagano *et al*. 2006). Some structures are not seen as well. The polar body, the shape of the oocyte and the diameter are somewhat covered by the cumulus cells. The oocytes can be divided into quality groups based on the cumulus cells. COCs with a complete and dense cumulus layer achieve good blastocyst rates (Figure 1). The COCs with incomplete or damaged layer have a lower outcome. Also, COCs which start expansion of outer cells and clumps in cytoplasm have good blastocyst rates. Even when the best COCs are used for IVP the blastocyst rates are ideally at 30-40% (Goovaerts *et al*., 2010).

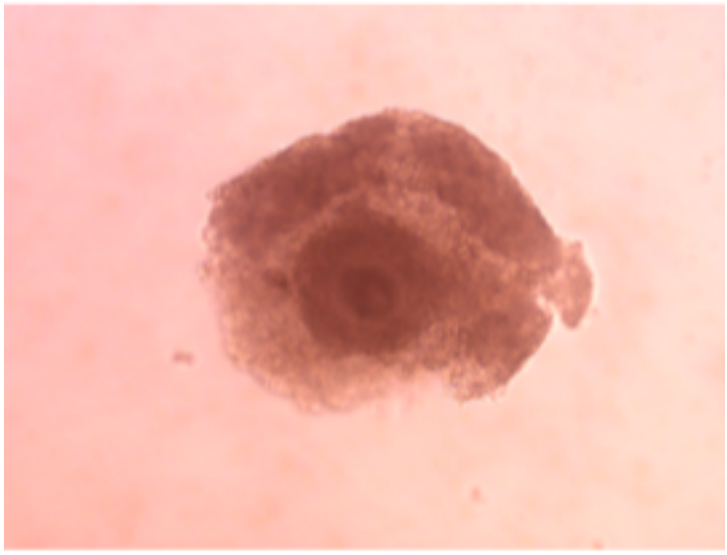

Figure 1. Oocyte with at least 3 layers of cumulus cells. (Photograph: E. Mark, 2017)

In comparison to the international embryo classification system (IETS) the classification of oocytes is more laboratory- specific (Stringfellow *et al*. 2010). They can be classified according to the morphology or amount of cumulus cell layers. Sometimes both are used in classification. The number of classification categories within a system varies commonly from 3 to 6. In OPU the classification is often focused on the COCs to monitor the quality of the OPU procedure. For example, the oocytes can be classified from I to III, were I is fully surrounded by cumulus-cells and III is without any cumulus cells. (Merton, 2013).

1.3. Body condition score and fertility

The body condition score (BCS) of cattle has an effect on the quality of oocytes and embryos. A study was made about the quality of preimplantation embryos in relation to donor animal body condition. The results showed that if the BCS is over 3.0 (Ferguson method) the incidence of poor oocytes is higher. There were more fragmented embryos and also fewer grade 1 embryos (Makarevich *et al*., 2015).

In over conditioned cattle the structure of the cells is different compared to cattle with a lower body condition score. The cattle with a body condition score 4.0 – 5.0 have a high lipid content in the cytoplasm (Olexikova *et al*., 2017). They have more lipid droplets in the cells and this can be one of the reasons for their subfertility and poor oocyte quality.

It is also not desirable that the cow has a body condition score of under 2.75 at the time of ovum pick up because BCS under this decreases fertility on average. Cattle with a low body condition score have an increased risk for postpartum endometritis (Kadivar et al., 2013). Cattle with BCS 3.5 or higher at parturition had less endometritis compared to the ones who had BCS less than 3.5 at parturition. The BCS at 4 weeks after parturition was 2.75 or higher in the cattle with less endometritis. The group with more endometritis had BCS less than 2.75 four week after parturition according to the study. After endometritis the ovaries take longer time to return to normal activity. It can also cause subfertility after the disease has passed (Kadivar et al., 2013).

According to these articles a BCS of over 3.0 at peak lactation would impact the quality of embryos negatively (Makarevich *et al.,* 2015). Then again, the BCS below 2.75 at peak lactation would increase the risk of endometritis and thus subfertility (Kadivar *et al.,* 2013). When comparing the articles, it seems like the ideal BCS for the oocyte donor animals for OPU-IVP would be 2.75-3.0.

1.4. Anti-Müllerian hormone

Anti-Müllerian hormone (AMH) is produced by the gonads. In males it is secreted from the Sertoli cells. It is needed for embryonic sex differentiation, causing the regression of the Müllerian ducts (Josso *et al.,* 1993). In females it is released by the granulosa cells, that are produced by growing ovarian follicles, also known as antral cells or secondary follicles.

AMH has two functions. It inhibits the growth of primary follicles from the follicle reserve, so they would not all be used prematurely. It also reduces the response to FSH for preantral follicles, so they can still continue to develop. AMH is a marker for the ovarian reserve of follicles. This is because AMH is only released by healthy and growing follicles. It means that it indirectly indicates the amount of healthy growing follicles. (Mossa *et al*. 2017)

The plasma levels of AMH have been positively associated with many different measures of fertility. Cattle with high AMH levels had a higher pregnancy rate, and thus they were less

frequently culled after the first calf. The levels of AMH are also an indicator of the superovulation response. (Souza *et al*. 2015)

After birth the level of plasma AMH increases in the first months and then decreases until puberty. At puberty the levels increase and stay quite stable. There is no correlation in AMH levels and parity. Unlike humans or mice, the levels do not decrease as the cow ages (Mossa *et al*., 2015). The levels of AMH in individual animals stay rather stable within the estrus cycle. There is a small decrease in the levels of AMH a week after ovulation. This decrease is so small that cattle with clearly high and clearly low plasma AMH levels can't be differentiated. (Vernunft *et al*., 2015)

The AMH levels of an individual can be lower due to negative factors which occurred during fetal development. These factors are for example the low body condition score of the mother, mastitis during pregnancy or because of the excess of testosterone. The factors cause the ovarian reserve to be negatively programed and in consequence the AMH and fertility are also decreased. The amount of AMH is typically higher in beef cattle compared to dairy cattle. The amount is also dependent on the breed or genetic group in question. (Mossa *et al*., 2017)

According to literature prenatal programming could also have an effect on the future fertility of the calf. Programming of the fetus is affected by the environment that the fetus lives in. It has an effect on several factors for the adult cow, for example development, physiology, diseases and immunity. Stress factors like malnutrition or disease of the mother can have a big impact on the programming of the reproductive system of the unborn calf. (Mossa *et al*. 2017)

In a study calves born from mothers that have had a restricted diet in the first trimester of pregnancy have a smaller ovarian reserve (Mossa *et al*., 2017). Their AMH levels were lower and they had a lower antral follicle count (AFC). AFC is the number of secondary follicles growing per follicular wave. The calves whose mothers had a restricted diet also had a high FSH concentration. This is typical for animals with low AFC. Studies show that overfeeding also has an effect on the programming. The fetal ovarian follicle development is decreased in these cases and causes a lower AMH level in adulthood. These studies show that the

nutrition of the mother have a significant effect on the ovarian reserve and AMH in their offspring's adulthood. (Weller *et al*., 2016)

The AMH of the offspring is also affected when the mother has a chronic mastitis during the gestation, particularly in the cases where the mother has a high somatic cell count for a longer period during the gestation. The concentration of AMH in their daughters as adults is sub-average. If a cow has several measurements of the somatic cells count over 200 000 during the pregnancy the AMH is found to be lower in their daughters. This leads to their daughters having less high-quality oocytes in ovaries and less ovarian function. Their AMH concentrations in serum were 0.015- 0.04ng mL. The daughters of the non-chronic mastitis mothers had AMH serum concentrations of 0.06-0.11ng mL. (Ireland *et al*., 2010)

Alterations in AMH can be seen between breeds and types of cattle. For example, Holstein and Jersey cattle have some difference in the size of ovary, AMH concentration and number of follicles. The variances between beef cattle and dairy cattle are larger. The amount of AMH in blood plasma is thought to vary between different breeds and genetic groups of cattle. It is still unclear and unsupported by evidence if there is a correlation between the ovarian reserve and AMH between different breeds. (Ribeiro *et al*., 2014)

When discussing viable or transferrable embryos it is important to keep in mind that the high oocyte rate per OPU session is not always in correlation with high number of viable embryos. The oocytes must survive the transport, the spermatozoa must survive cryopreservation, oocytes must be fertilized and develop in *in vitro* conditions into the blastocysts. Many hormones, growth factors and different cell interactions affect this development into transferrable embryos. (Vernuft et al. 2015)

In literature there are slightly different results about the selection of oocyte donors for OPU based on AMH concentration in blood. Vernuft *et al*., 2015 found that there is a correlation between the plasma AMH levels and oocyte quality. But the difference is in the amount of correlation found. The Vernunft *et al*. article states that the results of AMH plasma correlations are too low to be used as a precise predictive parameter of successful selection of OPU donors. The authors implied that it can help to identify groups of very good and very poor donors (Vernunft *et al*., 2015). Guerreiro *et al*., *2014* found that AMH can be used as an accurate endocrine marker for *in vitro* embryo yield and a possible predictor of *in vitro*

embryo production per donor animal. The question is whether AMH levels are better to be used as a marker for groups of animals or if it is useful also in selection of individual donor animals. The newest research implies that AMH measurement for a single animal would be useful for donor selection and this could help intensify breeding (Fushimi *et al*. 2019).

1.5. L-Carnitine

Carnitine has an important part in the energy metabolism of cattle. It is an amino acid and derivative of lysine. L-Carnitine takes part in the transport of long-chain fatty acids (LCFA) from the cytoplasm to the mitochondria. In the mitochondria the fatty acids are broken down. (Ringseis *et al*. 2018)

There are two types of carnitine, L-carnitine and D-carnitine. They are each other's enantiomers and L-carnitine is the active form of carnitine. Some of the carnitine can be made from lysine and methionine, but most of it comes from the feed. Also, the excretion of carnitine plays a role in regulation of it. L-carnitine is a general name for L-carnitines. There are several different carnitine compounds such as acetyl- L-carnitine and propionyl- L-carnitine. (Ringseis *et al*., 2018)

Some farmers use L-carnitine as a supplement in the feed of cattle. The studies show that the supplementation of carnitine is quite ineffective when used to increase the milk production (Ringseis *et al*., 2018). However, according to the studies in the case of fatty liver and prevention of fatty liver, the use of carnitine supplements can be useful, especially if the cow is in negative energy balance. The supplementation has been seen to be useful in the case of growing cattle where the feed balance is not correct. This is the case where the feed has too much non-protein nitrogen. If the diet has a sufficient amount of crude-protein, the carnitine for growing cattle is unnecessary. (Ringseis *et al*., 2018)

Studies have also identified carnitine to be a potential marker for disease before the clinical disease presents. Studies show that high carnitine levels in cattle can be indicative up to four weeks before clinical disease. The diseases that followed the high levels were periparturient diseases like metritis, mastitis and laminitis. (Hailemariam *et al*., 2014)

Fatty liver disease is a common disease affecting up to 50% of cattle about one-week postpartum. It occurs because the lipolysis increases due to declining dry matter intake and increased milk production. The lipolysis of adipose tissue causes the hepatic uptake of fatty acids to be too high. The liver starts to store the fatty acids. This in turn causes fatty liver and decreasing metabolic function of the liver. Fatty liver causes the health and reproductive functions of the animal to decline. (Olagaray K.E. *et al*., 2018)

Despite that L-carnitine is degraded in the rumen to some extent, the administration of L-carnitine in the diet is useful for the prevention of fatty liver. From 3 weeks precalving to 1 week post calving the plasma free carnitine decreases and acetyl carnitine increases. The total amount of carnitine is however decreased post-partum compared to precalving. The bioavailability of L-carnitine administered into the rumen is unclear, but studies show that abomasal administration has a better bioavailability. The ideal would be to use a rumen-protected product that is not over-encapsulated, so it could absorb from the abomasum and intestine. (Olagary *et al*., 2018)

The use of carnitine in *in vitro* growth medias has been widely researched. According to studies the use of L-carnitine in the *in vitro* maturation medias improves the development of less competent bovine embryos (Knitlova *et al*., 2017). L-carnitine is a lipid metabolism regulator. They can be added into the growth medias to improve the development and quality of embryos. The study had separate groups of meiotically more competent (MMC) and meiotically less competent (MLC) oocytes. The MLC oocytes are from small follicles, under 5mm in size. The study looked at maturation, fertilization and embryo development. According to the study, the L-carnitine has an effect on the oocyte maturation in the MLC group. In the group of MMC oocytes there was not a difference. The lipid content of the MLC is higher in the group that does not have added L-carnitine compared to the MLC group with additional L-carnitine in the growth media. (Knitlova *et al., 2017)*

The L-carnitine in the media also had an effect on the fertilization of the oocytes of the MLC class. The number of oocytes fertilized at the stage of syngamy was looked at. The syngamy stage is when two gametes form a zygote, also known as fertilization. In the MMC group there was no significant difference between the fertilized oocytes. Interestingly the media with L-carnitine in the MMC group had a smaller number of oocytes in the syngamy stage. (Knitlova *et al*., 2017)

The study also indicates that the effect of L-carnitine on embryo development was not seen on day 2 after cultivation. The cleavage rate was the same in MMC and MLC with or without carnitine. On day 7 in the MLC group the L-carnitine supplemented oocytes that had developed into early blastocysts were clearly higher compared to MLC group without L-carnitine. The same group had more blastocysts on day 8. In the MMC group there was no significant difference in the day 7 early blastocysts and day 8 blastocysts. Despite this in the MMC groups the number the of expanded blastocysts on day 8 compared to the total amount of blastocysts was higher in the group with L-carnitine in the media. The study also states that the L-carnitine in the media does not seem to have an effect on the differentiation of the blastocyst in either the MLC or the MMC groups. (Knitlova *et al*., 2017)

The function of L-carnitine in growth media is connected with the lipid content of the embryo. The cytoplasm has a darker color when the content of intra-cellular lipid content is high. It is believed that this dark color is because of mitochondrial function, that is not working well. The use of L-carnitine in the media reduces the amount of intracellular lipids due to the transport function. L-carnitine transports the intracellular lipids into the mitochondria that turns them into ATP energy. (Baldoceda *et al*., 2014)

The amount of reduction of lipid content is also dependent on the breed. Adding L-carnitine to growth media has a bigger impact on the Holstein cattle oocyte and embryo metabolism in comparison to Jersey cattle. The effect of L-carnitine in embryos obtained from Jersey cattle is weaker and more variable. There is a clear connection in the genetic background and embryo metabolism. (Baldoceda *et al*., 2014)

2. AIMS OF THE STUDY

The aim of the study was to evaluate the level of AMH and L-carnitine in Holstein OPU donor cows and compare the results with donor oocyte quality, follicle count, body condition score and pregnancy status. The second aim was to look for parameters which correlate with oocyte quality and quantity. The specific aims were:

1. To compare blood serum AMH and l-carnitine levels to oocyte quality, BCS and pregnancy status of a donor animal
2. To determine the factors affecting the number of follicles punctured during OPU, COCs aspirated and the quality of COCs

The hypothesis of this thesis was that there is a connection between oocyte quality, AMH and L-carnitine concentration in blood serum.

3. MATERIALS AND METHODS

3.1. Animals

The study was carried out in the end of 2018(31 October- 28 November 2018). It was conducted on 24 Holstein cows that were taking part in the ovum pick up program in a dairy farm in Tartu county. The farm has 630 cows and the milk production was in the year 2018 11937kg per cow. OPU was performed once a week. Nine of them were pregnant (day 44-85) and 15 non-pregnant (46-68 days post-partum). The cows were aged between 3 to 6 years old (1176-2255 days old). Body condition of the animals was evaluated on the day of OPU. The sample size was chosen by number of animals taking part in OPU in the study farm during the study period. The program had 6 donor animals per week for OPU. Some weeks previously sampled animals would come for OPU again. One cow would only be sampled once for this study. All together the number of samples was 24.

3.2. Blood collection and analyses

On the day of OPU blood samples were taken from the cows. Two 4ml vacuum clot-activator serum tubes (Becton Dickinson, Franklin Lakes, NJ, USA) were taken from each animal from the coccygeal vein. The samples were let to clot for 30 minutes. After clotting, the samples were centrifuged for 10 minutes at 1500xg. After this the serum was separated and stored at -20C until analyses.

For AMH evaluation the serum was sent to Laboklin (Laboklin, Bad Kissingen, Germany). AMH was determined with enzyme-linked immunosorbent assay (ELISA).

L-carnitine analysis was performed in University of Tartu using a mass spectrometry method. The protocol for L-carnitine analysis was taken from a study by Schulze A. *et al.* 2003 which was used to screen inborn errors of metabolism in newborn human babies by electrospray ionization-tandem mass spectrometry. It has also been used in a study by

Giesbertz *el al.* 2015 as a method to quantify acylcarnitine species. This method can also be used in cattle and has been used in studies about cows fatty acid metabolism (Rico et al. 2018).

3.3. Ovum pick up

To obtain oocytes from the donor cows the ovum pick-up technique is used. Before the procedure feces is removed from the rectum and the perineal area is cleaned with water. The donor cows are restrained and given epidural anesthesia to decrease peristalsis and discomfort with Xylazine (0.05mg/kg body weight) diluted in 5ml of saline. Follicular puncture was performed using the transvaginal ultrasound-guided OPU technique a real time B-mode ultrasound scanner (Hitachi Aloka Prosound 2) with a 7.5-MHz multiangle transducer. All visible follicles 5 mm and bigger were punctured using an 18-g disposable, single lumen needle (SUPRA® 18G1½1 1,20 × 75mm, Vivomed GmbH, Germany). Via a stainless-steel connector, the needle was connected to 90-cm siliconized tubing (ID 1.57 mm, OD 3.18 mm, NIFA, Netherland). Before aspiration, the tubing was flushed with OPU recovery medium. A vacuum of 50 mmHg, corresponding to a 20–25 ml/min flow rate, was used to aspirate the follicular content. The vacuum was created using a vacuum pump with continuous flow. The aspirated fluid was collected into a 50mL conical tube (Figure 2). The procedure of looking for the follicles includes magnification of the ovary in order to get a better view. Looking for the laminar flow in which the COCs and fluid moves is also essential. In case the vacuum is too high the laminar flow can turn into a turbulent flow, this exposes the COCs to uneven forces. After the oocytes have been collected, the tubing system is flushed with OPU recovery medium (Figure 3). Follicular fluid was kept in a portable incubator at 37 ^{0}C and taken within one hour to the embryo laboratory in Estonian University of Life Sciences. Oocytes were recovered under a stereo microscope.

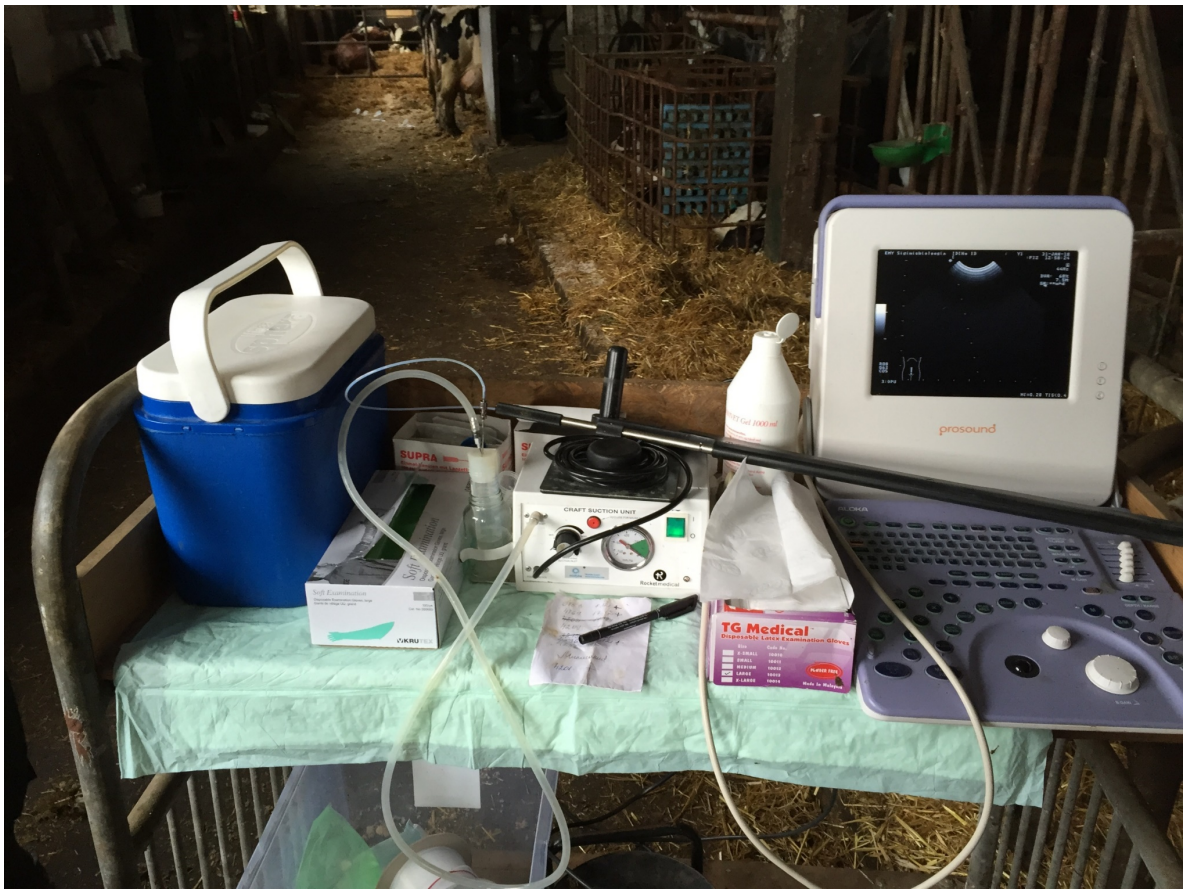

Figure 2. OPU on farm equipment: thermal case, conical tube, silicon tubing set, aspiration unit, micro-convex ultrasound transducer, ultrasound (Photograph: J. Kurykin, 2018).

Figure 3. OPU procedure at the farm. Transrectal fixation of the ovaries and transvaginal ultrasound guided aspiration of follicles with negative pressure vacuum-pump (Photograph: E. Mark, 2017).

Once in the laboratory the number of oocytes was counted as part of the *in vitro* maturation process. Oocytes were also morphologically classified into categories according to their quality. Morphological classification of the oocytes looked at the number of cumulus layers, colour, homogenicity, integrity, expansion of the cumulus cell and cytoplasm colour. The oocytes were categorized as Q1(high quality, at least 3 layers of cumulus), C2(Only 2 layers of cumulus cells), C1(only one layer of cumulus cells), C0 (no cumulus cells) and D(degenerated).

3.4. Body condition scoring

Body condition scoring of the donor animals were performed on the day of OPU at the farm. The method used was the Ferguson method, a 5-point scale with 0.25-point specificity. Score

1 is the lowest, 5 is the highest and 3 is an average condition. Animals with BCS 1 are the thinnest, they are described as having excessive loss of body condition. Cattle with BCS 5 are described as having excessive body condition. The focus of the evaluation is on the loin and rump. Certain landmarks like short ribs, hooks, thurl, pins, sacral ligament and tail head ligaments are used to make the differentiations. (Ferguson *et al*. 1994)

3.5. Statistical analysis

The data evaluated in statistical analysis included age, body condition score, last parturition, last insemination, pregnancy status non-pregnant or pregnant (NP or P), days postpartum, follicle count, oocyte number, AMH concentration, carnitine concentration and quality grade of oocyte. Figures were constructed from linear correlation analysis to measure the relationship between studied variables. LOWESS (locally weighted scatterplot smoothing) was used to discover potentially nonlinear relationships. The Wilcoxon test was used to evaluate statistical significance between pregnant and non-pregnant cows' differences.

The charts and tables were made with Microsoft Excel. All results were considered statistically significant at $P \leq 0.05$ and statistical analyses were performed with R version 3.5.3 (R Foundation for Statistical Computing, Vienna, Austria).

4. RESULTS

4.1. Ovum pick up, oocyte yield and quality

In total 61 oocytes were assessed for their quality. The most oocytes out of the 61 oocytes were in group Q1(31), C2 had 16, C1 9 and C0 2. There were 3 degenerated ones (table 1). There is a positive correlation between the quality of the oocyte and the serum AMH concentration. The correlation value is 0.73 (p=0.059). The carnitine concentration is correlated positively(r=0.31) with the oocyte quality, but it is not statistically significant (r=0.84).

Table 1. The quality of obtained oocytes

Q1	C2	C1	C0	Degenerated	Total
31	16	9	2	3	61

The mean number for punctured follicles per OPU session per pregnant donor animal was 6.4 ± 2.6. In nonpregnant cows the mean was 6.5 ± 4.3 punctured follicles per session (p=0.741 Wilcoxon test). The mean for recovered cumulus-oocyte complexes in pregnant animals was 3.3 ± 2.2 and for non- pregnant animals 2.1 ± 1.8 (p=0.171). In pregnant cows 58 follicles were punctured, and 30 cumulus-oocyte complexes recovered, this was 51.7% out of follicles punctured. In non-pregnant cows, where 98 follicles were punctured and 31 COCs aspirated, it was 31.6%.

4.2. Anti-Müllerian hormone concentrations

The concentrations of anti-müllerian hormones in the study group ranged from 0.03 to 0.62ng/ml. The average AMH concentration in serum was 0.25ng/ml. Most of the samples had an AMH concentrations in the range of 0.1-0.3ng/ml (Figure 4).

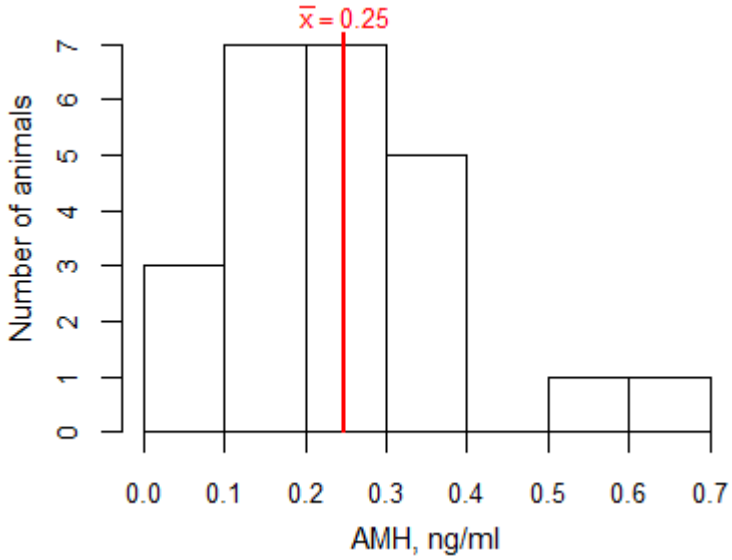

Figure 4. Distribution of AMH concentration; vertical red line and numerical value denote the average.

Pregnant animals had higher concentration of AMH in their serum. In pregnant animals the average AMH concentration was 0.28 ng/ml and in nonpregnant animals 0.23 ng/ml (p=0.255). The length of pregnancy had a positive correlation to AMH concentration in the serum (r=0.2), but it was not significant (p=0.354).

4.3. L-carnitine concentration

The concentrations of carnitine ranged from 1.87 to 4.66mmol/L. The average was 3.07mmol/L (Figure 5).

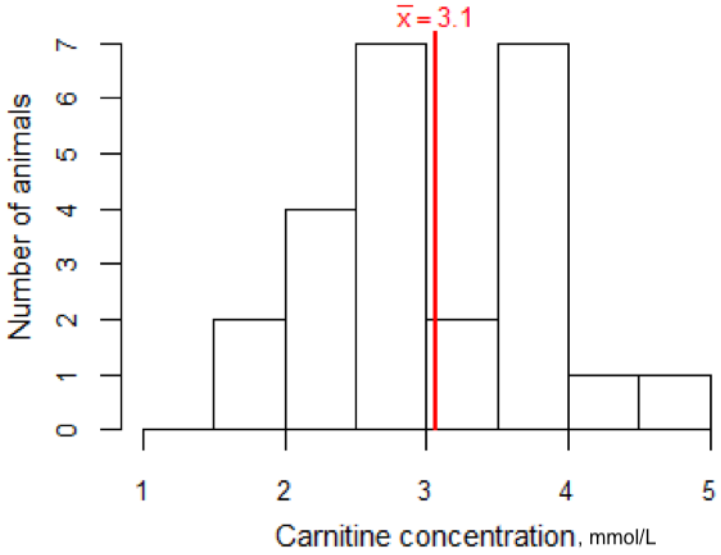

Figure 5 Distribution of carnitine concentration(mmol/L); vertical red line and numerical value denote the average.

There was a slight positive correlation (r=0.17) with L-carnitine and age that is statistically non-significant with the p-value 0.416 (Figure 6).

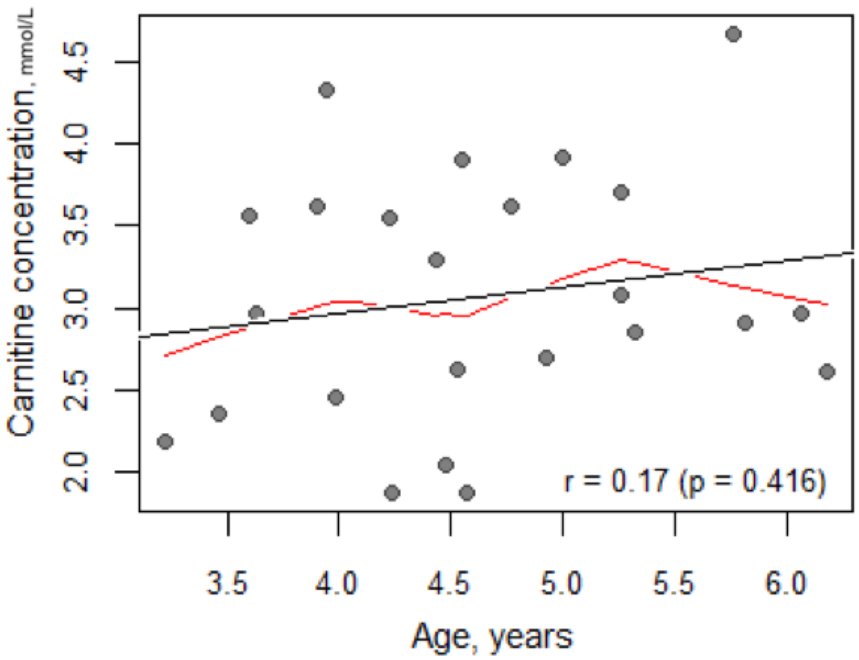

Figure 6. The relationship between L-carnitine(mmol/L) and age. The black line indicates linear relationship and red line fitted LOWESS curve; numerically are presented linear correlation coefficient with p-value.

The L-carnitine concentration increased in positive correlation(r=0.24) with pregnancy days, but it was not significant(p=0.255) (Figure 7).

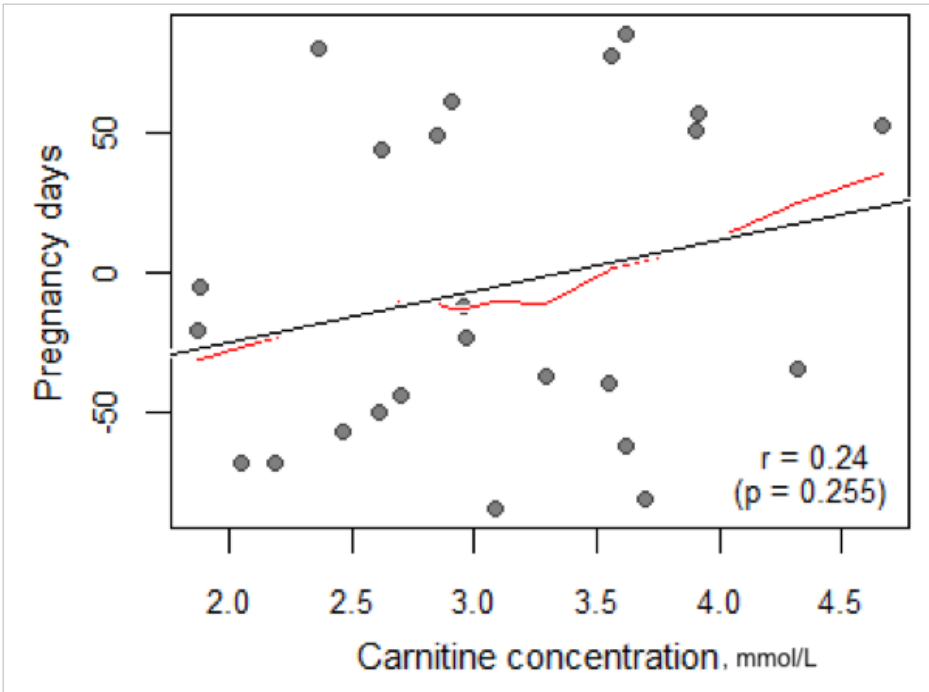

Figure 7. The relationship of carnitine concentration(mmol/L) and days pregnant.

There was a negative correlation (r=-0.35) between the follicle count and L-carnitine concentration in serum (Figure 8). The p-value between this correlation was p=0.091.

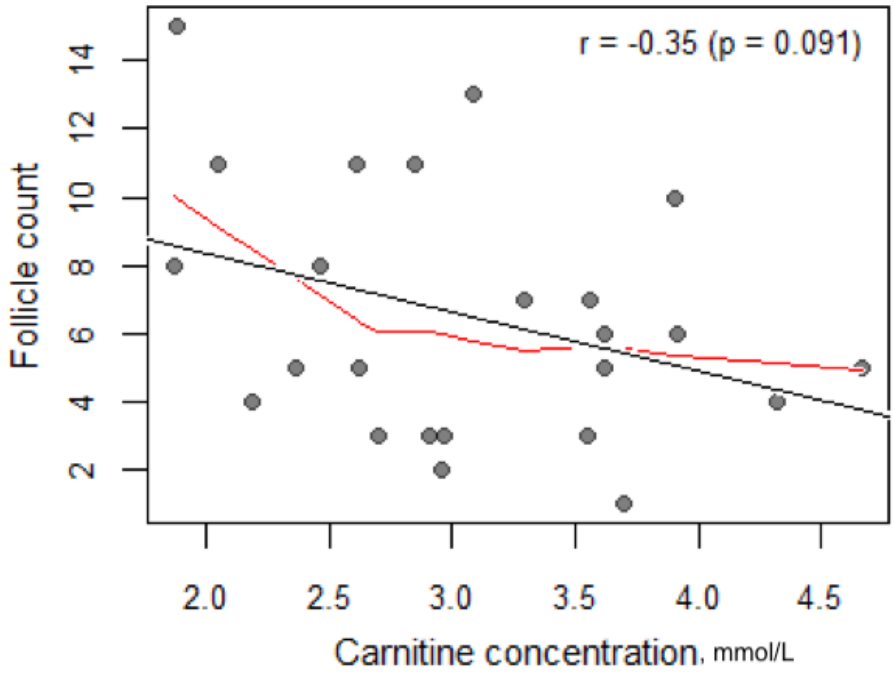

Figure 8. The relationship of carnitine concentration and follicle count.

4.4. Body condition score

The BCSs of the animals in the group ranged from 2.75 to 3.5. The strongest correlation for the body condition score was related to age. The correlation was negative, and it is statistically significant (r=-0.49, p<0.05). There was also a slight non-significant correlation for AMH concentration and BCS (r=0.13, p=0.560). BCS had a slight correlation with how far along the pregnancy was. The correlation value was 0.17 and the p- value 0.433.

5. DISCUSSION

Often the farms best animals are chosen for the OPU program, since multiple offspring want to be obtained from this animal. Criteria for choosing animals for the program can be for example production level, health, body conformation, temperament, high quality offspring, genetic background or a combination of them. Due to the costs and labor of producing embryos it is important that the success rate of the OPU would be as high as possible.

The study only used the same animals once, but it is a common practice to use the same donors repeatedly. According to a study high repeatability can be considered to increase *in vitro* embryo production. This study also states that the donors with high number of COCs retrieved would have a higher blastocyst rate (Monteiro *et al*. 2017). After the donor cows have been sampled for AMH and the oocytes graded, the best animals could potentially be used for several weeks in a row to maximize the outcome.

The limitations of the study were the number of animals used. Only a certain number of animals were used in the OPU program during the study period. It would be beneficial for the study to have more than 24 animals participating in it, so that more of the results would be statistically significant, especially when comparing sub-groups like pregnant and non-pregnant animals.

The body condition scoring was done mostly by one person. It is known that the Ferguson method guidelines are not fully objective. It would be preferable to have several people assess the BCS and then calculate the average or use a more objective method of evaluating the BCS. The Ferguson method has a moderate test value according to a statistic called Kappa. Despite this, if a 0.25 difference between evaluators is accepted the Ferguson method has a higher value as a diagnostic test (Ferguson, 1996). Also, the animals in this group did not have remarkable differences in BCS. They were all in the categories 2.75-3.5. Perhaps if there were larger differences for example a very high BCSs of over 4.0, the results could have been more significant.

According to literature, a good candidate for OPU would be within a body condition score of 2.75 and 3.0. This is because if BCS is too high (>3.0) the quality of oocytes is negatively impacted (Makarevich *et al*.2015). If BCS is too low (<2.75) there is an increase in the risk of endometritis and thus subfertility (Kadivar *et al*. 2013). When talking about BCS, it is important to note the time of the yearly cycle of the cows we are looking at. The cows in our study were in the early pregnancy and post-partum time. In this period the cows are at their thinnest. When evaluating the dry cows, the BCS is much higher. The body condition score did not have any significant relations to AMH or L-carnitine in this study. It did have a strong correlation to age, but this is quite self-explanatory.

According to the results the AMH concentration average was higher in pregnant animals(0.28ng/ml) than non-pregnant animals(0.23ng/ml), p=0.255. Despite the result not being significant, this is supported by the other results. The amount of aspirated COCs per number of follicles punctured. In pregnant cows where 58 follicles were punctured, and 30 cumulus-oocyte complexes aspirated, it was 51.7%. In non-pregnant cows 98 follicles were punctured and 31 COCs aspirated, it was 31.6%. The amount of COCs per punctured follicle is prosentually higher in pregnant animals. Previously studies have shown that the reproductive phase may influence the developmental competence of the recovered oocytes. The study also states that the quality of pregnant cows ooplasm in the oocytes is higher compared to non-pregnant animals ooplasm (Takuma *et al*. 2010). This supports the result of our study, that the pregnant cows would possibly be better OPU donors compared to non-pregnant cattle.

There is a great deal of literature and many studies done on L-carnitine as a supplement in feed and as a component in growth medias of *in vitro* embryos. However, there are currently no articles addressing whether there is a correlation between the amount of L-carnitine in blood serum and the oocyte or embryo quality. Carnitine concentration had a negative correlation to (r=-0.35, p=0.09) the follicle count. Despite this the quality of the oocytes had a positive, yet not significant correlation to carnitine. According to the results carnitine seems to have a negative connection with the follicle count in the ovaries. Studies have identified carnitine as a potential marker for diseases like metritis, mastitis and laminitis before the clinical disease itself. This can be explanatory for the lower follicle count since sick animals are less fertile. The number of follicles is not always directly an indicator for fertility like oocyte count and oocyte quality, but it can give us some indication. This is also

supported by the fact that cattle with a high antral follicle count have been shown to be more likely to develop embryos into blastocyst stage (Sakaguchi et al. 2018).

Animals with high AMH concentrations and animals that are pregnant seem to have better-quality oocytes. Also, animals with a high serum carnitine concentration are less likely to have a high number of follicles. The AMH result of our study is in line with a newly published study from Fushimi *et al*. 2019 that concluded that single measurement of plasma AMH is an informative way of choosing OPU-donors in breeding programs. Animals with high AMH concentration have been shown to have more healthy follicles in ovaries. This is because AMH is only released by healthy, growing follicles. The measurement of serum AMH is also a very reliable indicator since the levels stay quite stable in an individual animal during different phases of estrus (Vernunft et al. 2015).

AMH clearly has a correlation between many factors of fertility. It is also proven that the blood serum concentration of AMH has a positive correlation to donor cow oocyte and embryo quality. It has been unclear if the parameters should be used for dividing different oocyte donor animals into groups of likely good donors and likely bad donors or is it also useful for selecting individual donor animals for OPU. According to the results of this study and other recent studies it seems that AMH could be reliably used at individual animal level.

The cattle used for OPU should be of good general health because diseases like mastitis, endometritis and feet problems effect fertility. According to studies ovarian, embryonic and uterine function is affected negatively. They are affected directly by bacterial products like endotoxins or indirectly by inflammatory mediators like cytokines, nitric oxide and this causes oxidative stress. The direct or indirect effects of endometritis and inflammation in general can disturb reproduction in many crucial stages (Gilbert, 2011). In the future it would be interesting the look at some inflammatory markers in comparison to AMH, L-carnitine and oocyte quality.

According to the results of our study the number of follicles was lower in animals that had a high L-carnitine concentration in their serum. The fewer follicles there are to puncture during OPU, the less likely it is to obtain oocytes. Health problems in cattle can cause lower fertility. This is in parallel with studies that say that high values of carnitine are indicative of future health problems of up to four weeks before clinical disease (Hailemariam *et al*.,

2014). Diseases like metritis, mastitis and laminitis have a connection with low fertility. Measuring the L-carnitine of OPU candidates could also be potentially beneficial, but there are not many commercial tests that are easily available at the present time. This also needs to be studied further, since there are not many studies about serum carnitine levels in cattle in relation to fertility. Perhaps, in the future, there will be a simpler commercial test for measuring serum carnitine.

When choosing animals for the OPU program the genetics of the animal also has an important role, since the ultimate goal of OPU is to get superior offspring from the best cattle. Along with these genetic factors it would be advisable to look at the general health, AMH level, and possibly carnitine concentration, pregnancy status and body condition of the OPU candidate. Also, the latest studies, along with the results obtained above, suggest that a single AMH measurement for OPU donor selection could be advisable.

6.CONCLUSION

In conclusion, this study partly supported the hypothesis that there will be a connection with the oocyte quality and AMH. The correlation was 0.73 and the p- value 0.059. The study did not support the hypothesis of the connection between oocyte quality and L-carnitine since while the correlation was positive (r=0.31), the result was non-significant (p=0.84).

The AMH concentration average was 0.25ng/ml (0.03-0.62 ng/ml). The results also indicated that pregnant animals had a higher AMH in comparison to non-pregnant animals. AMH in NP animals was in average 0.23mg/ml and in P animals 0.28ng/ml (p=0.255). Pregnant animals also had a higher percentage of COCs aspirated out of follicles punctured (51.7%) compared to the non-pregnant animals (31.6%).

The L-carnitine concentrations average was 3.07mmol/L (1.87-4.66mmol/L). There was a slight positive correlation with L-carnitine and age of the donor animal (r=0.17, p=0.416). There was also a positive correlation with how far pregnant the donor animal was and L-carnitine (r=0.24, p=0.255). Comparison of blood serum carnitine revealed that follicle count was lower in animals that had a higher L-carnitine concentration in the serum. This result had a negative correlation (r=-0.35) and the p- value was 0.091.

BCS did not have a significant correlation with AMH or L-carnitine. There was a statistically significant correlation to the BCS and age (r=-0.49, $p<0.05$). Due to the small study group, especially in the pregnant and non-pregnant groups, a study with a larger sample size would be recommended to obtain more significant results.

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amh

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