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for International Students
Faculty of Veterinary Medicine and Animal Science
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The present thesis is a partial fulfilment of the requirements for a Master of Science Degree in Veterinary Medicine for International Students at the Swedish University of Agricultural Sciences (SLU), in the field of Animal Reproduction.

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To my family and all those who have made me smile…

ABSTRACT

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The repeat breeding problem in cattle is widespread among all the heavy dairy breeds in cattle and is being considered as a major problem throughout the world. The aetiology of the repeat breeding is of various origins. It is notorious for causing disturbance in the overall cattle health leading to lowered fertility and economic loss in dairy herds. Repeat breeding is caused by fertilization failure or early embryonic death, and the reasons for the reproductive failure may be due to various disturbances occurring at different sites along the genital tract, including the delicate hormonal regulation system. There are many diseases and disturbances held responsible for repeat breeding. Even intrinsic factors, such as oocyte quality, have been found to cause repeat breeding. Body condition and body weight also have an impact on fertility in cattle. Body condition scoring helps to isolate external scores and relate it to the internal condition of the animal and hence forms a good indication for grading the cattle. It has been observed previously that repeat breeder heifers (RBH) often are over weight and fat. For the present study, three RBH and two virgin heifers (VH, controls) of the Swedish Red breed were selected. The immature oocytes were collected by ovum pick-up twice weekly during five weeks. The oocytes were classified quality-wise and further examined under transmission electron microscope, TEM. Similar numbers of follicles were available for puncture in the ovaries of RBH and VH, but significantly more ($P<0.05$) of the follicles were aspirated for retrieval of oocytes in RBH (76.4 %) than in VH (64 %). There was a numerically higher occurrence (NS) of low quality oocytes collected from RBH (60 %) than from VH (52 %). The occurrence of cytoplasmic vacuoles and lipid droplets was similar in oocytes from RBH and VH with a tendency ($P = 0.07$) for more lipid droplets in RBH. The VH oocytes as a group were more uniform compared to RBH that displayed a great variation especially in numbers of lipid droplets. The most prominent difference between RBH and VH oocytes was the significantly higher total lipid amount present in the cytoplasmic lipid droplets in RBH oocytes ($P<0.001$), which was clearly visible as heterogeneous cytoplasm already under the stereomicroscope and confirmed by TEM. The results indicate that there are morphological differences in the appearance of RBH and VH oocytes, particularly due to higher lipid content in the cytoplasm, which can indirectly explain the low oocyte quality and the sub fertility in RBH.

Keywords: *repeat breeding, dairy heifer, body condition scoring, oocyte quality, lipids*
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Background

Repeat breeding in general

Repeat breeding cattle is the worst nightmare of the dairy owner. The main economics of the farm depends on the milk supply of that farm. As the modernization was taking place, so a bigger infrastructure was needed to organise a chain of distribution of the livestock resources in a large scale to the increasing population. The emergence of the selective breeding and genetic up gradation was being applied to increase the quantity of products from the livestock. But in the high lactating dairy cows there is a strong unfavourable genetic relationship between the milk production and the fertility (Oltenacu et al. 1991). This was noted far in future that the high yielding dairy cows are more prone to be repeat breeder. But to cater the demands of the increasing population more high producing cattle are put in for milk production. The increased demand of the milk due to globalisation and the other dairy products was getting disturbed by several reasons. There are plenty of reasons which pose a hindrance in the smooth functioning of the farm. Apart from the clinical, metabolic, physical etc reasons, one of the problems was recognised which was found to be the most prevalent in dairy cattle throughout the farms was repeat breeding. Repeat breeding has been described as a major concern in cattle throughout the world (Lucy 2001). Repeat breeding itself can have a wide definition as it may encompass all the problems which affect the reproducing capacity of the animal. The current definition of the repeat breeder animal is besides being inseminated three times or more without becoming pregnant (but returning to oestrus within normal intervals), they had no apparent pathologies explaining the repeat breeding, neither at palpation per rectum or at ultrasound examination of the genital tract (Båge et al. 2002a; Båge 2002).

Repeat breeding in dairy cattle

The phenomenon of the repeat breeding challenges the normal reproductive output of the farm and reproductive efficiency is essential in the profitable dairy farms (Nebel and Jobst, 1998). The repeat breeding subject was broadly studied around the world. In broader terms, repeat breeding in cattle is a multidimensional malady; it encompasses several aetiologies and signs under its domain. These include structural or chromosomal disorders, infections implicating in transient or permanent infertility, embryonic mortality, hormonal imbalance, environmental conditions, lactation, nutrition, management etc. In Sweden about 10 % of the dairy cows are culled annually because of reproductive failure and the main reason for this is repeat breeding. (Gustafsson and Emanuelsson, 2002). It is found that the main causes of the repeat breeding are fertilization failure and early embryonic death.

The repeat breeding in the animals can also be due to ill management practiced in the farm. The impact of poor oestrus detection is well known as negligence in detection can cause an animal to go empty without any service, which is uneconomical for the farm. Poor oestrus detection is an important factor to influence upon the reproductive performance of the animal (De Kruif 1976; O Farrell et al. 1983).

Various infections have been pointed out for the reason for the repeat breeding in cattle. Several of the infections have a considerable interference in the breeding and reproductive performance of the animal. Bovine viral diarrhoea virus has found to be an infective agent, colonising the reproductive organs for its proliferation and therefore interfering with the reproductive performance of the cattle. This infection induces malfunctioning of the reproductive system: conception failures, early embryonic death, still birth etc.. Some of the protozoan infections have also known to cause the repeat breeding in animals and thereby affecting the normal reproducing ability in animals e.g. *Trichomonas foetus.* Several studies have pointed out the importance of the deficiency of proper nutrition playing the role to influence the fertility of the cattle (Wathes et al. 2001.). Ovarian disorders are also found to be the cause of the repeat breeding in dairy cattle (Wiltbank et al. 2002). Some studies have indicated that the in few cases the crux of the repeat breeding lies in the animals itself (Gustafsson and Emanuelsson 2002) and therefore in repeat breeders it has been pointed out that the oocyte quality might be responsible for the repeat breeding in the cattle.

Some studies have pointed out several reasons as well; there may be genetic factors involved in the repeat breeding and these have been pointed out by the 5-10 % embryos having chromosomal aberrations (King 1990). Even inbreeding has been registered as an aspect of increasing the negative affects on the reproductive aspects of the dairy cows (Hermas et al 1987). Stress also plays a role in inhibiting the normal reproductive behaviour in the cattle leading to impaired fertility and reproductive efficiency (Dobson et al 2001).

There are instances where the internal anatomy of the repeat breeder animal's reproductive system has been pointed out as a culprit for the repeat breeding as well. Båge et al (2002b) has reported that a great distribution of progesterone receptors in the tubal isthumus may form a sort of hindrance for the sperm transportation. Even the tubal epithelium of the RBH, when subjected to ultrastructure examination, has revealed altered morphology of the secretory cells and micro villi (Båge et al 2002b). It has also been stated that hormonal imbalance, e.g. too high progesterone concentrations during oestrus and a delay in the LH surge, is supposed to interfere with oestrous symptoms and be responsible for delay in ovulation (Gustafsson et al 1986; Båge et al. 2002a). The uterine milieu in the RBH is also shown to be less supportive for embryo development (Albihn et al 1991a). Also a longer interval between the deposition of the spermatozoa in the female and the ovulation in that female is attributed for the cause of the repeat breeding in cattle (Salisbury and Flerchinger, 1967). It has been shown that this problem can be prevented and that conception rates can be improved in RBH when frequent inseminations are performed repeatedly during oestrus until spontaneous ovulation occurs (Singh et al. 2005).

Body condition and body weight in cattle: Its relation to fertility

Body condition scoring

Body condition scoring of cows can be an effective management tool for enhancing reproductive performance within the cow herd. The conception in the animal is depended on the body confirmation of the animal (Lowman 1973). Lowman et al. 1976 introduced the 5-point basis grading system for the body scoring of the animals. This method involves a visual and physical assessment of fat reserves of the body; it is basically a measurement of the fattiness in the animal or the degree of visible fat present. In a nutshell, body scoring helps to elucidate the herd management at the farm level for better reproduction record. The influence of the body scoring is apparent in judging the fertility in animals as suggested by Berry et al. 2003. Pryce et al. 2001 asserted that body condition scoring has merit as a potential management and selection tool for improving fertility.

Weight of the cattle

The weight of the cattle is related genetically to the body condition score of the animal (Berry et al.2002), and the body weight and the body condition are together associated with the reproductive performance of the animal. (Buckley et al. 2003). By the virtue of the genetic correlations between the body weight and the fertility traits indicated that genetically heavier cows gets service quickly but requires more services for conception. Even this was noticed by Hansen et al. 1999 albeit instead of weight his finding stated that cows with great body size required more services for conception than cows having small body size. Body weight of the dairy cattle has been found to be related to the number of services cattle receives for conception (Berry et al 2003). Berry et al. 2003 also concurs Hansen et al. 1999, that there is a positive co relation between the first service and the interval of the conception in the cattle. Thereby it is possible to state that there exists a relation between the body weight and the reproduction capacity of the animal.

OPU as a tool for oocyte retrieval in repeat breeding heifers

The ovum pick up (OPU) in bovines has its roots in the human medicine, wherein the 1970 first OPU was attempted in a woman through laparoscopy, this gave a vent for the animal reproduction workers to attempt the same technique in animals as well. As the artificial insemination was getting clouded with some of its shortcomings as a result this new breakthrough was readily accepted.

The first attempt was done in The Netherlands where the procedure of OPU was carried out successfully but by Pieterse et al (1988) and of course he was the torch bearer for the veterinarians/animal researchers who readily gave in themselves for this new technology, so then the research was carried on further. This study gave new dimensions for the futuristic approaches in the animal reproduction; new icons were added to this domain such as multiple ovulations (super ovulation by the hormone FSH (Wooliams, 1989) discrete study of the ovum as well as the

embryo-transfer technology study etc. brought revolutionary changes in the field of animal reproduction. The OPU hereby was still done by the laparoscopy which was tedious as well as time consuming, and it also had the problem of the animal takes a long time to recover, so search for a new alternative to this procedure was being carried out with hot pursuit. For a long time the most common source of oocytes were from offal ovaries from the abattoirs (Longergan *et al.* 1991) A new technique came up with a device combining trans-vaginal ultrasonography, as a basic tool for the visualisation of follicles, and a separate column for a needle for the puncture of follicles and aspiration of follicular fluid and the oocyte by negative pressure (Callesen *et al.* 1987; Pieterse *et al.* 1988). This technique was applied in the horses as well (Bruck et al. 1992).
The emphasis of the OPU in the long run is to obtain large number of oocytes from the meritorious animals which in turn can be utilised for in vitro production of embryos both for study purpose and its proliferation. OPU is a technique which may cause little stress as well as minor lesions on the ovary leading to hardening and fibrosis in some cases (Pieterse et al 1991). Petyim et al 2002 has also reported that there are no major morphological changes in the ovaries except for the increase in the ovarian connective tissue in the tunica albuginea resulting in slight hardening of the ovarian consistency. Moreover, the procedure does not cause any significant effect on the well being of the animal if done under proper epidural anaesthesia (Petyim et al 2002).

Follicular development

The follicle formation on the oocyte takes place, anywhere on the plane of ovarian cortex. The various types of the ovarian follicles represent different stages of follicular development and maturity. The process is called as folliculogenesis.
The follicles present in the oocyte are known as

- Primordial follicle
- Primary follicle
- Secondary follicle
- Antral follicle

Primordial follicles: These are the microscopic, immature stage found in the ovary. The oocytes surrounded by a thin layer of flattened squamous cells.
Primary follicles: This stage is a little advanced stage of the primordial stage, here the oocyte is covered with a single layer of the cuboidal epithelium cells.
Secondary follicle: The secondary follicle here has the oocyte under the one or two layers of the follicle cells, broadly it can be said that during this stage the oocyte is surrounded by a relatively thick translucent of layer called as zona pellucida.
Antral follicle: This stage is characterized by fluid filled cavity called the antrum. The fluid is known as follicular fluid, and is also known as tertiary follicle. These stages can be seen by the naked eyes on the surface of the oocyte. They appear as blister-like structures that varying in the size.
This follicle consists of three distinct cell layers. The innermost cell layer is consisted of granulosa cells responsible for nourishment of the oocyte, they are

enclosed in a thin layer of extracellular matrix, the basal lamina, which role is to envelope the follicle. The furthermost layers are theca interna and theca externa. There is a network of capillary vessels between these two thecal layers and circulate blood to and from the follicle.

Oocytes

Good quality oocytes are supposed to have a dense cumulus as well as an intact zona pellucida with a homogenous cytoplasm. The cumulus cells are a mass of cells surrounding the oocytes and they form a multilayer arrangement around the periphery of the oocyte and play an important role during its maturation. Their function is to facilitate nourishment of the oocyte from the gap junctions between the cumulus cells and the oocyte. (Mori. et al.; Tatemoto et al. 2000; Fatehi et al. 2002). The quality of the cumulus cells plays a crucial role in maturation of the oocyte. (Pocar et al. 2005).

Introduction to the research report

Repeat breeding in dairy heifers is caused by fertilization failure or early embryonic death, and the reasons for the reproductive failure may be due to various disturbances occurring at different sites along the genital tract including the delicate hormonal regulation system. One crucial detail is oocyte quality which is critical for the development of a viable embryo after mating or AI. There are indications for an impaired oocyte quality in RBH compared to normal heifers, an important finding that needs to be investigated and further explained. The morphological features that indirectly indicate the quality of the oocyte need to be connected to other characteristics of a RBH (e.g. body condition and weight) in order to be included in a greater context.

Aims of the study

The aim of the study is to assess oocyte quality (indirectly judged by the appearance of immature oocytes collected by OPU) in two categories of animals; over-conditioned repeat breeder heifers and normal virgin heifers.

Research Report

Excessive lipid contents in immature oocytes from repeat breeder dairy heifers.

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ABSTRACT

Repeat breeding in cattle is a major problem throughout the world. It comprises of numerous causes leading to lowered fertility in the dairy herds. Repeat breeding in dairy heifers is caused by fertilization failure or early embryonic death, and the reasons for the reproductive failure may be due to various disturbances occurring at different sites along the genital tract including the delicate hormonal regulation system. One crucial detail is oocyte quality which is critical for the development of a viable embryo after mating or AI. For the present study, three repeat breeder dairy heifers (RBH) and two virgin heifers (VH, controls) of the Swedish Red breed were selected. Body condition was scored and body weight recorded. Immature oocytes were collected by ovum pick-up twice weekly during 5 weeks. The oocytes were classified quality-wise and further examined under transmission electron microscope. Similar numbers of follicles were available for puncture in the ovaries of RBH and VH, but significantly more ($P<0.05$) of the follicles were aspirated for retrieval of oocytes in RBH (76.4 %) than in VH (64 %). There was a numerically higher occurrence (NS) of low quality oocytes collected from RBH (60 %) than from VH (52 %). The occurrence of cytoplasmic vacuoles and lipid droplets was similar in oocytes from RBH and VH with a tendency ($P = 0.07$) for more lipid droplets in RBH. The VH oocytes as a group were more uniform compared to RBH that displayed a great variation especially in numbers of lipid droplets. The most prominent difference between RBH and VH oocytes was the significantly higher total lipid amount present in the cytoplasmic lipid droplets in RBH oocytes ($P < 0.001$), which was clearly visible as heterogeneous cytoplasm already under the stereomicroscope and confirmed by TEM. The results indicate that there are morphological differences in the appearance of RBH and VH oocytes, particularly due to higher lipid content in the cytoplasm, which can indirectly explain the low oocyte quality and the sub fertility in RBH.

Keywords: *repeat breeding, dairy heifer, body condition scoring, oocyte quality, lipids*

Acronyms and their meaning: RBH = Repeat Breeder Heifer; VH = Virgin Heifer; OPU = Ovum Pick Up;

Introduction

Repeat breeding is a major concern in cattle, throughout the globe (Lucy 2001). For many years, fertilisation failure and early embryonic death have been issues of concern in the research on reproductive wastage in Swedish dairy heifers (Linares 1980a; Linares 1980b; Linares 1981a; Gustafsson 1985a; Gustafsson 1985b; Gustafsson and Larsson 1985; Albihn et al. 1989; Stanchev 1991; Albihn 1991a; Albihn 1991b; Albihn et al. 1991a; Båge et al. 2001; Båge et al 2002; Gustafsson and Emanuelsson 2002; Singh et al 2004). Repeat breeding has always been a challenge to the dairy industry. Repeat breeding cattle in the dairy farm is uneconomical as the assets involved in sustaining the repeat breeding cattle are considerable.

And a proper approach is needed in alleviating this phenomenon in the dairy cattle population. At least 10% of the Swedish dairy cows are culled annually due to reproductive failure, and the main reason behind it is the repeat breeders (Gustafsson & Emanuelsson 2002). Many studies have so far attempted to isolate myriad causes of the repeat breeding in dairy cattle. The causes can be management, nutritional, of genetic origin, hormonal as well as infectious. Management can alone be a strong cause of repeat breeding, e.g. in cases of failure to detect oestrus and mis-timed artificial inseminations (AI). Nutritional status and body condition will have direct as well as in-direct effects on reproductive performance (Wathes et al. 2001). Over-conditioned or fat heifers may have impaired oocyte quality due to effects of metabolic hormones or substances on ovarian function. Recently, it was verified that effects of diet on oocyte quality depends on animal body composition, i.e. the level of nutrition interact with degree of heifer body fatness and affects the post-fertilisation developmental potential of oocytes (Adamiak et al, 2005). Moreover, in the aforementioned study, moderately obese animals fed an energy-rich diet presented hyperinsulinaemia in combination with impaired oocyte quality. Thus, some causes of repeat breeding are ingrained within the animal itself and could be related to oocyte quality.

The study also involves the role of body condition and body weight of the animals in order to relate these two entities with the domain of the repeat breeding in cattle. Lowman et al. 1973 strongly suggested the importance of the body condition on the conception of the animal. It has been suggested by some researchers that it can be a medium to judge the improvement for fertility in cattle (Pullan 1978; Berry et. al. 2003). In this study an attempt is being done to demonstrate the importance of body condition on heifer fertility. The weight of the cattle also plays a major role in influencing the fertility in the animal. Berry et al. 2003 showed that the heavier cattle tend to get, the more services are required for conception.

The study here deals with an attempt to isolate the causes of the repeat breeding on the oocyte level in over-conditioned dairy heifers. These are being conducted by investigating the details of some oocyte components from repeat breeder heifers (RBH) and compare them with oocytes obtained from virgin heifers (VH). A good-quality oocyte is supposed to have an intact cumulus investment with several

layers of granulosa cells and have a homogenous cytoplasm. Various organelles are present within the oocyte, and e.g. membrane-bound vesicles or vacuoles have been described (Hyttel et al. 1986a; De-Loos et al. 1989; H. Nili et al. 2004). These vacuoles may contain glycogen (Nesbit Fleming and Saacke 1972). Some differences have been found in the lipid content of the oocyte, the differences recorded were in sizes and numbers of the lipid droplets (Nili et al. 2004). Lipid droplets are considered to be the source of energy and they have a role in oocyte maturation (Brown 2001). It has previously been demonstrated that lipids can be toxic to the oocyte (Adamiak et al. 2006), and as such excess of lipids in the oocytes exert a deleterious effect in the cryopreservation of the oocytes (Otoi et al. 1997). Not only the content but also the spatial distribution of organelles in the oocyte may reflect the status of the oocyte. The distribution of lipids inside the oocytes have been found to be as such that a central distribution of lipids has been characteristic of immature oocytes, and peripheral distribution has been found in mature oocytes (Hyttel 1986a; Båge 2002). Quality differences between oocytes from RBH and VH have been pointed out in previous studies based on morphology and ultra-structure (Båge et al. 2002). In general, oocytes from VH had normal, homogenous cytoplasm while heterogeneous cytoplasm was observed more frequently in RBH (Båge et al. 2002a; 2001).

The objective of the current study was to compare oocyte appearance between RBH and VH with the hypothesis that RBH oocytes are inferior to VH oocytes in quality.

Materials and Methods

Animals

The animals selected for the experiment belong to the Swedish Red Breed (SRB). Three RBH and three VH were procured from the Swedish University of Agricultural Sciences farm, Uppsala, Sweden. All the heifers selected were sexually mature and free from diseases, particularly bovine viral diarrhoea virus (BVDV) and bovine leucosis. A RBH was defined as a heifer, returning to oestrus within normal intervals besides being inseminated three times or more without becoming pregnant, not having apparent pathologies explaining the repeat breeding, neither at palpation per rectum or at ultrasonographic examination of the genital tract (Båge et al 2002). A VH was defined according to the following criteria: the heifer was sexually mature, never inseminated or mated and was exhibiting normal oestrus cycles. The heifers were kept tethered in the same barn, fed hay twice daily (5 kg at each time), 8 kg of green roughages, ½ kg of concentrates twice daily and *ad libitum* water, and minerals licks.

Six animals were originally included in the trials; however, one the three VH was detained from the study. The heifer detained from the study was found to be highly non co-operative. Therefore in all there were five animals on which the study was conducted upon.

Three RBH (age 2.2-2.5 years) with mean weight as 568 kg (range 480 – 625 kg) Two VH were selected (age 1.5 years) with average weight as 399 (range 389 – 409 kg). The body weight of the heifers was taken every two weeks during the experimental period.

Body condition scoring of the heifers was done after the guidelines for the grading the cattle on a 5-point basis *(*Lowman et al 1976). It was performed three times: at the arrival of the heifers and at the onset and final of the OPU period.
The Ethical Committee for experimentation with Animals, Uppsala, Sweden, approved the experimental protocol before the commencement of the study.

Oestrus detection and ovarian status monitoring
The heifers were checked daily for the signs of the oestrus: excitement, vocalization, licking, lordosis, oedema and redness of vulva, and the presence of vaginal mucous discharge. This was done before the trial in order to maintain and acclimatize the animals with the new handling as well as surrounding. Further, normal cyclicity was assured by monitoring of apparent oestrus signs and ovarian activity, as monitored by rectal palpation and trans-rectal ultrasonography of the ovaries (Pie Medical 485 Anser, Philipsweg 6227 AJ, Maastricht, The Netherlands). The oestrus cycles were synchronised with single shot of 2 mL prostaglandin $F_{2\alpha}$ analogue (0.25 mg/mL Estrumate® Vet; Schering-Plough Animal Health, Schering-Plough Corporation New Jersey, USA).

Counting of the follicles and Ovum pick-up (OPU)
The counting of the follicles was done with the help of a vaginal ultrasound probe (described below), which was used for the OPU, After a proper lubrication the device was inserted through the vagina of the heifer and pressed against the vaginal fornix, and with other hand inserted inside the rectum; the ovary was brought in close contact with the probe. The number of follicles greater than 4 mm in diameter were counted and classified by its diameter as small (4-6 mm), medium (7-10 mm) or large (> 10 mm). Then the OPU was performed. This procedure was conducted twice a week on fixed week-days for a total of 10 sessions during five weeks. Prior to the actual OPU as mentioned above the synchronisation of the oestrus was done. The procedure was started by the fixation of the heifer and then by the application of the epidural anaesthesia (5 mL Lidocaine hydrochloride, 20 mg/mL, Xylocaine®; AstraZeneca, Södertälje, Sweden) was performed uniformly on all the animals. At the later stages of the study some animals required some sedation (0.1 to 0.5 mL Xylazine Hydrochloride, 20 mg/mL, Narcoxyl® Vet, Intervet AB, Danderyd, Sweden), by intravenous administration in the tail. The methodology followed for the accomplishment of the follicular puncture was accessed by Petyim et al. 2000, which in turn was based on the altered version of the procedure described by Pieterse et al 1991. The equipment used was a 7.5 MHz multi-angle transducer of a real time B-mode ultrasound scanner (Scanner 200- VET®, Pie medical equipment, B.V. Maastricht, The Netherlands) that was connected to a 9-inch monitor. The transducer was placed in an OPU holder and through the needle guidance system, all visible follicles >4 mm in diameter were punctured with a 20 gauge disposable needle (Terumo® Europe N.V.3001, Leuven, Belgium). A vacuum pump (Cook, William & Cook Pty Ltd., Brisbane, Australia) of 50 mm Hg, corresponding to a 12 to 15 mL/min flow-rate, was created with a vacuum pump activated by a foot pedal..
After a clear visualization of the ovary, the follicles were punctured with the needle and then aspirated into a filter (EmCon, Immuno Systems, Inc., WI, Spring

Valley, USA) containing phosphate buffered saline (PBS, pH 7.4, 300 mOsm) supplemented with heparin (Heparin Leo; 5000 IE or 1 mL per 1000 mL of PBS, Leo Pharma, Ballerup, Denmark) and bovine serum albumin (BSA 3 mg/mL, Sigma Albumin Bovine, Sigma Chemicals, Steinheim, Germany)

Collection, isolation and quality scoring of the oocytes

After follicular puncture, the filter cup was rinsed with PBS. The oocytes were collected and accumulated in Petri dishes and examined under the stereomicroscope (Type MDG 17; Wild Heersburg, Switzerland). The recovered cumulus oocyte complexes (COCs) were counted and quality scored based on COCs investment and integrity of the cytoplasm (Marquant-LeGuienne, 1999). The grades were given as 1 to 4, with grade 1 signifying very good quality. Grade 5 was given to oocytes not eligible to the classification regime. In the further handling of oocytes, grade 1 and 2 oocytes were denominated as "good" oocytes and grade 3 and 4 oocytes as "bad".

The best and the worst COCs from both VH and RBH were isolated and fixed in 3% glutaraldehyde in 0.067 M sodiumcacodylate buffer for studies of oocyte ultra structure under a transmission electron microscope (TEM).

Preparation of the oocytes for transmission electron microscopy

The five best and the five worst COCs (in total 20 oocytes) from the pooled VH and pooled RBH oocytes were isolated and fixed in 3% glutaraldehyde in 0.067 M sodiumcacodylate buffer for studies of oocyte ultra structure under a transmission electron microscope (TEM).

The embedding procedure for TEM starts with rinsing 2-3 times in sodium-cacodylate-buffer. Following post-fixation in OsO_4 (2% in buffer) for one hour at room temperature, they are washed in buffer. The oocytes are then dehydrated in increasing concentrations of ethanol, followed by10 min wash in acetone 3 times. Thereafter they are embedded in agar 100 plastic resin (Agar-Aids, Essex, UK) polymerised under 60°C.

After localisation, the oocytes were cut in ultra-thin sections at their greatest diameter and collected on copper grids and then stained with uranyl-acetate and lead citrate before examination in TEM (JEOL JEM -1230 Electron Microscope, Akishima, Japan) at 60 kV

The vacuoles and lipid droplets present in the cytoplasm of the oocytes were counted in at least 5 sections and the count was subjected for calculations. The lipid droplets present in the oocytes were considered for their area occupancy in the oocytes. This procedure was done by the help of a special machine (DIGIPLAN Messergate GMBH Kontron, Munich, Germany). The probe was used to measure the area occupied by the lipid droplets by encircling it around the circumference.

Statistical analyses

The Statistics Analysis Systems Package (SAS Institute Inc., Cary, NC, USA, Version 8) was used for the statistical calculations. The data are presented as

arithmetic means and standard deviations (mean ± SD). The Fishers Exact Test was used for determining the oocyte quality between the RBH and VH. The GLM was used for the analysis of the follicle data. The frequency procedure was used for the comparison between the lipids and vacuoles. The calculations were done between the vacuoles and the lipids separately. The significance level was set at 95% ($P < 0.05$).

Results

3.1 Body condition scoring and body weight

At the onset of the study, the three RBH had a numerically higher average body weight than the two VH (625 kg, 600 kg, 480 kg vs. 409 kg, 389 kg, NS) and body condition score (4, 3 and 3 in RBH vs. 2.5 and 3 in VH, NS). During the experimental period, when the heifers were feed with identical ratios, all animals gained weight; RBH gained on average 5.4 % and VH 9.3 %, and BCS increased to 5, 3.5 and 4 in RBH, while both VH remained on 3.

Follicle analysis and oocyte quality evaluation

Results from ultrasonographic examination of the ovaries, OPU and oocyte grading and evaluation are presented in table 1.

Table 1. Numbers of follicles >4 mm in diameter available in the ovaries and numbers of follicles aspirated during ten OPU sessions in virgin heifers (n=2) and repeat breeder heifers (n=3), respectively (the proportion of aspirated out of available follicles is presented as percentages). Numbers of retrieved oocytes and oocyte quality grading after collection.

Groups				Oocyte grading after collection	
	No. of follicles in the ovaries at OPU	No. of aspirated follicles (%)	No. of retrieved oocytes (%)	Good	Bad
VH (n=2)	114	73 (64.0)	27 (37.0)	13	14
RBH (n=3)	199	152 (76.4)	67 (44.1)	27	40
P value	0.1504	0.0252	0.1305		

Out of the follicles present in the ovaries, 64 % of the follicles were aspirated for the retrieval of the oocytes from the VH and 76.4% from the RBH ($P < 0.05$). The results also exhibit that numerically less oocytes ($P = 0.13$) were retrieved from the puncture of the available follicles in VH (37.0 %) as when compared to the

proportion of retrieved oocytes from RBH (44.1 %). There was however no difference between VH and RBH regarding total numbers of retrieved oocytes. The quality scoring of the oocytes was done after the collection, and there was a numerically higher (NS) proportion of bad oocytes collected from RBH (60 %) compared to VH (52 %). The oocytes were kept for preservation in PBS awaiting microscopical examination and the loss of oocytes during preservation was 22.2% in the VH and 49.2%, in the RBH (NS).

Ultra structural examination of oocytes

Table 2. Numbers of vacuoles and lipid droplets present in transmission electron micrographs of good and bad quality oocytes (n=5 in each group) from virgin heifers (n=2) and repeat breeder heifers (n=3) (presented as means ± SD).

Groups	No. of vacuoles		No. of lipid droplets	
	Good oocytes	Bad oocytes	Good oocytes	Bad Oocytes
VH	43.9 ± 34.9	44.9 ± 33.0	3.4 ± 2.6	5.1 ± 5.6
RBH	41.9 ± 28.0	61.2 ± 43.8	4.5 ± 5.3	8.1 ± 9.4

In table 2, the numbers of vacuoles and lipid droplets in the cytoplasm of good and bad quality scored oocytes are presented. Good quality oocytes from RBH contain more lipid droplets, though the value is not significant ($P = 0.07$), while bad quality oocytes from RBH have numerically (but not significantly) more lipid droplets. It is obvious that VH oocytes (especially the good quality oocytes) are more uniform concerning numbers of lipids droplets present compared with RBH oocytes that have a great variation in this aspect.

Numbers of vacuoles were similar in RBH and VH (NS), barring that the ratio between of vacuoles and lipids within VH oocytes were significantly different from the proportion in RBH oocytes ($P < 0.01$).

Concerning the total amount of lipids present in the lipid droplets, the area (in units) of the lipid droplets was found to be significantly higher in RBH ($P < 0.001$), as presented in figure 1-3.

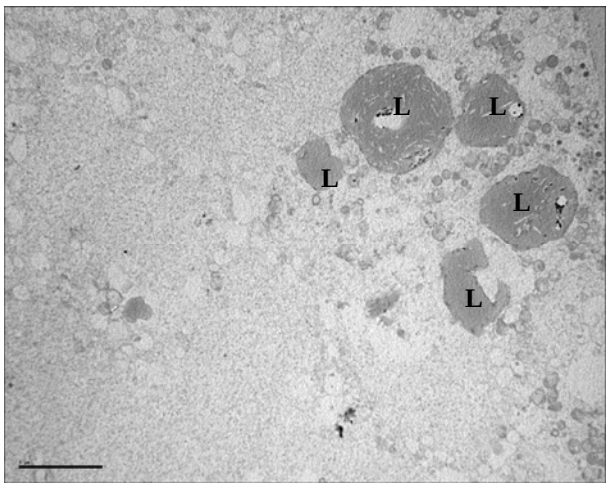

Figure 1. Transmission electron-micrograph of bad quality oocyte with large lipid droplets (L) from RBH (x 3,000).

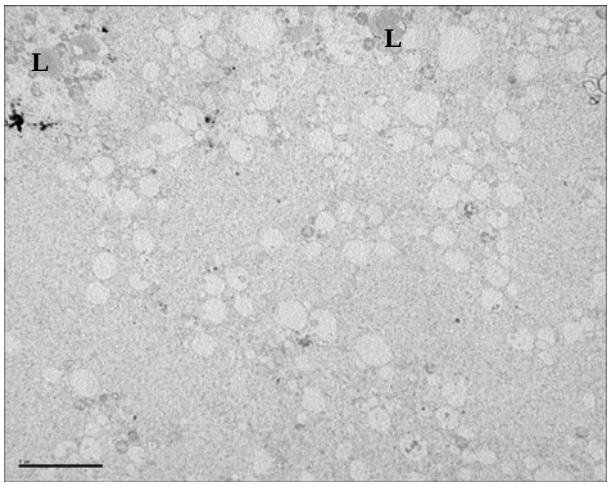

Figure 2. Transmission electron-micrograph of a good quality oocyte with small lipid droplets (L) from VH (x 3,000).

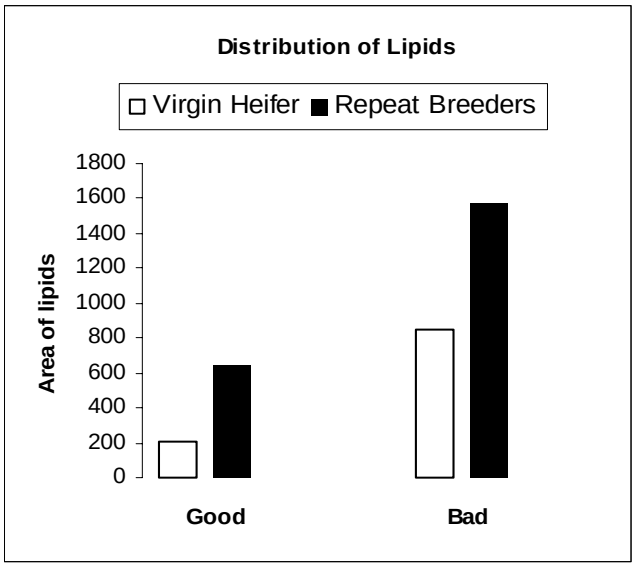

Figure 3. Total lipid content in cytoplasmic lipid droplets of good and bad quality oocytes from virgin heifers (n=2) and repeat breeder heifers (n=3).

Discussion

Body condition scores and body weights of the heifers

Body condition scoring is a tool which helps in investigation for the improvement in fertility (Pullan 1978; Berry et al. 2003). In the present study the heifers maintained approximately similar BCS or increased slightly during the course of the study. The scores of the VH were however of an ideal average heifer, whereas the RBH were over-conditioned already at the start of the experiment and increased to even higher BCS. Moreover, they tended to have higher body weights than the VH from the commencement of the study till termination. Some previous studies have reported delayed first oestrus, late conception and more services per conception if the body score of the animal is more than 3.5 (Garnsworthy and Topps 1982; Treacher et al. 1986; Garnsworthy and Jones 1987). Several studies have noticed similar relations between the body condition and the reproductive performance of the animal (Lowman et al. 1973; Whitman et al. 1975; Dunn and Kaltenbach, 1980; Osoro and Wright 1992; Buckley et al 2002). Naturally, the body condition is related to the feeding management and also the weight of the animal (Dingwell et al. 2006), and there are many reports available on detrimental effects of overfeeding on oocyte quality and blastocyst rate after both *in vivo* and *in vitro* embryo production (Humblot et al.,1998; Boland et al, 2001;Webb et al, 2004). Interestingly, the negative effects of overfeeding seem to be intensified in over-conditioned heifers, and as it was recently reported by Adamiak et al (2005), in this case hyperinsulinemia could be connected to poor oocyte quality.Even the cows that have higher body size have been pointed out that they tend to require

more services for conception than the cows of low body size (Hansen et al. 1999). The body weight with consideration from the genetic correlations, the heavier cows required more services and have a longer interval from the first service to conception. (Berry et al. 2003). This helps in understanding the repeat breeding and its relation to the weight of the animal. So here we can suggest that the higher the body weight in heifers as well as higher body condition scores are more prone to have more services to achieve conception.

All heifers in the present study increased in body weight, although this increase was more pronounced in VH however not matched by an increase in BCS. For RBH, on the contrary, the increase in weight was slower but the already high BCS still increased further. One explanation could be that the RBH were older and, had already reached mature body size while the younger VH were still growing in size and not primarily increasing their depots of subcutaneous fat. It might also reflect that individual heifers have different metabolism, which in turn may be one of the underlying causes to repeat breeding. The heifers those are prone to gain weight or rather, to gain high BCS, may have detrimental effects of their metabolism on fertility and subsequently end up as repeat breeders.

Number of follicles available throughout ovum pick-up procedure

The RBH and the VH were subjected to the regime of the continuous, twice-weekly OPU. In the present study, this continuous OPU puncture schedule was attempted for the first time in defined RBH. An attempt was made to judge the number of available follicles throughout the ten sessions of the OPU. Some previous work shows that the depletion of the follicle occurs in the VH under a continuous OPU schedule (Petyim et al., 2003). Here we are unable to ascertain the same in the RBH as the sessions we conducted were too few to corroborate this theory in RBH.

The number of follicles available for puncture in both the RBH and the VH were found to be similar (NS). It has been stated earlier that the ovarian activity has found to be heightened in the RBH when compared with the VH, and more number of the follicles has been found to be available for the puncture in the RBH. Hence a higher proportion of the available follicles were aspirated from the RBH, but the number of retrieved oocytes was similar in RBH and VH.

Oocyte quality

When the oocytes were subjected for examination and quality grading under the stereomicroscope, the bad quality oocytes from the RBH were found to have a chequered appearance due to the heterogeneity of the cytoplasm of the oocyte. We would like to suggest that the lipid droplets could be to the cause for the chequered appearance of the oocytes. Thereafter, when the oocytes were further examined under TEM, large lipid droplets were revealed in the cytoplasm of the RBH oocytes. Lipid droplets are present in large numbers in the bovine oocytes (Abe et al. 1999). Lipids have been pointed out as the source of energy (Brown et al., 2001; Kim et al. 2001; Kikuchi et al. 2002), and they seem to play an important role in oocyte maturation, fertilisation and early embryo development. It has been suggested that the lipid droplets are sensitive to the cryopreservation procedures (Mohr and Trounson , 1981; Liebo and Loskutoff, 1993) or in other words they

seem to interfere with the freezing ability of the oocytes or embryos (Otoi et al. 1997; Kikuchi et al. 2002) making the preservation of the oocytes to be difficult. Lipid accumulation in the oocytes reduces their quality and cryotolerance (Abe et al 2002). Hence the lipid analysis of the oocytes puts forth the fact of the superiority of the VH oocytes when compared with the RBH oocytes.
It has been proposed by some researchers that the oocytes themselves are of the inferior quality in repeat breeders (Harrison et al. 1990; O'Callaghan and Boland 1999; Gustafsson and Emanuelsson 2002; Båge et al. 2002; Horan et al 2005). As in humans too, a research showed that poor oocyte quality is the cause of the infertility in a significant number of the 1.6 million US couples, which were unable to conceive (CDC/SART, 1999; Krisher 2003). Hence we can propose the theory that the prominence of the lipid content in the oocytes of the RBH signifies a probable reason for an RBH oocyte being categorised as the bad quality oocyte. This also explains as one of the reasons of the repeat breeding syndrome found in some heifers.
The numerically high (but NS) loss of oocytes during preservation in PBS awaiting microscopical examination can be considered for representation of the sturdiness of the VH oocytes in preservation compared to the fragile oocytes from RBH. This is an interesting finding that may involve not only the detrimental lipid content but also zona pellucida fragility and requires further investigation.

Conclusions

The results yielded from the study conducted manifest the importance of the body condition of the animals and the influence of body weight on the reproduction capacity of the animal and its fertility as well. However more studies are still anticipated in future in a good light for more accurate yardsticks, in this stream. The quality grading of the oocytes and the study of lipid organelles of the oocyte in particular has pointed and clarified more the difference between the VH and RBH. The lipids analysis asserts a view in favour of the VH oocytes over the RBH oocytes, the corollary of the analysis is as such the RBH oocytes are significantly richer in the lipid content, which is deleterious in excess. Thereby it reiterates the potency of the developmental capacity of the VH oocyte against RBH oocyte. Hence a suggestion is put forth in favour for the superiority of the VH oocytes over RBH oocytes.

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Future prospects

The study done has attempted to be descriptive about the differences between the repeat breeding heifers and the virgin heifers, on the basis of the body condition scoring, body weight and vociferously on the lipid content of the oocytes. The body scoring and the body weight together they need more attention in order to explain the relation of the duo with reproductive performance of the animal. The yardstick for application of the body scoring and the body weights can be applied to the animals in a large scale as these quality parameters need a large sample size. The lipids in the oocytes if present in excess interfere the freezing ability of the oocyte, this study provides a striking benchmark between the oocytes obtained from the virgin heifers and the repeat breeders, This will help further in the research involving the freezing ability of the oocytes obtained from various animals and the a proper outcome for the best survival of the oocytes can be planned provided if the history of the animal is known in concern with its reproduction and fertility records. This clearly indicates the fact for the cryopreservation of the VH lipids, there will be less difficulty due to few lipids found in them. The processing of the oocytes of the VH will be less tedious and has more chance of survival hence can be studied further for epigenetic studies and in vitro fertilization.

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Rest is silence.