Triglyceride content of bovine oocytes and early embryos

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A microfluorescence technique was used to measure the triglyceride content of a minimum of two bovine oocytes or preimplantation embryos up to the hatched blastocyst stage. Embryos were produced *in vitro* from abattoir-derived ovaries and grown in medium containing synthetic oviductal fluid, amino acids and BSA (SOFaaBSA medium); 10% fetal calf serum was added to some of the embryos at the four-cell stage. Before maturation, the triglyceride content of oocytes was 59 ± 1.37 ng and it decreased ($P < 0.05$) after maturation to 46 ± 0.85 ng. A decrease in triglyceride content ($P < 0.05$) was also observed after fertilization with the formation of the two-cell embryo (34 ± 1.80 ng). In the absence of serum, the triglyceride content remained relatively constant from the two-cell to the hatched blastocyst stage. The triglyceride content of blastocysts produced *in vivo* was similar (33 ± 0.70 ng) to that of blastocysts produced *in vitro* in the absence of serum. In contrast, the triglyceride content of embryos grown with 10% fetal calf serum increased steadily from the 9–16-cell stage to a value in hatched blastocysts (62 ± 1.14 ng) almost double that in serum-free conditions. These results indicate that triglyceride may act as energy source during bovine oocyte maturation and fertilization and that the presence of serum causes excessive synthesis or accumulation of triglyceride in early embryos.

Introduction

Studies on energy metabolism in early mammalian embryos have focused on the consumption and use of exogenous nutrients provided in the culture medium. For example, bovine embryos consume substrates such as glucose, pyruvate and amino acids from the zygote to the blastocyst stage (Partridge and Leese, 1996; Thompson *et al.*, 1996).

In contrast to exogenous nutrients, the potential importance of intracellular stores to energy metabolism in the early bovine embryo has been largely ignored. Kane (1979) showed that one-cell rabbit ova were able to develop to viable morulae in the absence of extracellular nutrients in a simple salt solution containing defatted BSA, indicating that endogenous stores may be important in rabbit embryo development. There are three such energy stores within the embryo: glycogen, protein and lipid. Flynn and Hillman (1978, 1980) reported that when mouse embryos were cultured with radioactive glucose and fatty acids, the major class of labelled lipid was triaclyglycerol, the major biological function of which in adult tissues is to serve as an energy store. However, it is not known whether endogenous triglyceride is used to provide energy during early embryo development. In the mouse embryo, the total lipid content is almost 4 ng (half of which consists of cholesterol and its esters), which as a percentage of the dry mass is only 10% (Lowenstein and Cohen, 1964). If endogenous lipid in the

form of triglyceride acted as a source of energy in the mouse embryo, it could only sustain development for 4–5 h (Leese, 1991).

Quantitative measurements of total lipid have been made in sheep and pig oocytes. McEvoy *et al.* (1997) reported that pig oocytes contain 75 ng triglyceride of a total lipid content of 156 ng, which also includes phospholipids, cholesterol esters and free fatty acids. Coull *et al.* (1997) reported that the triglyceride content of sheep oocytes was 25 ng of a total lipid content of 89 ng. However, in both of these species, the triglyceride content was only measured at the oocyte stage and 1000 oocytes were required for analysis. Triglyceride content has also been measured in day 10 and day 11 pig embryos and values of 113 and 141 ng, respectively, were reported (Youngs *et al.*, 1994).

Serum is commonly added to media used to grow bovine embryos *in vitro* since it increases the proportion of zygotes that develop to the blastocyst stage. However, the presence of serum in culture medium for early sheep embryos can induce premature blastulation and the accumulation of cytoplasmic 'lipid-like' inclusions, and affect the duration of gestation and the birth weight of lambs after embryo transfer (Thompson *et al.*, 1995). Thompson *et al.* (1995) suggested that lipid globules that accumulate after the addition of serum contain significant amounts of triglyceride derived from the serum.

The aim of this study was to quantify the triglyceride content of bovine oocytes and preimplantation embryos up to the hatched blastocyst stage using a microfluorescence

method developed recently to measure the triglyceride content of two or three bovine oocytes or early embryos.

Materials and Methods

Production of embryos in vitro

Bovine embryos were derived from oocytes matured and fertilized *in vitro* using the method of Thompson *et al.* (1996) with modifications. Ovaries were collected from a local abattoir and transported to the laboratory at 27°C in PBS. The ovaries were washed in PBS containing 50 mg kanamycin sulfate l^{-1} (Sigma, Poole) and tap water to remove any debris and blood. Oocytes were obtained by dissection. Each ovary was cut into two halves starting at the hilum, and these were placed in a Petri dish containing Hepes buffered TCM-199 ($\times 10$) (Sigma) plus 50 mg kanamycin sulfate l^{-1}, 40 mg heparin l^{-1} and 400 mg BSA fraction V l^{-1} (Sigma). The top layer of the ovary was removed (mesenchymal part) and care was taken not to burst the follicles and to leave them clearly visible. The tissue was cut into small pieces using a scalpel and forceps, and the individual follicles were punctured. Oocytes with an intact unexpanded cumulus oophorus and evenly graduated cytoplasm were washed twice in Hepes buffered TCM-199 and once in maturation medium comprising bicarbonate buffered TCM-199 with Earle's salts and L-glutamine (Sigma) supplemented with 10% fetal calf serum (FCS, Sigma) and 10 ng ml^{-1} epidermal growth factor (Carolan *et al.*, 1996). This technique enables between 10 and 30 cumulus–oocyte complexes to be obtained per ovary, depending on its size. The oocytes were placed in groups of about 50 per well and incubated under humidified 5% CO_2 in air for 24 h.

After maturation, the cumulus–oocyte complexes were washed twice in Hepes buffered Tyrode's albumin lactate pyruvate medium (Hepes–TALP) (Lu *et al.*, 1987) and once in fertilization medium. Spermatozoa were prepared from frozen–thawed semen samples obtained from a single bull of proven fertility. The contents of one straw were thawed in a waterbath at 37°C, layered on a Percoll (Pharmacia Biotech, St Albans) gradient (45:90) and centrifuged at $2100\,g$ for 25 min. The motile sperm pellet was washed once in Hepes–TALP, centrifuged at $1200\,g$ for 10 min and resuspended in bicarbonate buffered fertilization TALP supplemented with 0.01 mg heparin ml^{-1}, 0.3 ng penicillamine ml^{-1} and 0.01 ng hypotaurine ml^{-1}. An aliquot (100 µl) of sperm suspension was added to each well containing 400 µl fertilization TALP and approximately 50 cumulus–oocyte complexes resulting in a final sperm concentration of 1×10^6 spermatozoa ml^{-1}. Cumulus–oocyte complexes and spermatozoa were incubated for 24 h in a humidified atmosphere of 5% CO_2 in air at 39°C.

After fertilization, the putative zygotes were vortexed for 2 min to remove any remaining cumulus cells and washed twice in Hepes buffered synthetic oviductal fluid (H-SOF) comprising 20 mmol Hepes l^{-1}, 5 mmol $NaHCO_3$ l^{-1}, 0.33 mmol pyruvate l^{-1}, 3.30 mmol D-L lactate l^{-1} and 4 g BSA l^{-1} (Fraction V BSA, Sigma). The putative zygotes were washed in bicarbonate buffered SOF plus minimum essential

medium (MEM), essential and non-essential amino acids (Gibco Life Technologies, Paisley) and containing 3.30 mmol D-L lactate l^{-1}, 0.33 mmol pyruvate l^{-1}, 1.0 mmol glutamine l^{-1}, and 8 g BSA l^{-1} (fatty acid free, Sigma) (SOFaaBSA). Approximately 20 putative zygotes were placed in each 20 µl drop of SOFaaBSA medium and incubated under humidified 5% CO_2, 5% O_2 and 90% N_2 at 39°C. In some experiments, 10% FCS was added on day 2 of culture.

Embryo recovery in vivo

Day 7 bovine embryos were obtained by non-surgical collection from superovulated and inseminated cows at ADAS High Mowthorpe (Duggleby, Malton, North Yorkshire). The donor cows had either a progesterone-releasing intravaginal device (PRID) or controlled internal drug release (CIDR) implanted in the vagina to achieve oestrous synchrony. On the morning before removal of the PRID or CIDR, each cow was injected i.m. with 4 ml Estrumate (prostaglandin; Intervet, Cambridge); this drug is effective in the induction of oestrus in cattle that are between day 5 and day 15 of the oestrous cycle. The cows were then injected with FSH to bring about superovulation and were artificially inseminated with two straws of semen. Embryos were recovered by flushing the uterus with warm ovum culture medium through a catheter placed in the uterus. The embryos were washed twice in Hepes–SOF and placed in sterile eppendorf tubes containing Hepes–SOF transport medium with 1.0 mmol pyruvate l^{-1}, 10.0 mmol D-L lactate l^{-1} and 4 g fraction V BSA l^{-1}, and transported to the laboratory at 39°C. The embryos were washed in Hepes–SOF, allocated to culture drops and removed for extraction at the blastocyst stage.

Selection of bovine embryos

In vitro produced bovine embryos can vary widely in quality and rate of development. Therefore, embryos that had cleaved at least once in the first 30 h after fertilization, which are considered to have a higher chance of forming compact morulae (Van Soom *et al.*, 1992), were pooled in culture drops, eliminating the potentially detrimental influence of dead oocytes. Embryos were then selected for measurement of triglyceride content at peak times of appearance for each stage as identified by Van Soom *et al.* (1992) and compared with non-pooled embryos that had not undergone the first cleavage in the first 30 h after fertilization.

Preparation and freezing of early preimplantation bovine embryos before analysis

Cumulus cells were removed from oocytes before and after maturation by repeated pipetting through glass pipettes of decreasing diameter in the presence of 2.5 mg hyaluronidase ml^{-1}. Freezing was carried out in 5 µl microcaps in a known volume of medium containing 0.1% fatty acid free BSA (Sigma) in PBS.

Table 1. Triglyceride content (ng per embryo) of *in vitro* produced bovine embryos grown with and without the addition of serum from the four-cell stage

Stage of development	With serum	n	Without serum	n
Oocytes before maturation	–	–	59 ± 1.37^e	13
Oocytes after 24 h maturation	46 ± 0.85^c	10	–	–
Two-cell embryo	–	–	34 ± 1.80^{af}	12
Four-cell embryo	–	–	33 ± 2.77^a	11
Five- to eight-cell embryo	33 ± 0.65^a	21	33 ± 0.43^a	15
Nine–sixteen-cell embryo	37 ± 0.75^{bf}	16	33 ± 0.51^a	15
Compact morula	39 ± 0.94^b	10	33 ± 0.59^a	10
Early blastocyst day 7	45 ± 0.68^c	10	33 ± 0.66^a	10
Early blastocyst day 8	46 ± 0.42^c	12	36 ± 0.48^{ab}	10
Expanded blastocyst day 7	52 ± 0.89^d	12	34 ± 0.76^{af}	11
Expanded blastocyst day 8	51 ± 0.52^d	11	35 ± 0.74^{af}	10
Hatched blastocyst day 7 or day 8	62 ± 1.14^e	10	36 ± 0.40^{ab}	10
Total number of samples (oocytes and embryos)	–	112	–	127

Values are mean ± SEM.
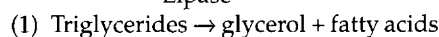n denotes the number of oocytes or embryos measured for each stage.
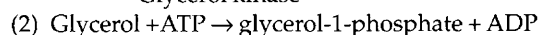Values without a common superscript are significantly different ($P < 0.05$).

Measurement of triglyceride content

The triglyceride assay was based on the hydrolysis of triglycerides to glycerol and free fatty acids followed by enzymic measurement of the glycerol released.
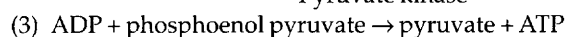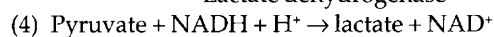
Lipase
(1) Triglycerides → glycerol + fatty acids

Glycerol kinase
(2) Glycerol + ATP → glycerol-1-phosphate + ADP

Pyruvate kinase
(3) ADP + phosphoenol pyruvate → pyruvate + ATP

Lactate dehydrogenase
(4) Pyruvate + NADH + H$^+$ → lactate + NAD$^+$

The amount of glycerol in the sample is proportional to the amount of NADH oxidized to NAD in reaction (4) and may be quantified using a fluorescence microscope with photometer and photomultiplier attachments (Leese and Barton, 1984; Gardner and Leese, 1986). The assay uses two reagents (A and B), which were obtained from Sigma. Reagent A contains ATP, lactate dehydrogenase, lipase, NADH, phosphoenol pyruvate, pyruvate kinase, buffer and non-reactive stabilizers and fillers. Reagent B contains glycerol kinase and non-reactive stabilizers and fillers.

The assay was scaled down to microlitre values to allow measurement of the triglyceride content of a minimum of two embryos. Reagent A (5 μl) was taken up into a 5 μl graduated polymerase chain reaction (PCR) micropipette (Drummond, Broomall, PA) and the end of the pipette was sealed immediately with parafilm to prevent evaporation. The tubes were placed in an incubator and warmed to 37°C. The contents of each tube were then expelled in turn onto a siliconized slide and the frozen embryos contained in 1 μl extraction medium were added. The drop was immediately taken back up into the PCR tube and resealed with parafilm.

The tubes were incubated at 37°C for 10 min to allow reactions (1), (3) and (4) to occur. Initial fluorescence readings were taken for each tube at three different places and the average was calculated. The contents of the tube were then expelled onto the siliconized slide and 1 μl reagent B was added. The drop was immediately taken back into the PCR tube which was sealed and incubated for 7 min. Finally, triplicate readings were again taken for each tube. The change in fluorescence was related to the amount of triglyceride present in the sample from a standard curve of triglyceride in FCS (range 0–0.57 μg μl^{-1}), which was prepared each time the assay was carried out.

Embryo triglyceride content was measured in unfertilized and fertilized oocytes and embryos grown in the presence and absence of serum. Comparisons were made with *in vivo* day 7 blastocysts. The embryos used for analysis were from at least ten different culture weeks. Further comparisons of triglyceride content were made between pooled and non-pooled embryos and those with and without the zona pellucida, which was removed by placing the embryos in acid Tyrode's at 37°C for about 1 min.

Statistical analysis

Comparisons were made between all embryo stages grown with and without the addition of serum. Data were analysed by one-way analysis of variance and Fisher's test using Minitab.

Results

The data on the triglyceride content of bovine oocytes and early embryos to the hatched blastocyst stage are summarized (Table 1). Some embryos were grown in the presence of serum from the four-cell stage. Measurements
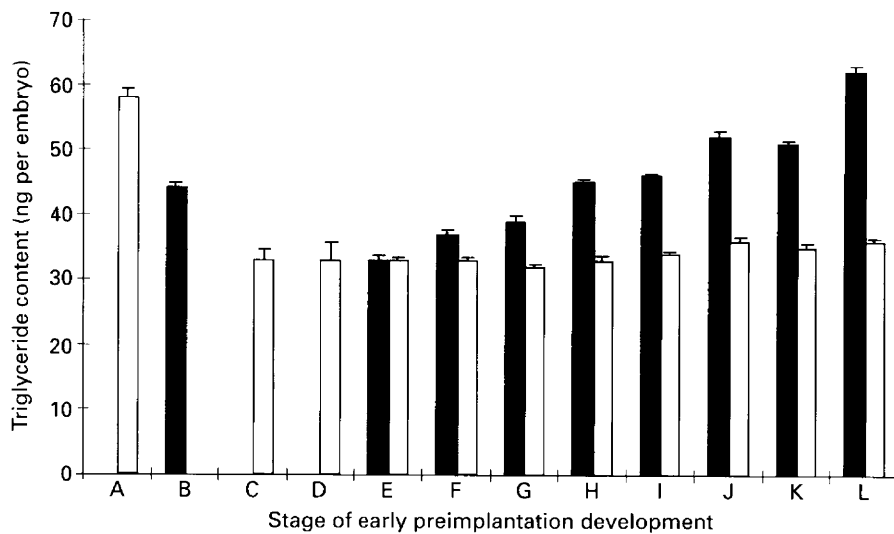
Fig. 1. Triglyceride content (ng per embryo). Mean ± SEM of *in vitro* produced embryos grown in the (■) presence or (□) absence of serum from the four-cell stage. A, oocytes before maturation; B, oocytes after 24 h maturation; C, two-cell embryo; D, four-cell embryo; E, five–eight-cell embryo; F, nine–sixteen-cell; G, compact morula; H, early blastocyst day 7; I, early blastocyst day 8; J, expanded blastocyst day 7; K, expanded blastocyst day 8; L, hatched blastocyst day 7 or day 8.

were made on groups of two or three embryos over more than ten different culture weeks.

The triglyceride content of oocytes before maturation was 59 ± 1.37 ng and decreased ($P < 0.05$) to 46 ± 0.85 ng after 24 h of maturation (Fig. 1). A decrease ($P < 0.05$) in triglyceride content was also found after fertilization with the formation of the two-cell embryo. The triglyceride content of embryos cultured in the absence of serum did not change from the two-cell stage to the hatched blastocyst, and the amount (approximately 33 ng) was similar to that of *in vivo* blastocysts obtained by flushing of the uterus on day 7 (33 ± 0.70 ng). In embryos grown in the presence of FCS from the four-cell stage, the triglyceride content remained at approximately 34 ng to the five- to eight-cell stage. However, an increase ($P < 0.05$) in triglyceride was observed after the five- to eight-cell stage to the hatched blastocyst stage (62 ± 1.14 ng) (Fig. 1).

Triglyceride content in pooled embryos that had undergone the first cleavage in the first 30 h after fertilization was compared with that in non-pooled embryos that had undergone the first cleavage after this time. The triglyceride content of pooled five- to eight-cell embryos and expanded blastocysts was 33 ± 0.87 ng and 51 ± 0.86 ng, respectively, compared with 32 ± 1.12 ng and 50 ± 0.9 ng, respectively, for embryos that underwent the first cleavage 30 h or more after fertilization. These results show that timing of the first cleavage division had no effect on triglyceride content. The triglyceride content of embryos with and without the zona pellucida were compared at two stages. The triglyceride content of four-cell and five- to eight-cell embryos with a zona pellucida was 34 ± 0.94 ng and 34 ± 0.7 ng, respectively, compared with 36 ± 1.01 ng and 33 ± 1.25 ng, respectively, for embryos without a zona pellucida. These results indicate that triglyceride is not detectable in the zona pellucida.

Discussion

Bovine embryos consume exogenous substrates, but the importance of intracellular energy sources is unknown. Although there is morphological evidence for changes in lipid content during preimplantation development (Fleming and Saacke, 1972; Betteridge and Flechon, 1988), the present study is the first to quantify the triglyceride content of bovine oocytes and preimplantation embryos produced *in vitro* and grown in the presence and absence of FCS from the four-cell stage.

Before maturation, the triglyceride content of bovine oocytes was 59 ng, a value intermediate to that reported for pig (75 ng; McEvoy *et al.*, 1997) and sheep (25 ng; Coull *et al.*, 1997) oocytes.

The triglyceride content decreased significantly to 46 ng after oocyte maturation, indicative of a metabolic role. The data from the present study also indicate a significant decrease in triglyceride content during fertilization and the first cleavage. Fleming and Saacke (1972) suggested that lipid may be used as an energy source during fertilization in bovine oocytes due to the close association of the endoplasmic reticulum and mitochondria with lipid droplets. Kruip *et al.* (1983) found that the *in vivo* maturation of bovine oocytes was associated with a change in mitochondrial location, from a peripheral to a cortical position. This movement of organelles may reflect a change to a reliance on an intracellular energy source as the oocyte loses contact with follicle cells. During maturation, there is intensive clustering of mitochondria, in association with lipid droplets and elements of the smooth endoplasmic reticulum, and ribosomes appear in the cytoplasm. The lipid component is degraded during maturation (Kruip *et al.*, 1983). These findings indicate that during maturation of bovine oocytes,

lipids may be used as a source of the ATP required for protein synthesis on the increasing number of free ribosomes (Kruip *et al.*, 1983). It has been suggested that such protein synthesis is essential for the continuation of meiosis and cytoplasmic maturation. There are major changes in protein synthesis during this period in sheep (Warnes *et al.*, 1977, Moor and Warnes, 1978, Crosby *et al.*, 1981, Moor *et al.*, 1981), and these changes are thought to be important for full oocyte maturation and subsequent embryonic development.

The ultrastructural changes observed in cattle oocytes may be related to the findings of Reiger and Loskutoff (1994) who reported that pyruvate, glutamine and glycine metabolism by the Krebs cycle increased during the *in vitro* maturation of cattle oocytes, indicating that oxidative metabolism is the major site of cellular energy production at this time. Reiger *et al.* (1998) also found that the presence of growth factors (insulin-like growth factor I and epidermal growth factor), hormones (human menopausal gonadotrophin (hMG)) and serum during oocyte maturation stimulated oxidative metabolism. Pyruvate metabolism was highest when oocytes were matured with hMG and serum, and this was reflected in higher cleavage rates and development to the blastocyst stage, indicating that the stimulation of mitochondrial function favours, or is predictive of, developmental potential (Reiger *et al.*, 1998). These findings are consistent with the results of the present study, and indicate that fatty acids derived from the breakdown of triglycerides are oxidized via the Krebs cycle.

In embryos grown in the absence of serum, the triglyceride content remained at about 33 ng to the hatched blastocyst stage; the same value was observed in *in vivo* blastocysts on day 7. The triglyceride content of embryos grown in the presence of serum increased ($P < 0.05$) from the nine–sixteen-cell stage (37 ng) to a maximum value at the hatched blastocyst stage (62 ng). Although serum was added at the four-cell stage, an increase in triglyceride content could not be detected until the nine–sixteen-cell stage. Van Langendockt *et al.* (1997) reported that the presence of serum in the culture medium causes accelerated development of bovine embryos to the blastocyst stage. This accelerated development is only observed after activation of the embryonic genome, after the eight–sixteen-cell stage, even when serum is added as early as the four-cell stage. The mechanism of accumulation of triglyceride reported in the present study is unknown. It is possible that serum stimulates the embryo to synthesize its own triglyceride or, more likely, that serum is taken up by pinocytosis and the triglyceride released intracellularly. Dunglison *et al.* (1995) showed that insulin increases endocytosis in the trophectoderm of mouse blastocysts. Thus, it is possible that insulin present in the serum stimulates pinocytosis in the bovine embryo once the embryonic genome has been switched on.

Triglyceride was not detected in the zona pellucida; the triglyceride content of embryos with and without a zona pellucida was similar. This finding is in agreement with the conclusion of Gwatkin *et al.* (1980). A comparison of triglyceride content was also made between embryos that had undergone the first cleavage in the first 30 h after fertilization and those that had undergone the first cleavage

after this time. No difference in triglyceride content was observed. In embryos that undergo the first cleavage in the first 30 h after fertilization, the probability that they will form a compact morula is almost 50%, whereas in embryos that cleave for the first time at 48 h after fertilization, the probability is < 35% (Van Soom *et al.*, 1992). However, the difference in the time to the first cleavage did not appear to reflect different amounts of triglyceride.

The excessive accumulation of triglyceride that occurs when embryos are cultured in the presence of serum could be associated with the enlarged calf syndrome, but as yet there is no causal relationship. Serum causes premature blastulation, fragmentation and mitochondrial degeneration which could contribute to fetal oversize (Dorland *et al.*, 1994; Thompson *et al.*, 1995). In addition to metabolic defects, the accumulation of lipid increases cell volume and may pose osmotic problems for the embryo. The excessive accumulation of lipids observed when embryos are cultured in the presence of serum could also partly explain why such embryos are more sensitive to freezing and have a lower buoyant density than their *in vivo* counterparts. Leibo *et al.* (1993) examined the effect of intracellular lipids on chilling and freezing sensitivity of *in vitro* produced bovine embryos. Compact morulae that contained different amounts of intracellular lipids were produced by high speed centrifugation. The results indicated that differences in intracellular lipid content are at least partially responsible for the decreased buoyancy and increased chilling and freezing sensitivity shown by *in vitro* produced embryos (Leibo *et al.*, 1993).

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