Alterations in lipolysis activity during:

- **Ovine Pregnancy Ketosis.**
- **The transition period of dairy cattle.**

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Contents

Abstract

The most common metabolic disease affecting the adipose tissue in sheep and dairy cattle is ketosis. In these days it is thought that food (poor quality or an inadequate quantity) is one of the most important causes of ketosis. In this study we used ewes (2 ketotic and 2 control animals) and dairy cattle (6 Holstein Frisian dairy cattle) adipocyte tissue and blood.

The aim of the study in ewes is to see if the rate of lipolysis changes when we incubated the adipocyte tissue with isoproterenol, insulin and beta-hydroxybutyrate (BHB) and the combination of isoproterenol insulin and the combination of BHB and insulin is different between ewes with or without ketosis.

The aim of the study in dairy cattle is to see if there is an alteration in the lipolysis rate during the transition period (last week of gestation until the third week of lactation). We also tested of the differences are seen in the different incubations we did.

In the ewes isoproterenol stimulates the lipolysis rate, BHB inhibits the lipolysis rate and insulin had little or no effect. The combinations of insulin with isoproterenol also elevated the lipolysis rate and the combination of insulin and BHB reduces the lipolysis rate. Not any of these effects where significantly. The blood samples where taken to confirm if the ewe was ketotic or not.

In dairy cattle the incubations we tested are isoproterenol, insulin 50 pM, 100 pM, 500 pM, BHB and the combination of isoproterenol with insulin 50 pM and isoproterenol combined with insulin 500 pM. Isoproterenol stimulates the lipolysis, insulin had little or no effect, BHB caused a reduction in lipolysis activity and the combination of isoproterenol and insulin elevated the lipolytic activity. Only the reduction of lipolysis caused by BHB is significant.

Introduction

The most common metabolic disease affecting the adipose tissue in sheep and dairy cattle is ketosis (Henze, et al, 1994).
In ewes it appears normally at the end of gestation and is called ovine pregnancy ketosis (OPK), also called pregnancy tox(a)emia, twin lamb disease, pregnancy ketosis or sleeping ewe disease (Henze et al 1994 & 1998, Schlumbohm & Harmeyer 2008). OPK is seen more often in elderly ewes during late gestation, pregnant with multiple lambs (Kahn & Line 2005: 828-830).
In cows it mostly occurs in early lactation of dairy cow breeds. It is also called acetonemia or ketonemia (Kahn & Line 2005: 830-832).

The function of adipocyt tissue?

Lipid metabolism

The function of white adipose tissue is storage of triacylglycerol (TAG). TAG is a glycerol backbone with three fatty acids (FA) esterified and stored in the cytosol of adipose tissue (Roach et al, 2004 & Stoneham, 1989).
FA are an essential fuel and major energy source for the body because they are easily mobilized to provide energy during prolonged exercise or starvation (Roach et al, 2004).
In ruminants the liver, adipose tissue, lactating mammary glands and kidney's synthesize FA de novo out of volatile acids formed in the rumen by fermentation. This is called lipogenesis (Roach et al, 2004, Stoneham, 1989, Pethick et al 2005 & Berne et al 2004: 752-756). Lipogenesis is a series of reactions adding two carbon molecules of acetyl-CoA to a growing fatty acid chain (Roach et al, 2004). FFA synthesis de novo is principally out of acetate (Pethick et al 2005).
Lipolysis is the hydrolysis of TAG by a hormone sensitive lipase (HSL) (Pethick et al 2005 & Roach et al, 2004) at the C1 and C3 position which produces monoacylglycerol and two free fatty acids (FFA). A monoacylglycerol-specific lipase removes the remaining FA. This results in three FFA and one glycerol molecule (Roach et al, 2004).
Glycerol cannot be metabolized by the adipose tissue and goes to the liver. In the liver the glycerol will be metabolized to glycerol-3-phosphate and returns to the adipose tissue to form TAG or it is metabolized to glyceraldehyde-3-phosphate a precursor for gluconeogenesis (Roach et al, 2004 & Pethick et al, 2005).
FA are either re-esterfied to TAG in the adipose tissue or bound to serum albumin, then called FFA or non-esterified free fatty acids (NEFA) (we will refer to it as FFA), and travel to liver and muscle for oxidation in the cell (Roach et al 2004, Pethick et al, 2005 & Berne et al 2004: 752-756). FA also can be transported as TAG in the blood as part of very low-density lipoprotein (VLDL when produced by the liver) or chylomicrons (when produced by the gastrointestinaltract cells) (Pethick et al, 2005 & Vernon, 2005).
See figure 1 for a short overview of the lipolysis.

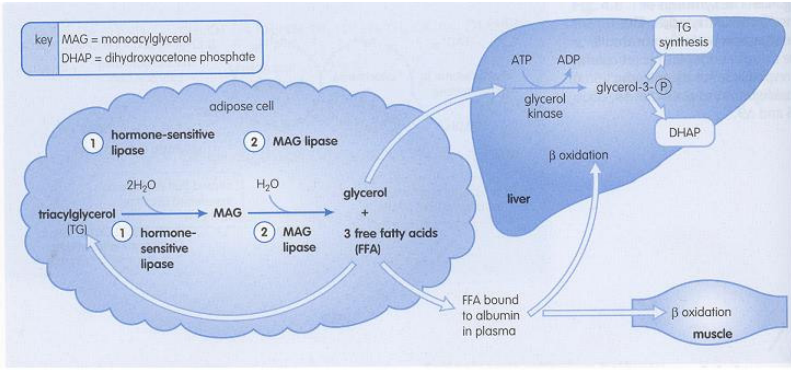


Figure 1: hydrolysis/lipolysis of TAG, glycerol and FFA (Roach et al, 2004: 64)

The adipose metabolism is influenced by a family of nuclear receptors; the peroxisome proliferator-activated receptors (PPAR). PPARγ is found in all adipose tissue (PPARα only on brown adipose tissue) and stimulates the synthesis, binding, transport and storage of FFA. This is because stimulation of PPARγ stimulates the transcription of phosphoenolpyruvate carboxynase mRNA (PEPCK) which increases the level of PEPCK in adipocytes which stimulates re-esterfication of FFA by a raise in glycerol-3-phosphate (Everts et al, 2008 & Kallen & Lazar, 1996). PEPCK caboxylates and phosphorylates oxaloacetate into glyceraldehyde-3-phosphate. Glyceraldehyde-3-phosphate can be converted to dihydroxyacetone phosphate which can be converted to glycerol-3-phosphate and this can be used to form TAG (Roach et al, 2004).
For our study the most important agonist of PPARγ are polyunsaturated fatty acids. They activate the PPARγ and therefore a decrease of insulin resistance, a decrease of leptin production and an increase of PEPCK. Another agonist of PPARγ are the thiazolidinediones. They activate the PPARγ receptor and therefore also PEPCK which stimulates the re-esterfication of FFA and reduction of leptin (Everts et al, 2008 & Kallen & Lazar, 1996). Thiazolidines are used to treat diabetes type II (non-insulin-dependent diabetes mellitus), because they lower the insulin resistance of cells, the mechanism is unknown (Kallen & Lazar, 1996, Vidal-Puig et al, 1997 &Nolan et al, 1994).
The expression of PPARγ is decreased by elevated levels of estrogen (Everts, R.R. et al 2008).

In the cell cytosol, activation of FFA occurs by attachment to CoA to form acyl-CoA. By the carnitine shuttle the acyl-CoA molecules are transported into the mitochondria. This is because the inner mitochondrial membrane is relatively impermeable to long-chain acyl-CoA (see figure 2). The carnitine shuttle is controled by manolyl-CoA → inhibits carnitine acyl transferase I → inhibiting of acyl groups entring the mitochondria. Manolyl-CoA is produced during acyl-CoA synthesis and ensures that newly produced FA do not enter the mitochondria to enter the β-oxidation. (Roach et al, 2004).
The acyl-CoA is split into several acetyl-CoA molecules by a cyclical sequence of four reactions: oxidation, hydration, oxidation, thiolysis called the β-oxidation. The β-oxidation is competing with the Krebs-cycle for NAD^+ and FAD because they are acting at the same time (Roach et al, 2004). β-oxidation of FA produces a major source of energy for the body (Berne et al, 2004: 586) (see figure 3).

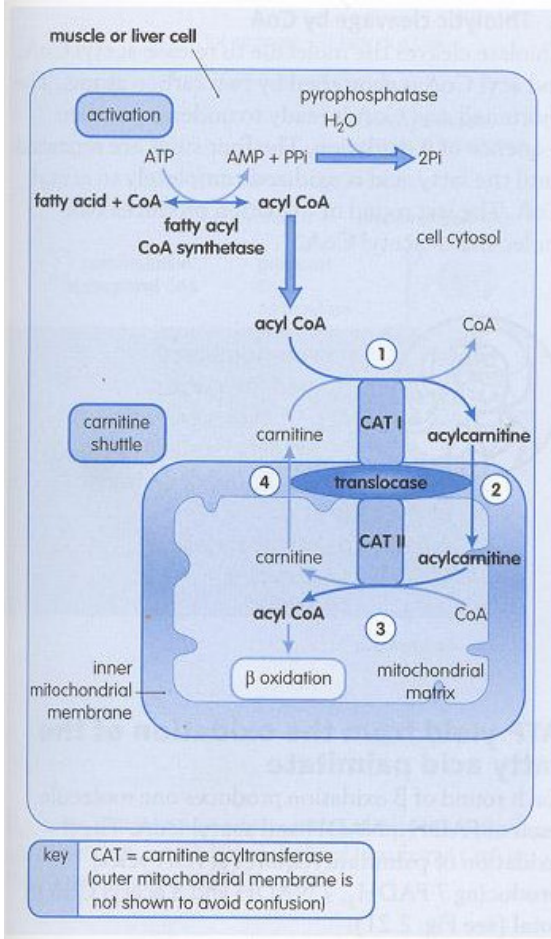


Figure 2: Transport of FFA into mitochondria by the carnitine shuttle (Roach et al, 2004: 65)

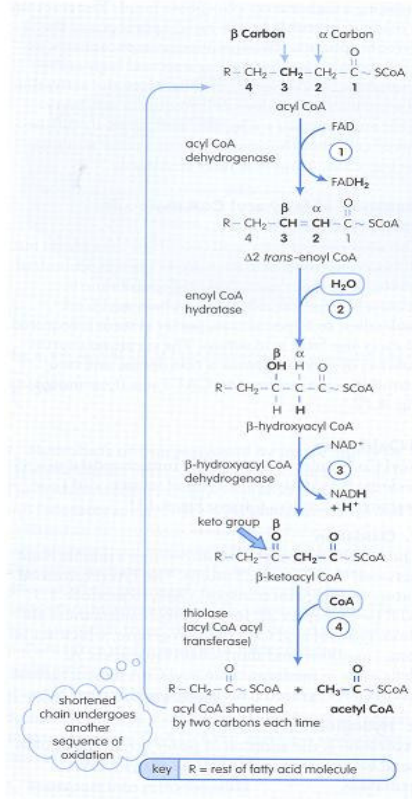


Figure 3: β-oxidation pathway (Roach et al, 2004: 66).

1). The oxidation introduces a double bond between the C2 and C3 atoms by acyl-CoA dehydrogenase.
2). The hydratation is the addition of H_2O across the double bond by Δ2 enoyl-CoA.
3). β-hydroxyacyl-CoA dehydrogenase converts the OH-group at C3 to a ketogroup.
4). Thiolase cleaves the molecule to release acetyl-CoA.
At the end of this four reactions the acyl-CoA is shortened by two carbon atoms and there is an acetyl-CoA formed. The remaining acyl-CoA undergoes another β-oxidation until it is completely oxidized to acetyl-CoA (Roach et al, 2004).

Overall the lipid breakdown is controlled at three levels: lipolysis, carnitine shuttle and β-oxidation.

Lipolysis

The lipolysis is controlled by the amount of hormone-sensitive lipase (HSL) which is regulated itself by reversible phosphorylation. Adrenaline, glucagon, adrenocorticotrophic hormone (ACTH), activate adenylate cyclase. Activation of adenylate cyclase increases the amount of cAMP in the cell. Increase of cAMP activates the cAMP-dependent protein kinase. cAMP-dependent protein kinase phosphorylates lipase (is activated) and phosphorylates of acetyl-CoA carboxylase (is deactivated). The lipase stimulates the lipolysis and acetyl-CoA carboxylase inhibits the FA synthesis (Roach et al, 2004).

HSL is activated by cAMP. cAMP itself is increased by stimulation of a membrane bound adenylate cyclase bound complex. The adenylate cyclase complex consist of a nucleotide binding site, a hormone receptor and a catalytic protein with stimulating and inhibiting GDP biding sites. Binding of catecholamines (dopamine and (nor)adrenalin) has a short but rapid stimulating effect on the lipolysis. This is because cAMP will raise. For example during exercise or stress. The binding of insulin to adenylate cyclase complex has a short and rapid suppressing effect on the lipolysis. The catecholamine-stimulated lipolysis increase when the insulin induced glucose utillisation by adipocytes reduces and vice versa (Pethick et al, 2005 & Vernon, 2005).

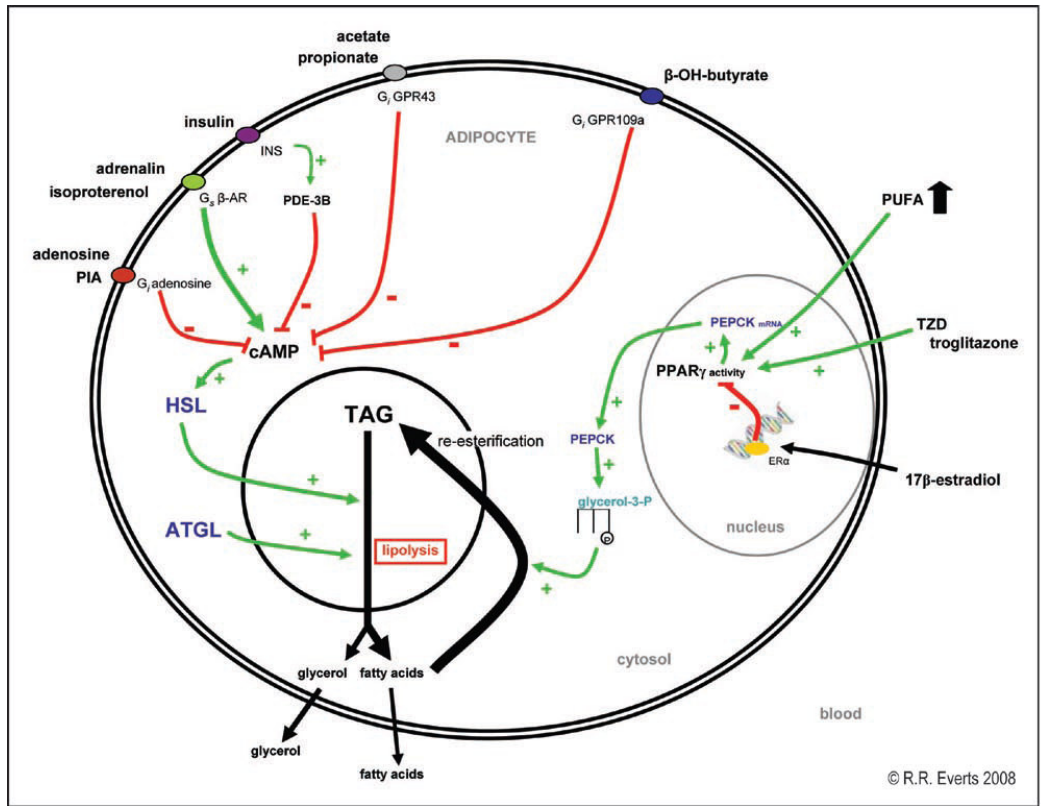


Figure 4: Schematic, simplified, overview of adipocyte metabolism. (Everts et al, 2008: 17)

Abbreviations: ATGL, adipose triglyceride lipase; β-AR, β-adrenergic receptor; cAMP, 3',5'-cyclic adenosine monophosphate; ERα, estradiol receptor α; G*i*, inhibitory G protein; G*s*, stimulatory G protein; HSL, hormone sensitive lipase; INS, insulin receptor; PDE-3B, phosphodiesterase 3B; PEPCK, phosphoenolpyruvate carboxykinase; PIA, N6-[R-(-)-1-methyl-2-phenethyl]adenosine; PPARγ, peroxisome

proliferator activated receptor γ; PUFA, polyunsaturated fatty acids; TAG, triacylglycerol

Stimulating the lipolysis

Cortisol/ACTH

Cortisol stimulates the gluconeogenisis in the liver by stimulating the breakdown of substrate, for example promoting lipolysis. Glycerol is used for gluconeogenesis by the hormones glucagon, epinephrine and growth hormone (GH). The insulin mediated glucose uptake by muscle, adipose tissue and other tissue that uses glucose is inhibited, which reduces the use of glucose in the body (Dunlop, 2005 & Berne et al, 2004: 895-899).

Cortisol is necessary for epinephrine, growth hormone and other lipolytic substances to stimulate the hydrolysis of stored TAG at maximal rates (Berne et al, 2004: 895-899 & Dunlop, 2005).

Ghrelin

Ghrelin is secreted by the gastrointestinal tract when there is a short or long-term nutritional shortage (Vestergaard et al, 2008 & Lely et al, 2004). In human adipocytes it is found that ghrelin is an endogenous ligand for the GHRH (growth hormone releasing hormone) receptor and stimulates the secretion of GH in vitro (Vestergaard et al, 2008, Everts et al, 2008 & Lely et al, 2004). The GHRH receptors of GH are present all over the body so there are several effects of ghrelin. These effects are; stimulation of ACTH secretion, stimulation of appetite, food intake and motility of the gastric motility. It is also known that it increases plasma glucose, FFA and glycerol serum levels by promoting the lipolysis. Also is there a reduction in disposal rate of glucose comparable to an insulin resistance which ensures glucose for glucose dependent tissue (Vestergaard et al, 2008).

Estrogen

Elevation of estrogen makes the adipocytes more sensitive for insulin. Increase of sensibility to insulin gives more inhibition of lipolysis when there are low levels of insulin (Pelt et al, 2006). But estrogen also inhibits the expression of PPARγ on the adipocytes so there is a reduction in re-esterfication speed of FFA ((Everts, R.R. et al 2008).

Adrenalin/Isoproterenol

In vitro stimulation of human adipocytes with adrenalin is known to stimulate the lipolysis (Galton & Bray, 1967) by raising the levels of cAMP in the cytosol of the adipocytes. But on the contrary adrenalin also stimulates the production of PEPCK which stimulates the reesterfication of FFA (Everts et al, 2008).

GH or somatotropin

GH is known to increase lipolysis by up-regulation of the β-adrenergic receptor (Everts et al, 2008, Vernon et al, 2005 & Pethick et al, 2005) and can induce resistance of adipocytes to insulin (Vernon et al, 2005). GH also mildly stimulate the lipolysis itself (Pethick et al, 2005).

As you can read above the secretion of GH is stimulated by ghrelin and growth hormone releasing hormone (GHRH) and inhibited by somatostatin in vitro in human adipocytes (Lely et al, 2004). GH secretion can be stimulated by 17β-estradiol secreted by placental tissue during pregnancy (Everts et al, 2008).

Glucagon

Glucagon is the primary counter-regualtory hormone of insulin. The ratio of insulin/glucagon is the most interesting. A low ratio means stimulation of lipolysis

and ketogenesis. A high ratio means stimulation of glucose use and lipogenesis in ruminant adipocytes in vivo (Radostits et al, 2007: 1661-1668, Brockman, 1979).

Leptin

In humans (in virto) and in rats (in vivo) leptin is secreted when the adipocytes are full with TAG and when there are high levels of cortisol and insulin (Berne et al, 2004: 759-763, Elimam et al, 2002 & Vernon 2005). The actions of leptin are: a decrease of food intake, increase of the metabolic rate, increase of the activity and a raise in body temperature acting on the hypothalamus. The overall effect of these actions is the decrease in amount of adipose tissue (Berne et al, 2004: 895-899 & Siegrist-Kaiser et al, 1997). Leptin is also known to stimulate the adenylate cyclase/G_i protein which on his turn stimulates the lipolysis (Elimam et al, 2002). Leptin is also considered to impair the metabolic actions of insulin on adipocytes. (Berne et al, 2004: 759-763, Elimam et al, 2002 & Vernon, 2005, Schlumbohm et al 1997 & Pethick et al, 2005), but stimulates the insulin-induced glucose utilization (Siegrist-Kaiser et al, 1997).

Leptin is down regulated when the PPARγ is stimulated (Everts et al, 2008, Berne et al 2004: 752-756 & Kallen & Lazar, 1996).

Inhibiting the lipolysis

Insulin

Insulin has a feedback mechanism for its secretion. When glucose, amino acids, ketoacids, potassium and FFA are high the secretion of insulin is stimulated. On the contrary when insulin is high the levels of glucose, amino acids, ketoacids, potassium and FFA will be lowered. This will cause less insulin secretion (Berne et al, 2004: 773 & 780). When the nutrient supply is inadequate there is a fall in serum insulin concentration. The decrease of insulin concentration will cause a reduction in anabolic processes, an increase of glucose (reserve) and FFA release and when there is starvation, there is muscle proteolysis (Vernon, 2005).

Insulin inhibits the lipolysis by actition of cAMP-phosphodiesterase which activates the breakdown of cAMP into AMP (Vernon, 2005), therefore HSL is not activated (Roach et al, 2004, Berne et al, 2004: 780-783 & Vernon, 2005). Insulin stimulates the transport of glucose into adipocytes by, activation of insulin dependent glucose transporter (Glut-4) on adipocyt membranes. The glucose is converted to dihydroxyacetone phosphate which is reduced by glucerol-3-phosphate dehydrogenase into glycerol-3-phosphate. So there is more glycerol-3-phosphate in the adipocytes to form new TAG. (Stoneham, 1989, Brockman, 1979 & Vernon, 2005).

Another function of insulin is dephosphorylation (activation) of pyruvate dehydrogenase which stimulates the production of acetyl-CoA. The acetyl-CoA is then carboxylated by acetyl-CoA carboxylase to manolyl-CoA. Acetyl-CoA carboxylase is stimulated by insulin (Stoneham, 1989). The little amount of ketoacids that is produced (mainly by visceral adipose tissue) is used by muscle, brain and heart, because high levels of insulin stimulates these tissues to use ketoacids as energy source (Berne et al, 2004: 780-783).

Adenosine

Adenosine can be secreted by the adipocyte itself out of AMP. In vitro studies on human adipocytes showed that adenosine inhibits cAMP accumulation and the lipolysis. It also stimulates the effect of insulin in its inhibiting processes and stimulates the glucose uptake of adipocytes. Adenosine can counteract stimulatory effects of lipolytic hormones because it lowers the levels of cAMP in the cytosol of

the adipocytes (Stoneham, 1989 & Heseltine et al, 1995). Adenosine especially suppresses the lipolytic effects of noradrenalin (Heseltine et al, 1995).
Adenosine deaminase breaks down adenosine and stimulates the lipolytic effects of isoproterenol (Stoneham, 1989).

Free Fatty acids

FFA released in the blood binds to serum albumin. The albumin has two high-affinity binding sites and five low-affinity binding sites for FFA. The concentration of albumin is 0,5 mM so when there is a concentration of 1,0 mM of FFA the high-affinity binding sites are saturated. This results in a decrease of FFA release when the concentration of FFA is above 1.0 mM (Vernon, 2005).
Increase in FFA decreases the insulin mediated glucose metabolism (Heseltine et al, 1995).

Out of control

There are several things that can go wrong. In this article only the ketogenesis is explained. Ketogenesis is the formation of ketone bodies out of acetyl-CoA in the liver, occurring in all the time in the body of mammals (Pethick, 2005 & Roach et al, 2004). See figure 5

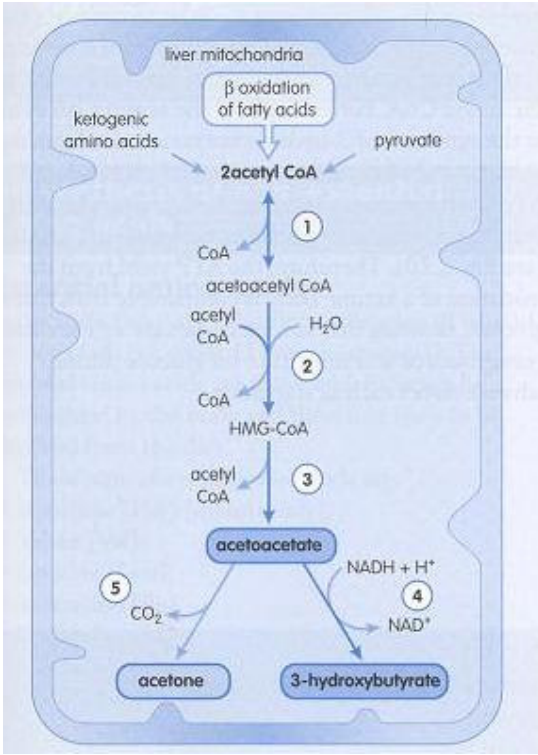


Figure 5: Ketogenesis (Roach et al, 2004: 81)
The ketogenesis occurs in the mitochondria of the liver cells.
1). Acetoacetyl-CoA is formed out two acetyl-CoA by the enzyme acetoacetyl-CoA thiolase.
2). Acetoacetyl-CoA is hydrolysed with another acetyl-CoA into HMG-CoA controlled by HMG-CoA synthase.
3). HMG-CoA is lysated by HMG-CoA lyase to acetoacetate.
4). Dehydrogenase of acetaoacetate into 3-hydroxybutyrate (BHB) by 3-hydroxybutyrate dehydrogenase
5). Spontaneous reaction. Acetoacetate converts into aceton

Ketone bodies (acetoacetate, acetone and 3-hydroxybutyrate (BHB)) are formed, when mammals are in several adverse states; starvation, a substantial negative energy balance (NEB), prolonged severe exercise or uncontrolled diabetes the ketogenesis is necessary to produce alternative energy for the brain, heart and muscle to lower the use of glucose by the body (Pethick et al, 2005, Vernon, 2005, Hayes, 2004 & Roach et al, 2004). Under certain circumstances there is overcompensation for the inadequate energy intake by the ketogenesis which can lead to ketoacidosis (Brockman, 1979, Pethick et al, 2005, Vernon, 2005, Hayes, 2004).

During adverse states; starvation, a substantial negative energy balance (NEB), prolonged severe exercise or uncontrolled diabetes there is an increase in FFA supply towards the liver, a decline in glucagon/insulin ratio and a decrease in feed intake (Pethick et al, 2005, Brockman, 1979 & Roach et al, 2004). The liver oxidizes the FFA and uses them for ATP production or releases them as ketone bodies into the circulation, which can be used by other organs. The liver also can re-esterfy the FFA into lipids. Some of the lipids are used in the liver for membrane turnover, but the most of it is secreted as VLDL into the blood (Vernon, 2005).

BHB has an inhibitoir effect on the lipolysis, in vitro on cattle adipocytes, so there are less FFA formed. BHB has an indirect negative feedback on its own production. The inhibitory effect of BHB is due to the lowering of cAMP levels in the adipocytes (Metz et al, 1974).

In sheeps and cattle there is a disease known as ketosis caused by high levels of ketone bodies in the blood.

Ethiology and Pathogenesis

Ovine pregnancy ketosis (OPK)

Until now it is thought that there are several predisposing causes of OPK. These are: 1) Inadequate nutrition during late gestation. 2) High demands of glucose by lambs and uterus 3) Poor (< 2,0) or high (>4,0) body condition score (BCS) (Kahn & Line, 2005: 828-830, Radostits et al, 2007: 1668-1670, Schlumbohm & Harmeyer, 2008 & Brockman, 1979). Nowadays we think there is also a predisposition in the metabolism of fat- and carbohydrates in some ewes (Everts et al, 2008).
Inadequate nutrition can results in a negative energy balance (NEB). The NEB can be caused by low energy density of the food, decreased rumen capacity during the last weeks of gestation caused by the lambs in the uterus. (Kahn & Line, 2005: 828-830, Radostits et al, 2007: 1668-1670 & Schlumbohm & Harmeyer, 2008). The energy reserves of the ewes that are being used to compensate for the shortage of glucose are glycogen (from the liver and muscles) and TAG (Everts et al, 2008, Kahn & Line, 2005: 828-830). The amount of glycogen in liver and muscle is small, so it can only be used for a short period of time. The TAG is used for the β-oxidation of the FFA. FFA can be converted to ketone bodies (betahydroxy butyraat (BHB), acetone and acetoacetaat) an alternative for glucose. However if the concentration of ketone bodies raises above 3,0 mM/L the ewe is going to be affected and develop OPK. The neurological signs seen in OPK are similar as the signs seen in hepatoencephalopathy (Everts et al, 2008 & Radostits et al, 2007: 1668-1670).
The influence of BCS not really understood because, in experiment there is no correlation between BCS and biochemical parameters in the blood shown (Henze et al, 1998), but in books they say there is a correlation between BCS and OPK (Kahn & Line, 2005: 828-830). In the experiment the BCS of the ewes will be registered.

Ketosis in cattle

There are five forms of ketosis in cattle. They have the same clinical signs but different causes (Radostits et al, 2007:1661-1667). In this paper we refer to the primary ketosis, which is due to a NEB because the demand for energy is much higher then the animal can consume (Radostits et al, 2007:1661-1667).
Cattle and other ruminants have to synthesize glucose de novo from propionate and amino acids, precursors coming from fermentation in the rumen. Also glycerol and

lactate is used, because the sugars present in the diet are fermented in the rumen (Radostits et al, 2007: 1661-1667). It is not really understood why bovine ketosis appears. Mostly it is suggested about the combination of intense adipose mobilization and a high glucose demand. In early lactation there is a negative energy balance (adipose mobilization) and a high glucose demand. As a result of adipose mobilization there are high levels of FFA in the blood. The liver converts the FFA into ketone bodies, especially when there is a high gluconeogenesis. Cows with ketosis have high levels of ketone bodies and FFA, but low levels of glucose (Kahn & Line, 2005: 830-832, Radostits et al, 2007: 1661-1667).

Clinical findings and pathology

OPK

Most cases of OPK occur 1-3 weeks before lambing in elderly ewes pregnant with multiple lambs (Clarkson, 2000, Bickhardt et al, 1989, Schlumbohm & Harmeyer, 2008, Kahn & Line, 2005: 828-830 & Straiton, 1998). If OPK occurs before 140 days of gestation it is seen that OPK is more severe and has a higher mortality rate (Kahn & Line, 2005: 828-830).

OPK has aspecific symptoms and therefore it is difficult to diagnose the disease merely by its symptoms. The most common signs are: separation from the flock, reluctancy to move, decreased aggressiveness at feeding and rejection of food (starts with concentrates). When OPK progresses; signs of listlessness, aimless walking, muscle twitching or fine muscle tremors, opisthotonus, teeth grinding and compulsive circling movement can be seen. This can be followed by: blindness, ataxia and finally sternal recumbency, coma, and death of the ewe if no or an inadequate therapy is applied (Clarkson, 2000, Kahn & Line, 2005: 828-830, Straiton, 1998 & Radostits et al, 2007: 1668-1670). The death of fetuses is very common (Clarkson, 2000 & Radostits et al, 2007: 1668-1670).

At the necropsy there are several findings. Fatty liver and kidney degeneration in seen in varying degrees. The liver and kidneys look pale, yellow and friable (Clarkson, 2000, Radostits et al, 2007: 1668-1670 & Kahn & Line, 2005: 828-830). The fatty liver is probably due to the uptake of FFA, proportionally to the concentration, from the blood. This is more than the liver can use for ketogenesis or β-oxidation and is therefore re-esterfied to TAG in the liver (Pethick et al, 2005).

Enlarged adrenal glands (pale or hyperaemic) can be seen (Clarkson, 2000, Kahn & Line, 2005: 828-830).

The brain can also be affected in several ways. There can be astrocystic nuclear swelling, hypertrophy and proliferation. Also, necrosis of the neurons cerebrocortical and vacuolation of cerebral and cerebellar white matter is seen at necropsy (Clarkson, 2000, Radostits et al, 2007: 1668-1670).

There are dead lambs mostly twins or triplets present in the uterus, at any state of degeneration (Radostits et al, 2007: 1668-1670).

Ketosis in cattle

Most cases of ketosis in cattle occur between the 10-28 days of lactation, whith a peak in the 2nd week of lactation (Kahn & Line, 2005: 830-832 & Radostits et al, 2007: 1661-1667). Multiparous dairy cows have a higher chance of developing ketosis (Radostits et al, 2007: 1661-1667). The average prevalence is between 5-16% but there are great differences between herds (Kahn & Line, 2005: 830-832 & Radostits et al, 2007: 1661-1667).

The first signs of ketosis are reduced feed intake (starting with grain) progressing to reduced milk production and lethargy and a decrease in body weight (Kahn & Line, 2005: 830-832 & Radostits et al, 2007: 1661-1667). By physical examination the cow may be slightly dehydrated. Rumen motility can be hyper- or hypoactive (Kahn & Line, 2005: 830-832 & Radostits et al, 2007: 1661-1667). The feces may be firm and dry (Radostits et al, 2007: 1661-1667). Neurological signs can be apparent in a minority of the cases and have a sudden onset. The sings can be abnormal licking and chewing with salivation, spreading or crossing the legs, walking in circles, incoordination, gait abnormalities, apparent blindness, head pushing or leaning against obstacles, aggression and bellowing (Kahn & Line, 2005: 830-832 & Radostits et al, 2007: 1661-1667).
Ketosis is usually not fatal in cattle but fatty degeneration of the liver and secondary changes in the adrenal glands may be present (Radostits et al, 2007: 1661-1667).

Diagnosis

OPK

The diagnose is set mostly after the results of the blood biochemistry are known. This is due to the aspecific signs of OPK (Kahn & Line, 2005: 828-830).
To confirm the suggestion of OPK *Siemens labstick* are used to test in the urine. If it is positive it is highly suggestive and if it is negative it rules out OPK (Clarkson, 2000).
To confirm the test on the urine test some serum for ketone bodies (BHB) and glucose. There are some other hormones, electrolytes and substances you can test for. See table 1 for the values.

Substance	***Concentration***[1]	***References***[2]
Glucose	2.15 mM	2.4-4.0 mM
BHB	3.63 mM	< 0.8 mM
Insulin	40 pM	91-117 pM
Estrogen	446 pM	286 pM
FFA	0.79 mM	0.38 mM
Cortisol	1860 nM	22-37 mM
Calcium	2.11 mM	2.5-2.9 mM
Sodium	148.2 mM	145-150 mM
Potassium	4.17 mM	4.7-6.5 mM

Table 1: Concentration of hormones, electrolytes and other substances in the blood of ewes with OPK.
[1]Henze, P. et al 1994, 1998, Schlumbohm, C., Hayes, A.M., 2004 & Harmeyer, J. 2008.
[2]GD-deventer referentie lijst & Henze, P. et al, 1998

Testing glucose and BHB after death is possible by testing a sample of cerebrospinal fluid. The values remain unaltered for 6 h after death (Clarkson, 2000, Radostits et al, 2007 & Kahn & Line, 2005: 828-830).
Insulin is very important during the OPK, because it stimulates the use of glucose and reduces the lipolytic activity. When ewes suffer from OPK they often show a hypocalcaemia and hyperpotassaemia. Hypocalcaemia can lead to insulin resistance of cells because insulin receptors are calcium dependent (Draznin et al, 1987). The insulin resistance reduces the transport of glucose into tissue and will reduce the rate of gluconeogenesis in the liver. The consequence of hypocalcaemia is that there is a relative insulin and relative glucose shortage (Schlumbohm et al 1997, Kahn & Line, 2005: 828-830). Hyperpotassaemia can lead to reduced responsiveness of the glucose induced insulin production, because hyperpotassemia will hyperpolarisate the cells (Schlumbohm et al, 1997).
When there is a (relative) shortage of insulin the fat-mobilization will be elevated and therefore an elevation of the FFA and ketone bodies (Henze et al 1998, Schlumbohm

et al 1997). Elevated levels of FFA in the blood is a specific biochemical sign of OPK. High levels of FFA can lead to hepatic lipidosis and a reduction of liver function (Kahn & Line, 2005: 828-830).

Ketosis in cattle

Mostly the diagnose of ketosis is set on the presence of risk factors, clinical signs and ketone bodies in milk or urine (Kahn & Line, 2005: 830-832). Testing the milk or urine on the presence of ketone bodies with a *Siemens Labstick* can be used to confirm the diagnose of ketosis. A negative test is negative, but when you test positive or a trace it is more likely that the cow has ketosis. Testing the milk is preferable, because the BHB concentration in the milk is less variable and more sensitive, so there are less false positives, then the concentration in urine samples (Radostits et al, 2007: 1661-1667).

See table 2 for the expected values and references of glucose BHB and FFA in the blood of dairy cattle suffering of ketosis.

Substance	***Concentration[1]***	***References[2]***
Glucose	1.9-3.8 mM	2.9-4.4 mM
BHB	> 1.4 mM	< 0.9 mM
FFA	> 1.0 mM	< 0.59 mM

Table 2: Concentration of glucose, BHB and FFA in cattle with ketosis.
[1]Radostits et al, 2007: 1661-1667
[2]GD-deventer referentie lijst

Treatment

OPK

The treatment of OPK is very unstatisfactory, the results are mostly between 60-80% of recovery (Bickhardt et a,l 1989, Henze et al, 1994, Henze et al, 1998, Schlumbohm & Harmeyer, 2008, Schlumbohm et al, 1997, Clarkson, 2000, Kahn & Line, 2005: 828-830, Straiton, 1998). Mostly the treatments are based on adding glucose, glucose precursors or stimulating the digestion and gluconeogenesis (Henze et al, 1994). See table 3 for the most common treatments. Most of the treatments are based on giving propylene glycol oral, glucose intravenously sometimes added with other substances see table 3 for an overview of possible treatments (Henze et al, 1998, Clarkson, 2000, Kahn & Line, 2005: 828-830, Straiton, 1998 & Radostits et al, 2007).

Treatment	***Purpose of treatment***
Propelene glycol (po) 60 mL for 3d	Propylene glycol is a precursor of glucose. Elevation of glucose levels.
Electrolytes mostly with dextrose (po) 160 mL for 3d	Prevention of hypocalcaemia or when there is hypocalcaemia raise the levels calcium
Insulin (IV) 0.4 IU/kg every other day	Raise the insulin concentration of the blood. Inhibition of the lipolysis
Recombinant Bovine Somatotropin (SC) 160 mg once + 160 ml Concentrated dextrose and electrolyte solution, 3/d (po)	Inhibition of lipolysis and elevation of the glucose levels in the blood.

Corticosteroids (IV) 15-20 mg once	Induction of parturition after 36-48h. After the parturition most ewes will recover Before 140 days of gestation most lambs are not viable so there will be abortion. After 140 days of gestation lambs are viable, but can be dead
Caesarian section	To save the ewe when they are very sick or valuable. Before 140 days of gestation most lambs are not viable so there will be abortion. After 140 days of gestation lambs are viable, but can be dead

Table 3: Treatments of OPK that are used.
Abbreviations: IU; international Units, IV; intravenously, po; orally, SC; subcutaneously
(Henze et al, 1998, Clarkson, 2000, Kahn & Line, 2005: 828-830, Straiton, 1998 & Radostits et al, 2007).

In a recent research they used flunixin meglumine (NSAID) in addition to propylene glycol and calcium. There was a much higher survival rate of the ewes and lambs, when the ewes were also treated with the NSAID (Zamir et al, 2009). Flunixin meglumine is not registered for sheep in the Netherlands.

Ketosis in cattle
The goal of the treatment is to reestablish a normoglycemia, and reducing the concentration of ketone bodies in the blood. See table 4 for a short overview of treatments that are listed.

Treatment	*Purpose of treatment*
Dextrose (or) 500 mL of 50% solution/d for 4d	Glucose precursor. Raise the levels of glucose
Propelene glycol (po) 250-400g 2/d for 2d then 110g/d for 2d	Glucose precursors. Raise the levels of glucose
Propylene glycol + electrolytes en propion acid for 4d	Glucose precursors. Raise the levels of glucose and the levels of the electrolytes
Propylene glycol + glucose (IV) 2/d for 4d	Raise the level of glucose in the blood.
Propylene glycol + insulin (IM) 150-300 IU/day + glucose (IV) 2/d for 4d	Raise the levels of insulin → inhibition of the lipolysis. The glucose is to prevent hypoglycemia.
Propylene glycol + glucocorticoid hormone (IV) 15-20 mg/d + glucose (IV) 2/d for 4-6d	Raise the levels of glucose in the blood and inhibition of the lipolysis.

Table 4: short overview for treatment of ketosis in dairy cattle.
Abbreviations: d; day, IV; intravenously, IM; intra muscular, IU; international units, po; orally
(Kahn & Line, 2005: 830-832 & Radostits et al, 2007: 1661-1667).

Hypothesis

Hypothesis ewes

During ketosis the basal lipolysis in adipocytes is elevated, because during late ketosis there is hypocalcaemia, hyperpotssaemia, high levels of cortisol and low levels of insulin, which will stimulate the lipolysis. Because of the high lipolysis rate the adipocytes of ketotic ewes are less sensitive for stimulating or inhibiting the lipolysis. Due to the fall in response to inhibiting substances the treatment won't give the expected effect.

Hypothesis cattle

After the parturition the basal lipolysis and ketogenesis is elevated, because of the negative energy balance that raises along with the lactation periode. The lipolytic activity remains elevated eventhough BHB and FFA have a negative feedback on there own production. The adipocytes also become less sensitive for inhibiting or stimulation of the lipolysis.

Materials and Methods

Ewes

From January until March 2010, samples of blood and adipose tissue were taken from four ewes (cross breed Texelaars). Two of the ewes had clinical ovine pregnancy ketosis and and testing ++++ on *Siemens labsticks* Ketone. The other two ewes were control animals, at the same period of gestation, showing no clinical signs of OPK and testing less then ++ on *Siemens labsticks* Ketone. The ewes were kept on sheep farms in the area of Utrecht, The Netherlands.
The blood and adipose sample were taken during OPK
We compared the OPK ewes with the control ewes as groups, but we also compared the ewes from the same farm with each other.
The experimental procedures were evaluated and approved by the Ethical Committee on Animal Experiments of Utrecht University.

Dairy Cattle

From January until March 2010, samples of blood and adipose tissue were taken from six healthy Holstein – Friesian cows at the University Clinic for Farm Animals of the Faculty of Veterinary Medicine, Utrecht University, The Netherlands. The first blood sample and adipose tissue biopsy were taken at approximately one week prior to the expected calving date of the cows (D). After parturition, cows were sampled in week 1 (L1), 2 (L2) and 3 (L3) of lactation, provided that the cow remained healthy and had sufficient adipose tissue available for biopsy.
During the experiment, cows were kept in tie stalls and milked twice daily, and fed according to requirements (CVB). All six cows delivered by caesarian section as part of the veterinary student training program in the clinic (no medical indication). Two of the six cows developed clinical ketosis while the other four cows remained clinically healthy.
For the statistical analysis we made three groups: group 1 we sampled from D-L3, group 2 we sampled from L1-L3 and group 3 the cows that developed ketosis during the trail. The groups are made because the cows in group 2 are not tested in the week

before parturition and if we combined group 1 and 2 we had a different number of cows in each group during the weeks of transition period.
The experimental procedures were evaluated and approved by the Ethical Committee on Animal Experiments of Utrecht University.

Blood Samples

Prior to sampling of adipose tissue, a blood sample (BD Vacutainer® serum tube) was drawn from the jugular vein of the cows for analysis of FFA and BHBA. Blood samples were centrifuged at 2800g for 10 minutes and serum was separated and frozen at –20 °C until analysis. Serum samples were analyzed for FFA and BHBA in a routine laboratory using Randox® test kits (NEFA: FA 115 kit; BHBA: Ranbut kit; Randox laboratories Ltd, Crumlin, UK).

Adipose Tissue Biopsy and Isolation of Adipocytes

Biopsies were taken from the subcutaneous fat tissue in the ischiorectal fossa, alternately on the left and right side of the tail base. Briefly, the area was shaved, washed and disinfected. Local anesthesia was performed by injecting approximately 40 mg of lidocaine hydrochloride + 20 μg adrenalin for sheep and 100 mg of lidocaine hydrolchloride + 50 μg adrenalin for dairy cattle (Alfacaine 2 % + adrenalin, Alfasan®) in the targeted site of incision. Subsequently, a small skin incision of 2 -3 cm was made with a scalpel. The subcutaneous adipose tissue was grasped with a forceps and approximately 5 cm^3 was cut from the surrounding connective tissue. The adipose tissue samples were then immediately put into a 0.9% NaCl-solution at 38 °C and transported to the laboratory for immediate analysis. The incision was closed with a single suture (Vicryl®) and covered with wound spray (Opsite®).
The adipocytes where isolated according to the method of Honnor (Honnor et al, 1985), with some modifications. Briefly, the procedure was as follows. Within 30 minutes after harvesting, the adipose tissue was dissected from connective tissue and blood vessels and about 2 g of adipose tissue was put into a scintillation vial containing 2.0 ml collagenase buffer (Krebs-Ringer-Hepes (KRH) supplemented with 3.5% BSA, 2 mM glucose, 200 nM adenosine and 1.0 mg/ml collagenase type 1a). The adipose tissue was then cut 25-30 times with a pair of scissors to favor digestion by the collagenase. After this, the scintillation vials were put in a 38 °C non-shaking water bath for 60 minutes.
After completion of the collagenase digestion, the adipocytes were separated from undigested material by filtration, one time through two layers and one time through three layers of wound gauze. The cells were centrifuged for 30 s at 130 g and washed two times in KRH. A needle with capillary tubing was put into the tube during centrifugation, so the infranatant could be removed easily without disturbing the floating adipocyte layer. After this, the adipocytes were washed once in DMEM and recentrifuged at 130 g for 30 s. All isolation and centrifuging steps were performed in a climate chamber at 38 °C, to prevent coagulation of the bovine adipocytes.
Collagenase from *Clostridium histolyticum* type 1a was purchased from Sigma-Aldrich, adenosine deaminase (ADA) was purchased from Roche Applied Science and low glucose (5.56 mm) Dulbecco's Modified Eagle Medium (DMEM, without phenol-red) was from Invitrogen.

Incubations

By the use of a hematocrit centrifuge, the lipocrit of the adipocyte suspension was assessed, and the cells were suspended at a 2.0% concentration in 35 mm culture

dishes in 1 ml DMEM and incubated at 37.5 °C and 5% CO_2. The incubation medium (DMEM) was supplemented with 10 mM HEPES, 1 U/ml ADA and 3% BSA (fatty acid free).

Incubations sheep

0.3 µM isoproterenol (DL-isoproterenol hydrochloride) 50 pM insulin 3.0 mM BHB all three were purchased from Sigma Aldrich. We also did the incubate with the combination of 0.3 µM isoproterenol + 50 pM insulin and 3.0 mM BHB + 50 pM insulin.

Incubations dairy cattle

The incubations we did are 0.3 µM isoproterenol, 50 pM insulin, 100 pM insulin and 500 pM insulin, 0.3 µM isoproterenol and 3.0 mM BHB. We also incubate with a combination of 0.3 µM isoproterenol + 50 pM insulin and 0.3 µM isoproterenol + 500 pM insulin.

After incubation, the adipocytes were separated from the medium via a Pasteur capillary pipette filled with glass fiber. The incubation medium was collected in eppendorf cups and stored at -20 °C for further assays. All incubations were performed in triplicate. The incubation medium was tested by a coupled glycerol assay according to the method of O.H. Wieland.

Statistical analyses

The statistical analyses are done with a one-sample t-test. It is assumed that there is a normally distribution and we want to know of the elevation or inhibition caused by the incubations is significant.

Results

Ewes

Adipocytes

The control incubations are compared with the other incubations. All the incubations stood for 5h in the stove. Chart 1 shows the results. From the ewes on farm 1 we only had enough adipocyte tissue to do control and the first three incubations.

The glycerol production in comparison with the control incubation of the sheep adipocytes is reduced when the samples are incubated with 3.0 mM BHB and the combination of 3.0 mM BHB and 50 pM of insulin. In both groups (OPK and clinical healthy), is the reduction in glycerol production not significant. The inhibition of glycerol production by the combination of BHB and insulin is not significant lower than the inhibition of glycerol production by BHB alone.

When the adipocytes are incubated with 0.3 µM isoproterenol and the combination of 0.3 µM isoproterenol and 50 pM insulin there is an elevation of glycerol production. This elevation is not significant in both groups. The elevated glycerol production caused by the combination of isoproterenol and insulin is not statistical higher than the incubation with isoproterenol alone.

The incubation with 50 pM insulin alone had little or no effect on the glycerol production. In two samples there was a little raise in glycerol production and in the other two samples there was a little reduction in the glycerol production compared to the control incubations.

When we compared the OPK sample with the control sample from the same farm there was a significant difference (P=0.0186) in reaction to incubation of the samples from farm 1.

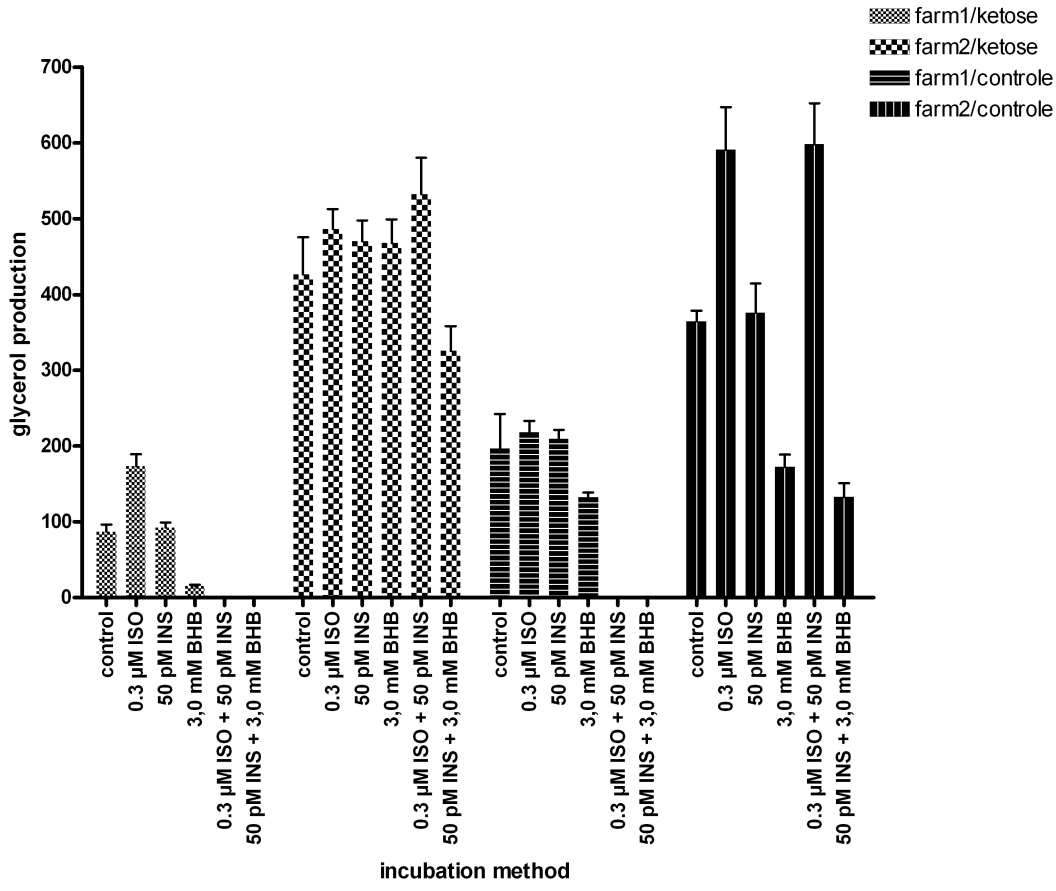


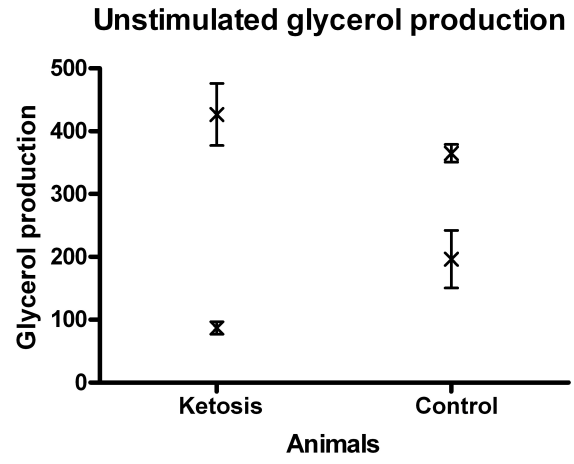
Chart 1: Line chart of the glycerol production of adipocytes during the 5h incubations.

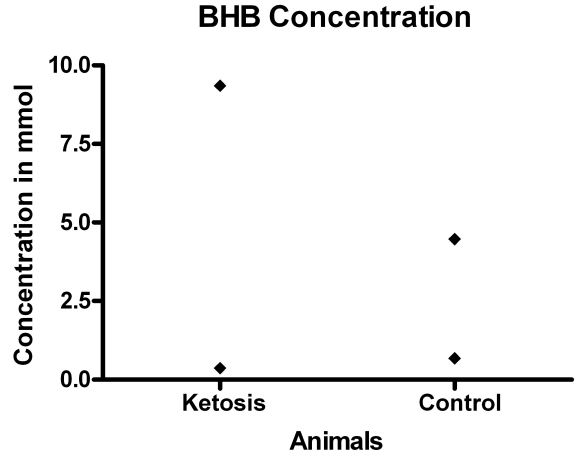
Blood samples

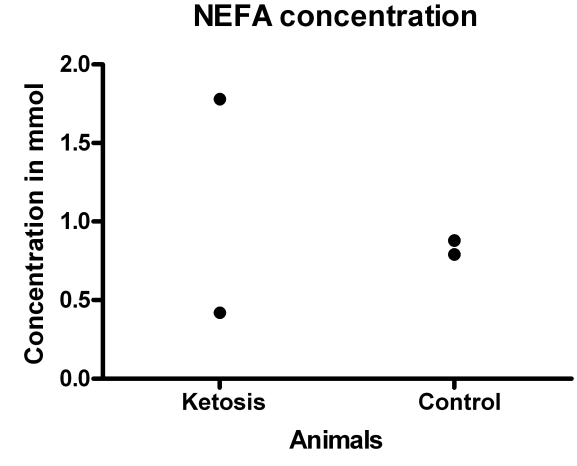
Blood was taken from the ewes as well and the levels of BHB and FFA in the blood where measured. Chart 2 shows the results.

The concentration of BHB in the blood ewes suffering from OPK is not significant different then the concentration of BHB in the blood of the control ewes (P> 0.05).
Also the concentration of FFA in the blood of ewes suffering from OPK is not significant different then the concentration of FFA in the blood of the control ewes (P>0.05).

Chart 2: overview of the concentration of BHB and FFA in the blood of the ewes compared to the glycerol production of the adipocytes







Dairy Cattle

Adipocytes

We tried to test the cows during the transition period from the last week of gestation towards the third week of lactation, considered that the cows remain healthy and had enough adipocyte tissue to do all the incubations. This was not always possible so we formed three groups; group 1 tested from D until L3, group 2 tested from L1 until L3 and group 3 developed ketosis during the trail, all the three groups include two cows because some cows died or had not enough adipocytes tissue to complete all four weeks of testing.

Chart 3 shows an overview of the glycerol production, per group of cows, in the different incubations.

In the group 1 (tested from D until L3) there is an elevation of glycerol production when the adipocytes are incubated with isoproterenol. This is not significant with P=0.0722. However the incubation with isoproterenol and insulin (50 pM) gives a significant raise in glycerol production with P=0.0104 just like the incubation with isoproterenol and insulin 500 pM with P=0.0302. However the incubation with insulin alone gives no significant raise of glycerol production compared to the control incubation. There is no significant difference between the incubation with isoproterenol alone and the combination of isoproterenol and insulin (50 and 500 pM). The incubation with BHB inhibits the glycerol production significantly with P=0.0261.

In the group 2 (tested from L1 until L3) there is not any significant difference between the control incubation and the incubations we tested.

In the ketosis group we compared the glycerol production of the adipocytes taken in the week before ketosis with the glycerol production of the adipocytes taken during the first week of ketosis. The glycerol production of the adipocytes taken in the week before ketosis is not significant different then the glycerol production of the adipocytes taken during the first week of ketosis in any of the incubations we did. Although that the adipocytes taken in the week before ketosis show more reaction on the incubations, more inhibition by BHB and more stimulation by isoproterenol and isoproterenol + insulin there is no significant proof. It looks like the adipocytes taken in the first week of ketosis do not react on isoproterenol and insulin and the combination of both at all.

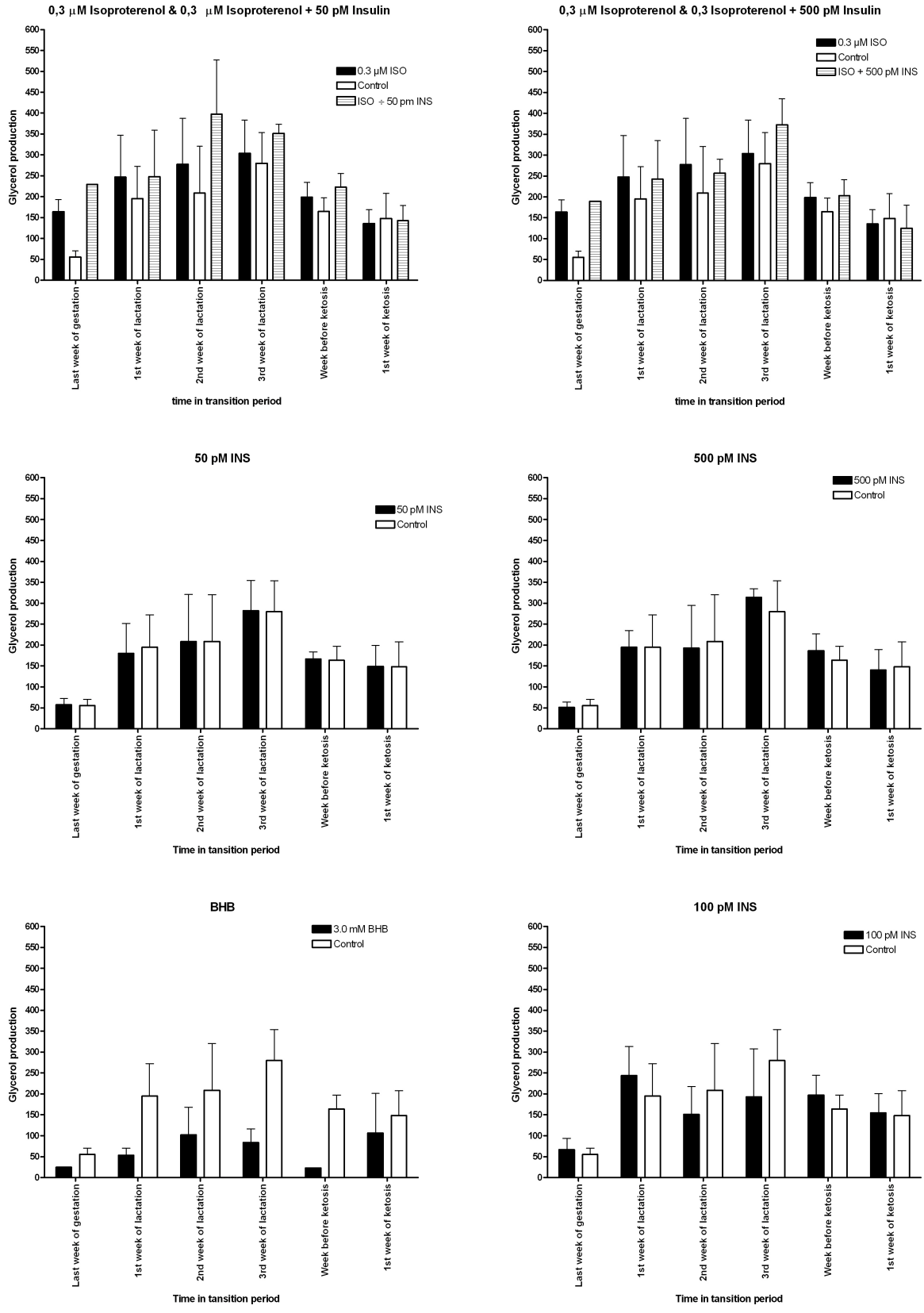
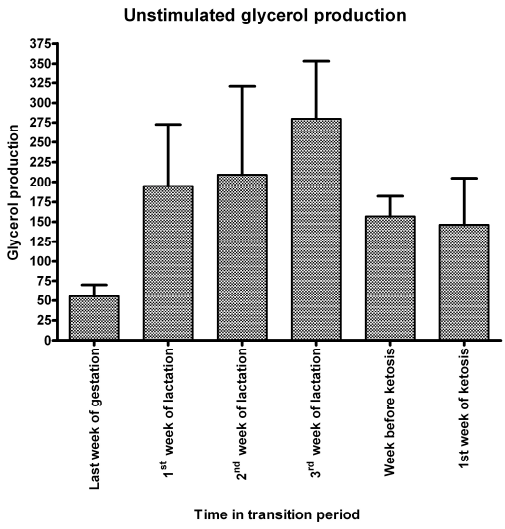
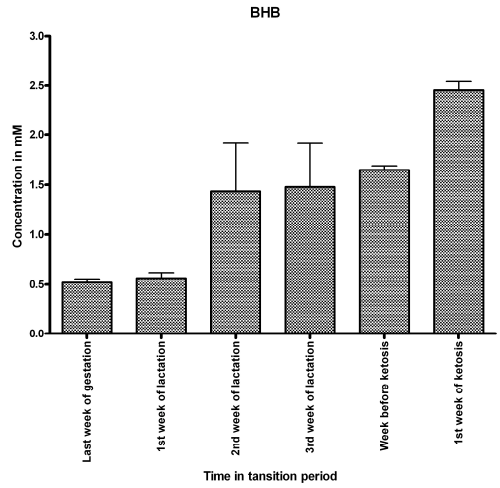
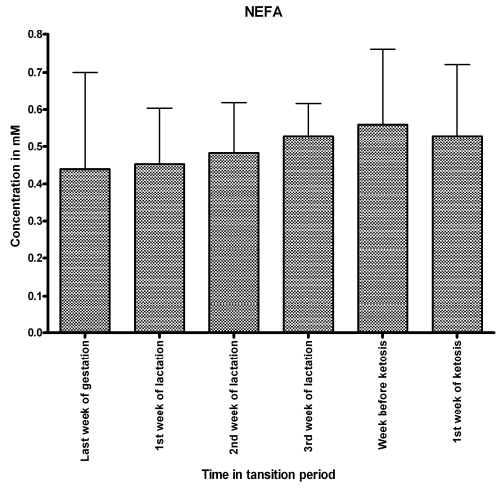


Chart 3: Glycerol production of cattle adipocytes during 5h incubations







Blood samples

In the blood of the cows was only tested for BHB and FFA. See chart 4 for the results.

Chart 4: Overview of the concentration of BHB and FFA compared to the glycerol production of the dairy cattle adipocytes

The BHB concentration in the blood is elevated in addition to the length of the lactation period. The elevation is seen in all the three groups. In the group with ketosis the raise of BHB in the first week of ketosis is significant with P=0.0133 compared to the BHB concentration in the week before ketosis. There is not a connection between the concentration of BHB in the blood and the production of glycerol by the adipocytes in all the three groups.

The FFA concentration in the blood is variable and there is no significance alteration in the values in all three groups. There is not a connection between the concentration of FFA and the production of glycerol by the adipocytes in al the three groups.

Discussion

Adipocytes

When the adipocytes (from sheep and dairy cattle) are incubated with 3,0 mmol BHB (concentration seen in ketotic animals) there is drop in the glycerol production (stands for lipolysis). We conclude that BHB has a negative feedback on its own production by inhibiting the lipolysis. This is in agreement with the conclusions Metz et al, 1974 & Taggart et al, 2005 did in their research.
For the incubation of adipocytes in the presence of BHB combined with insulin (only tested in the ewes) we saw a further decrease of glycerol production. BHB and insulin both inhibit the lipolysis in adipocytes (Metz et al, 1974 & Vernon, 2005). The reason why BHB inhibits the lipolysis is because of the feedback on its own production (Metz et al, 1974 & Taggart et al, 2005).
As expected isoproterenol, a known agonist of adrenalin, gave an elevation of the glycerol production (Galton & Bray, 1967).
The combination of isoproterenol and insulin (in ewes and dairy cattle) also gives an elevation in glycerol production, the elevation is even bigger than the incubation with isoproterenol alone. This seems strange because, insulin is known to inhibit the effects of isoproterenol (Galton and Bray, 1967).
The incubation with insulin alone had no or little effect on the glycerol production. Insulin is an anabolic hormone and inhibits the lipolysis (Berne et al, 2004: 773 & 780, Brockman, 1979, Roach et al, 2004, Stoneham, 1989, & Vernon, 2005), so there was an insulin induced inhibition expected, it is possible that by adding ADA, in the incubation medium the effect of insulin couldn't be seen because, it is known that the inhibition effect of insulin in the body is enhanced by adenosine, a residue from the break-down of AMP produced by the adipocytes. Adenosine itself has an inhibiting effect on the lipolysis, to make sure that there is no effect of adenosine on the lipolysis there is adenosine deaminase (ADA) added to the incubation medium. (Stoneham, 1989 & Heseltine et al, 1995). Maybe because of the ADA we added the inhibiting effect of insulin on the lipolysis is less then you should expect?

The ketotic animals and clinical healthy animals do show some difference. With regard to the literature there are explanations why there should be a difference between ketotic and non-ketotic animals. For example, the lack of effect of insulin in ketotic animals in vivo can be due to the insulin resistance induced by the elevated levels of cortisol, growth hormone (GH), leptin, adenosine deaminase (ADA) and ghrelin (Henze et al, 1998, Vernon et al, 2005 & Vestergaard et al, 2008). This explanation is not likely, as we washed the adipocytes and this should remove the cortisol, GH, leptin and ghrelin. Why do the adipocytes of ketotic animals react less on the insulin? In ewes suffering from OPK there is often a hypocalcaemia and hyperpotassaemia, both known to have a negative influence on the ability of cells to react on the effects of insulin. Hyperpotassaemia causes hyperpolarisation of the cells and calcium is necessary for the receptor to react on the binding of insulin. So when there is hypocalcaemia the receptors for insulin will not work properly (Draznin et al, 1987, Schlumbohm et al 1997, Kahn & Line, 2005: 828-830). Dairy cattle need a lot of glucose and energy to produce milk, so there is a hypoglycemia and a NEB, which stimulates the ketogenesis (Radostits et al, 2007: 1661-1667)

Why do the adipocytes react less on the stimulation of isoproterenol? Is there some intracellular change when the animals become ketotic? Why is there still lipolysis despite the high levels of BHB and FFA, because both inhibit the lipolysis (Metz et al, 1974, Taggart et al, 2005 & Vernon, 2005). Ketone bodies are an alternative energy source when the levels of glucose are not sufficient enough. When the levsl of glucose not elevate the lipolysis will continue, so there is substrate to form ketone bodies.
It is known that in ketotic ewes the levels of estrogen cortisol and potassium are elevated and the levels of glucose, insulin, calcium and are decreased (Henze, P. et al 1994, 1998, Schlumbohm, C., Hayes, A.M., 2004 & Harmeyer, J. 2008). Estrogen and cortisol are known to enhance the lipolysis in adipocytes (Berne et al, 2004: 895-899, Dunlop, 2005, Everts et al, 2008 & Pelt et al, 2006). Do estrogen and cortisol overrule the inhibiting effect of BHB and FFA on the lipolysis and are the decreased levels of glucose, insulin, calcium and potassium the results of there elevation?
When there is hypoglycaemia in dairy cattle the acetyl-CoA, formed out of the β-oxidation of FFA in the liver, is catabolized to ketone bodies (Yameogo et al, 2008). This can be the explanation why the ketotic cows have high levels of BHB and a lower glycerol production then the clinical healthy cows. But this should be confirmed with the values of glucose in the blood.
An explanation of the lack of significance of the incubations can be due to the little groups we used. The groups of ewes and dairy cattle exist of two animals.

Blood samples

The levels of FFA in the blood are what we expected (both in ewes and dairy cattle), because FFA have a (indirect) negative feedback on its own production. The liver takes up the FFA out of the blood to lower the levels of FFA. The FFA are either re-esterficated into TAG or used in the β-oxidation to form acetyl-CoA. The TAG either leaves the liver in VLDL, or is stored in the adipocytes (fatty liver). The acetyl-CoA can be metabolized completely in CO_2 and H_2O or is used to form ketone bodies such as BHB, which can be used as an alternative energy source by brains, heart and muscles. And high levels of BHB and FFA inhibit the lipolysis (Roach et al, 2004, Yameogo et al, 2005 & Vernon, 2005).
The levels of BHB in the blood of the ewes where not what we expected, because the level of BHB in the ewe from (farm 1) suffering from OPK was not above 3,0 mmol. But the ewe had clinical signs, but forgot the *Siemens Labstick,* so maybe the ewe had milk fever and not OPK. On farm 2 both ewes had levels of BHB higher then 3 mmol (above 3 mmol and clinical signs is called OPK. This is expected because most ewes have elevated levels of BHB in their blood at the end of gestation (Radostits et al, 2007: 1668-1670).
The level of BHB in the blood of the dairy cattle is what we expected, in the literature it is known that the levels of BHB raise along with the lactation period. There is a peak of BHB around week 2&3 (depends on the severity of the NEB) of the lactation and if the cow develops ketosis the level of BHB will raise also. The negative energy balance is due to the high demand of energy from the body and the insufficient rumen capacity, the levels of glucose and therefore insulin are low and the body is in a catabolic state. The body starts to use the energy reserves, such as glycogen (from liver and muscle) and TAG (Roach et al, 2004, Yameogo et al, 2005 & Vernon, 2005).

Because we only took one blood sample we cannot do any statistical test on the levels of BHB and FFA in the blood.

Recommendations of further studies

1. More animals in a group so there is a more precisely mean calculated.
2. Test all the cows from the last week of gestation until the third week of lactation or when they develop ketosis first then until the first week of ketosis, So the groups are more evenly spread and the statistics have more value.
3. Take also an adipocyte and blood sample during the last two week of lactation until the first two weeks of the dry period, to have a better indication of the basal lipolysis activity in cows. For better comparison alterations in the lipolysis activity.
4. Measure the concentrations of other hormones, electrolytes and other substances in the blood during ketosis in cattle. And test in incubations if they have effect on the lipolytic activity.
5. Test more animals from the same farm and do this on two to four farms, so you can compare the farms including the management of the farms. To find out the effects of management on the prevalence and severity of ketosis in ewes or cattle.
6. Effects of treatment. Make four groups of animals: two control groups treated and non-treated and two groups with OPK ewes treated and non-treated. Sample the animals several times in a row (for example every two days) to see if there is any difference, in lipolytic activity or BHB and FFA concentration, when the animals are treated or not and if there is any difference in effect between healthy ewes and OPK ewes.
7. Trials with insulin. Have physiological levels of insulin enough lipogenetic activity to reduce the lipolysis during OPK in vitro? Is adenosine a key factor for insulin to have an inhibiting effect on the lipolysis?

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