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Influence of Day-0 and Day-7 Superovulated Cow Serum During Development of Bovine Oocytes *in vitro*

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Abstract. Oocytes were matured in medium supplemented with 5% serum collected from superovulated cows at oestrus (Day-0 SCS) or at the time of embryo collection (Day-7 SCS), or in medium supplemented with fetal calf serum (FCS). After insemination using frozen–thawed sperm, oocytes were cultured *in vitro* with medium supplemented with 5% Day-0 SCS or 5% Day-7 SCS or 5% FCS. The proportions of embryos that cleaved were not significantly different among treatments, whereas development of the embryo to a blastocyst was significantly higher in the presence of SCS than FCS. When the four possible combinations of Day-0 SCS and Day-7 SCS were used in the maturation and culture media, there were no differences among treatments, except that the cleavage rate was significantly higher ($P < 0{\cdot}05$) with Day-0 SCS in the maturation medium and Day-7 SCS in the culture medium than with Day-7 SCS in the maturation medium and Day-0 SCS in the culture medium. The proportions of embryos that cleaved and developed to blastocysts were not related with the level of progesterone and luteinizing hormone in the serum added to the maturation and culture media. However, the use of serum with low concentrations of glucose, fatty acids and cholesterol in the maturation medium and the culture medium tended to be associated with a higher rate of cleavage and blastocyst development.

Extra keywords: IVM, IVF, IVC, superovulated cow serum.

Introduction

In many laboratories, bovine blastocysts are routinely obtained from oocytes after *in vitro* maturation, fertilization and culture (IVM, IVF, IVC). Oocytes collected from ovarian follicles can undergo spontaneous nuclear maturation in medium containing serum. Bovine oocytes have matured in medium containing either serum or a more defined protein source such as bovine serum albumin (BSA) or fetal calf serum (FCS) (Liebfried-Rutledge *et al.* 1986). Although nuclear events characterizing maturation can occur in the absence of serum (Suss *et al.* 1988), fertilization and development of oocytes into blastocysts was superior after IVM and IVC in the presence of serum. Blastocyst development from bovine follicular oocytes was stimulated following IVM and IVC in medium supplemented with cow serum obtained at pro-oestrus (Younis and Brackett 1991), at oestrus (Schellander *et al.* 1990; Peli and Schellander 1993) or from Day-7 superovulated cows (Suzuki and Shimohira 1985; Matsuoka *et al.* 1992; Geshi *et al.* 1993). The establishment of ideal culture conditions for early embryonic development is important for the study of factors involved in this process as well as for technologies such as IVF, nuclear transplantation and gene transfer.

We examined the development *in vitro* of fertilized bovine oocytes into blastocysts and cultured in medium supplemented with superovulated cow serum (SCS) collected on Day 0 (oestrus) and on Day 7 (when embryos developed *in vivo* were collected).

Materials and Methods

IVF Bovine Embryos

Maturation of oocytes. Bovine ovaries obtained from a local abattoir were placed in a physiological saline solution (0·9% (w/v) NaCl) containing penicillin-G (100 I.U. mL^{-1}) and streptomycin sulfate (0·2 $\mu g\ mL^{-1}$). Ovaries were held at 30–32°C and transferred to the laboratory within 3 h. Oocytes within follicles 2–5 mm in diameter were aspirated with an 18G needle connected to a 5-mL syringe. The resultant oocyte suspensions were mixed with modified-PBS (m-PBS; Embryotec, Nihon Zenyaku, Fukushima, Japan) supplemented with 50 $\mu g\ mL^{-1}$ gentamycin sulfate (Sigma, St Louis, MO, USA). The oocyte preparations were then pooled into a 10-mL plastic tube and maintained at 37°C.

Oocytes were rinsed once with m-PBS and three times in maturation medium. This medium consisted of medium-199 (TCM-199 with Earle's salts, L-glutamine, 2200 mg mL^{-1} sodium bicarbonate and 25 mM HEPES buffer; Gibco, Grand Island, NY, USA) supplemented with: 5% SCS (collected on Day 0 or Day 7) or 5% heat-inactivated fetal calf serum (FCS; Gibco); 0·01 mg mL^{-1} follicle-stimulating hormone (FSH; Denka Pharmaceutical, Kawasaki, Japan); and 50 $\mu g\ mL^{-1}$ gentamycin sulfate. Oocytes (100–200) that were surrounded by cumnulus cells for more than one-third of

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their surface were cultured in this medium for 21 h at 38·5°C under 5% CO_2 in air. The medium (2·5 mL) in a polystyrene culture dish (35 mm diameter, Falcon 1008; Becton Dickinson, Oxnard, CA, USA) was overlaid with mineral oil (E.R. Squibb & Son, Princeton, NJ, USA).

In vitro fertilization. Frozen–thawed semen from a single bull was used for fertilization *in vitro*. Frozen spermatozoa were thawed in water (25°C) and washed twice by centrifugation (500*g*, 5 min) in 2·5 mM caffeine in Brackett and Oliphant's medium (Caff-BO; Brackett and Oliphant 1975). The resultant sperm pellet was resuspended in Caff-BO supplemented with 1% bovine serum albumin (BSA; Sigma) and 20 μg mL^{-1} heparin (Shimizu Pharmaceutical, Shimizu, Japan) to a final concentration of 5×10^6 spermatozoa mL^{-1}. A 100-μL aliquant of the sperm suspension was overlaid with mineral oil and preincubated for 1 h at 38·5°C under 5% CO_2.

Oocytes matured *in vitro* were transferred into sperm microdrops (20–25 oocytes per microdrop) for insemination. After 5 h, oocytes with adherent cumulus cells were washed by repeated pipetting in culture medium and transferred for further development into a polystyrene dish (4-well multidish; Nunclon, Roskilde, Denmark) containing: medium-199 supplemented with either 5% SCS or 5% FCS, 5 μg mL^{-1} insulin (Wako Pure Chemical Industries, Osaka, Japan) and 50 μg mL^{-1} gentamycin sulfate; the culture medium (0·5 mL) was then overlaid with mineral oil (0·5 mL).

Adherent cumulus cells surrounding the embryos were removed by repeated pipetting 48 h after insemination. In contrast, the monolayer of cumulus cells adherent to the surface of the culture dish was not disrupted and embryos were cultured on this layer. The culture medium was replaced with fresh medium 4 days after insemination.

Collection of superovulated cow serum (SCS). SCS was collected from superovulated cows treated with a total of 20 mg FSH. Prostaglandin was administered 48 h after FSH injection to induce luteolysis (Matsuoka *et al.* 1991). Blood was collected on Day 0 (at oestrus; artificial insemination was performed) and on Day 7 (when embryos were collected). Whole blood samples were centrifuged twice (950*g*, 10 min, 5°C) and the serum obtained was then heat-inactivated (56°C, 30 min) before use.

Experiment I

During IVM of oocytes and IVC of bovine embryos fertilized *in vitro*, the medium was supplemented with 5% SCS collected on Day 0 or Day 7 or with FCS. Each treatment was repeated five times, the sample of SCS and FCS remaining constant throughout.

Experiment II

Maturation and culture media were supplemented as follows: (*1*) Day-0 SCS for both IVM and IVC; (2) Day-0 SCS for IVM and Day-7 SCS for IVC; (*3*) Day-7 SCS for IVM and Day-0 SCS for IVC; and (*4*) Day-7 SCS for both IVM and IVC. For each treatment, Day-0 SCS and Day-7 SCS were collected from the same cow within one oestrous cycle.

Experiment III

Day-0 SCS or Day-7 SCS collected from individual cows were used for IVM and IVC of bovine oocytes. Concentrations of progesterone, luteinizing hormone (LH), glucose, fatty acids and cholesterol in the heat-inactivated SCS were measured as described by Matsuoka *et al.* (1991) before addition to the media. LH concentrations were measured by solid-phase [^{125}I]-labelled LH radioimmunoassay; progesterone concentrations were determined by enzyme-linked immunosorbent assay (ELISA) using Denka kits (Denka Pharmaceutical); concentrations of glucose, fatty acids and cholesterol were estimated by spectrophotometry with enzymatic test kits (Wako Pure Chemical Industries).

Embryo Evaluation

The proportion of embryos that had developed to the 2-, 4-, and 8-cell stage were recorded 48 h after insemination. Blastocyst development was assessed on Days 7, 8, and 9 and, when counted, blastocysts were removed from the culture dish to avoid a double counting. Data were analysed by the χ^2-test (Experiments I and II) and by the General Linear Model of least squares analysis of variance (Experiment III; Anon. 1985). $P = 0{\cdot}05$ was considered significant.

Results

The development of embryos in the presence of Day-0 SCS or Day-7 SCS or FCS during IVM and IVC of bovine oocytes is shown in Table 1. The proportion of embryos that had cleaved 48 h after insemination of matured oocytes and culture of fertilized oocytes was not significantly different among treatments (60%, 62% and 60% for Day-0 SCS, Day-7 SCS and FCS respectively). However, embryonic development to the blastocyst stage was significantly higher in the presence of Day-0 SCS (25%) and Day-7 SCS (27%) than in the presence of FCS (16%).

The proportion of embryos that had cleaved and developed into blastocysts when medium supplemented with various combinations of SCS was used for IVM and IVC is shown in Table 2. There were no significant differences among treatments except that the cleavage rates were significantly higher ($P < 0{\cdot}05$) with Day-0

Table 1. Development of bovine oocytes matured and cultured *in vitro* in medium supplemented with superovulated cow serum (SCS) or fetal calf serum (FCS)

Values in parentheses are percentages based on initial no. of oocytes present

Sera[A]	No. of oocytes initially present	No. of embryos cleaved 48 h after insemination	No. of blastocysts present on Day 9[B]
Day-0 SCS	202	122 (60)	50 (25)[a]
Day-7 SCS	204	126 (62)	55 (27)[a]
FCS	180	108 (60)	28 (16)[b]

[A] SCS was obtained on Day 0 (at oestrus) and on Day 7 (when embryos developed *in vivo* were collected).

[B] Within columns, means with different superscripts are significantly different ($P < 0{\cdot}05$).

Table 2. Development of bovine oocytes matured and cultured *in vitro* in medium supplemented with various combinations of superovulated cow serum (SCS)

Values in parentheses are percentages based on initial no. of oocytes present. IVM, *in vitro* maturation; IVC, *in vitro* culture

Day on which SCS collected for IVM[A]	IVC[A]	No. of oocytes initially present	No. of embryos cleaved 2-cell	4-cell	8-cell	Total[B]	No. of blastocysts present[B]
Day 0	Day 0	809	65	138	287	490 (61)	205 (25)
Day 0	Day 7	816	79	160	276	515 (63)[a]	214 (26)
Day 7	Day 0	759	54	141	237	432 (57)[b]	178 (23)
Day 7	Day 7	818	42	146	317	505 (62)	217 (27)

[A] SCS was obtained on Day 0 (at oestrus) and on Day 7 (when embryos developed *in vivo* were collected).

[B] Within columns, means with different superscripts are significantly different ($P < 0.05$).

Table 3. Development of bovine oocytes matured and cultured *in vitro* in medium supplemented with superovulated cow serum (SCS) collected from individual cows

Values in parentheses are percentages based on initial no. of oocytes present

Cow[A]	Progesterone ($ng\ mL^{-1}$)	Luteinizing hormone ($ng\ mL^{-1}$)	Glucose ($mg\ dL^{-1}$)	Fatty acids ($\mu Eq\ L^{-1}$)	Cholesterol ($mg\ dL^{-1}$)	No. of oocytes initially present	No. of embryos cleaved[B]	No. of blastocysts present[B]
1	0·26	0·77	45·9	186·0	80·9	226	155 (69)	62 (27)
2	0·45	0·06	65·2	169·4	95·4	295	177 (60)	78 (26)
3	0·68	0·55	72·3	182·3	121·9	331	185 (56)	66 (20)
4	23	0·68	51·9	197·3	85·4	222	132 (59)	55 (25)
5	23	10·84	67·0	338·4	111·9	210	120 (57)	35 (17)
6	26	0·69	91·0	287·2	126·1	256	132 (52)	53 (21)

[A] For Cows 1–3 SCS was collected on Day 0 and for Cows 4–6 SCS was collected on Day 7; in each case, the same preparation of SCS was used for IVM and IVC of oocytes.

SCS for IVM and Day-7 SCS for IVC than with Day-7 SCS for IVM and Day-0 SCS for IVC.

Table 3 shows the cleavage and blastocyst rates of bovine oocytes matured and cultured *in vitro* in medium supplemented with SCS collected from individual cows. Concentrations of progesterone, LH, glucose, fatty acids and cholesterol in SCS were measured before it was used for IVM and IVC. The proportion of embryos that had cleaved and developed to the blastocyst stage was not related to the concentration of progesterone present. Both the addition of Day-0 SCS (low concentration of progesterone) and Day-7 SCS (high concentration of progesterone) to IVM and IVC media resulted in a high proportion of fertilized oocytes developing to blastocysts. Similarly, the concentration of LH present in Day-0 SCS and Day-7 SCS was not related to the production of 2-, 4-, and 8-cell (cleavage) stage embryos 48 h after insemination or to blastocyst production. In contrast, when low concentrations of glucose, fatty acids or cholesterol were present in SCS, higher cleavage rates and rates of blastocyst development tended to be observed.

Discussion

Hormonal and follicular factors affect maturation of sheep oocytes *in vitro* and their subsequent developmental capacity (Moor and Trounson 1977). Bovine gametes matured *in vitro* show full embryonic competence whereas oocytes matured in hormone-free medium show a limited developmental competence (Hensleigh and Hunter 1985). The present results suggest that SCS contains substances that enhance the capacity of bovine oocytes matured and fertilized *in vitro* to develop into blastocysts.

The beneficial effect of SCS observed in both IVM and IVC systems was not related to the time of collection. These results are supported by previous studies (Matsuoka *et al.* 1992; Geshi *et al.* 1993) in which SCS was used for maturation, fertilization and culture of bovine oocytes.

Analysis of SCS collected from individual cows for IVM and IVC of bovine oocytes indicated that the concentrations of progesterone and LH in the serum did not affect the capacity of bovine oocytes to undergo cleavage and develop into blastocysts. Previously, Zuelke and Brackett (1990) reported that a high concentration of LH during IVM enhanced cleavage and blastocyst development. In the present study, even at a low concentration of LH, the cleavage rate and rate of blastocyst development of oocytes fertilized *in vitro* was high. Peli and Schellander (1993) also reported that different concentrations of LH present in cow serum had no effect on blastocyst development.

In contrast, a high concentration of glucose in SCS added to medium in IVM and IVC systems inhibited

early development of bovine embryos to the 2-, 4-, and 8-cell stage as well as the blastocyst stage. It has been previously reported that the presence ofr glucose during early development and beyond to the morula stage inhibits bovine embryo development (Nakao and Nakatsuji 1990; Kim *et al.* 1993). Since the metabolism of glucose increases markedly from the 2-cell to the blastocyst stage, the inhibitory effect of glucose on early development of bovine embryos may be related to glucose metabolism (Rieger 1992). The presence of high concentrations of fatty acids and cholesterol in IVM and IVC systems may also inhibit embryo development. This suggests that the use of Day-0 SCS or Day-7 SCS serum with a low concentration of glucose, fatty acids and cholesterol may be beneficial to the survival of bovine embryos cultured *in vitro.*

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Direct rehydration of in vitro fertilised bovine embryos after vitrification

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VITRIFICATION is a valuable embryo freezing technique because it is simple, quick, economic (Rall and Fahy 1985), reliable and reasonably easy to apply under field conditions (Niemann 1991). Vitrification offers considerable promise for simplifying and improving the cryopreservation of cells because controlled-rate freezing equipment is not required and the potential injury associated with the formation of ice is eliminated (Rall 1987). The first successful vitrification of bovine embryos was achieved by Massip and others (1986). The survival of vitrified embryos is influenced by the type of cryoprotectant used and the exposure procedures (Yang and others 1992). Leibo (1989) suggested that embryos can be successfully vitrified in either glycerol-based or propylene glycol-based solutions. Sucrose and other carbohydrates like trehalose are effective in preserving the structural and functional integrity of membranes at low water activities (Massip and others 1987). Compact morulae seem to be the most suitable stage for vitrification, and the survival rate of expanded blastocysts can be increased up to 10-fold by using Massip solution and by equilibration of 4°C (Cseh and others 1992).

In the present study, in vitro fertilised embryos were cryopreserved in a simpler way by using direct rehydration in modified phosphate buffered saline (PBS) after vitrification.

Oocytes were aspirated from the ovaries collected from a local slaughterhouse and matured for 20 to 22 hours at 38·5°C in 5 per cent carbon dioxide in air. These matured oocytes were fertilised by in vitro capacitated sperm and cultured in vitro until blastocysts formed. The blastocysts were used for the vitrification experiment on days 8 and 9.

Three types of vitrification solutions were used. In the first type the embryos were kept in 10 per cent ethylene glycol for five minutes and then in 10 per cent ethylene glycol in 0·3M trehalose for five minutes. Finally, the embryos were kept in 40 per cent ethylene glycol in 0·3M trehalose for one minute. The first two media were at room temperature but the third was pre-cooled on ice. The embryos were placed in 0·25 ml straws which were immersed horizontally in liquid nitrogen after being held in the vapour for 20 seconds. The frozen embryos were thawed in a waterbath at 20 to 25°C. After thawing, the embryos were placed directly into the modified PBS at room temperature for five minutes and washed two or three times, and then placed in the culture medium. The second and third types of vitrification solution contained an additional 5 per cent and 10 per cent Percoll, respectively, in the final solutions. All the solutions were prepared with PBS supplemented with 0·3 per cent bovine serum albumin (w/v) and 50 mg/ml gentamicin.

Twenty embryos were used with each type of vitrification medium, and the results are shown in Table 1.

TABLE 1: Viability of bovine embryos following vitrification

Type of vitrification solution	Number of embryos	Survival after thawing (%)	Survival after 24 hours (%)	Number of hatched embryos (%)
1	20	16(80)[a]	10(50)	3(15)
2	20	13(65)	8(40)	4(20)
3	20	8(40)[b]	2(10)	–

a, b Within columns, means with different superscripts are significantly different (P<0·01)

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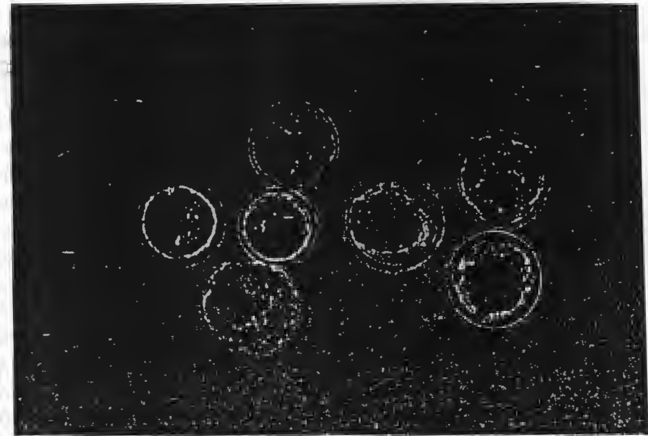

FIG 1: Day 8 and day 9 vitrified blastocysts after thawing (type 1)

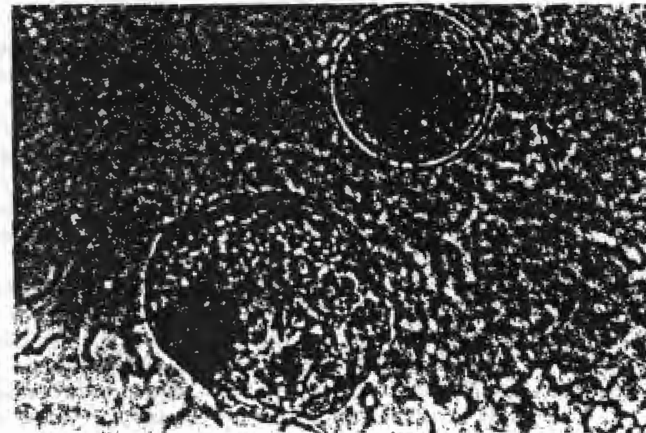

FIG 2: Hatched blastocyst after 48 hours of in vitro culture (type 1)

In the first five minutes the cryoprotective solutions used for equilibration contained 10 per cent ethylene glycol, and in the next five minutes they contained 10 per cent ethylene glycol with 0·3M trehalose. These two steps were done at room temperature. In the next step the embryos were put into solutions pre-cooled on ice and containing 40 per cent ethylene glycol with 0·3M trehalose for about one minute. During this gradual equlibration, substantial amounts of ethylene glycol entered the embryonic cells, and at the same time trehalose helped to dehydrate the embryonic cells, a process which is very important for successful vitrification. In the experiment in which 5 per cent Percoll was used the proportion of embryos which survived was similar, possibly because the Percoll helped the embryo to overcome cold shock, but when 10 per cent Percoll was used significantly fewer embryos survived, possibly because at this concentration Percoll was toxic to the embryo. The viability of the embryos was determined visually as the number which recovered from the dehydrated state (survival after thawing), the number of blastocysts which survived (survival after 24 hours) and the number of blastocysts which developed to hatched blastocysts (number of hatched embryos).

With the type 1 solution, 80 per cent of the blastocysts survived after thawing (Fig 1) and after 24 hours of culture the survival rate was 50 per cent. This result was lower than the value observed by Ishimori and others (1992) who used in vivo day 7 morulae, and a little lower than the value observed by Dobrinsky and others (1991) who used in vivo day 6 late morulae or early blastocyst stages, however, the survival rate was higher than that observed by Van Der Zwalmen and others (1989). After 48 hours of culture three (15 per cent) hatched blastocysts were obtained (Fig 2). The results obtained with the type 2 solution were not significantly different from those obtained with the type 1 and type 3 solutions, but the results obtained with the type 3 solution were significantly less satisfactory than those obtained with the type 1 solution.

These results suggest that the direct rehydration of in vitro fertilised bovine embryos after vitrification is useful. Most of the embryos survived the process of thawing, but their later development in the culture medium depended on the quality of the embryos. However, the direct rehydration in modified PBS made the procedure simpler and less time consuming.

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Fat absorption in female boxer dogs with undiagnosed hormonal alopecia

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CANINE hormonal alopecia may be associated with hyperadrenocorticism, hypothyroidism, growth hormone deficiency, imbalances of oestrogens, androgens and progesterone and diabetes mellitus (Muller and others 1989). In some cases, particularly of seasonal flank of ovariectomised female dogs (Scott 1990), despite extensive evaluation of the dog's hormonal status, the nature of the imbalance remains undetermined. Disturbances of fat metabolism associated with hyperadrenocorticism, hypothyroidism and diabetes mellitus result in profound alterations of the fat absorption curve (Simpson and van den Broek 1990, 1991). Imbalances of oestrogens, androgens and progesterone also influence fat metabolism (Kissebah and Schectman 1987) and may therefore be accompanied by alterations in the fat absorption curve. In this study quantitative fat absorption was evaluated in six female boxer dogs presented at the Royal (Dick) School of Veterinary Studies with undiagnosed hormonal alopecia.

Quantitative fat absorption was studied in two groups of dogs.

Group 1 was made up of 10 clinically normal dogs comprising seven neutered and three entire female animals aged between two and nine years (median age five years one month) and the following breeds: German shepherd dog (three); labrador retriever (two); springer spaniel (two); rough collie (one); Yorkshire terrier (one); crossbred (one).

Group 2 consisted of six boxers aged between two and seven years (median age five years four months). One of the boxers was an entire female, three had been spayed after the first season and two after the second season. All cases presented with a non-inflammatory, non-pruritic bilaterally symmetrical, hyperpigmented area of alopecia which had originated in the sublumbar region and in some cases had extended to the dorsal lumbar area or the lateral aspect of the thorax. The age at which the alopecia was first observed ranged between one and a half and six and a half years and, in the neutered animals, occurred six months to four years after ovariohysterectomy. In four cases there was a history of spontaneous hair regrowth which had occurred at different times of the year in each dog.

All the dogs were submitted to the following investigation: skin scrapings and biopsies were taken from affected sites, thyroid and adrenocortical function were assessed, respectively, by the thyroid-stimulating hormone response (Thoday 1990) and adrenocorticotrophic hormone or dexamethasone suppression tests (Feldman 1983). Plasma oestradiol, testosterone and progesterone concentrations were estimated and growth hormone status evaluated by xylazine stimulation (Lothrop 1989).

Quantitative fat absorption was determined using the method described by Simpson and van den Broek (1990). After a 12-hour fast each dog was fed 3 ml/kg corn oil. Venous blood samples (92 ml) were collected in anticoagulant-free Vacutainer tubes (Becton Dickinson) immediately before and 60, 120, 180 and 240 minutes after feeding corn oil. The serum was harvested and the triglyceride concentration determined with a Technicon 500 auto-analyser, using a commercial kit (Technicon).

The mean concentration of triglyceride in serum at each of the sampling times and the area under the curve were calculated for each group of dog. Differences between the two groups were compared by the Student's two sample *t* test using separate variance estimation for each group.

In the dogs with hormonal alopecia, no evidence of fungal infection or parasitic infestation was detected by microscopic examination of skin scrapings. Histopathological examination of skin biopsies revealed hyperkeratosis, increased pigmentation of basal cells, reduced numbers of hairs in the hair follicles, follicular keratosis and reduction in the size of sebaceous (four cases) and apocrine (three cases) glands. In all the dogs, evaluation of the hormonal status gave results within the normal range.

The number of normal dogs and dogs with undiagnosed hormonal alopecia, together with the mean, standard deviation and range of values at each sampling time are given in Table 1. The mean concentration of triglyceride in the serum of dogs with undiagnosed hormonal alopecia was significantly lower than that of

TABLE 1: Concentration (mmol/litre) of serum triglyceride and AUC for normal dogs and dogs with undiagnosed hormonal alopecia

Time (mins)	Parameter	Normal (n = 10)	Alopecia (n = 6)
0	Mean	0·56	0·49
	Range	0·38-0·77	0·38-0·62
	sd	0·10	0·10
60	Mean	0·91	0·55*
	Range	0·44-1·89	0·38-0·78
	sd	0·42	0·14
120	Mean	1·51	0·76***
	Range	0·62-2·26	0·50-0·98
	sd	0·48	0·17
180	Mean	1·90	0·98**
	Range	0·74-3·05	0·55-1·84
	sd	0·72	0·47
240	Mean	1·62	1·23
	Range	0·63-2·48	0·65-1·90
	sd	0·63	0·49
AUC	Mean	1·35	0·79**
	range	0·78-2·05	0·56-1·03
	sd	0·38	0·19

AUC Area under the curve
sd Standard deviation
Significant differences from normal are indicated by $^{*}P<0·05$, $^{**}P<0·01$, $^{***}P<0·001$

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