Serum albumin concentration of donor cows as an indicator of developmental competence of oocytes

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Highlights

- Serum albumin level of donor cows independently and significantly predicts developmental competence of oocytes.
- Adjusting the levels of protein supplementation in *in vitro* culture protocols based on donor serum albumin may be warranted.
- Albumin levels in follicular fluid are not correlated, indicating regulatory mechanism that determines follicular fluid albumin level.

Abstract

Adequate nutrition is required for maintenance of normal reproduction in cattle. Albumin, the best marker and fundamental part of nutrition, most abundant plasma protein and major component of fetal bovine serum, is the best predictor of malnourishment in South African cattle. The aim of this study was to determine if serum albumin concentrations of donor cows predict the developmental competence of oocytes, and if additional protein supplementation of the *in vitro* culture media improves embryo outcomes in oocytes from cows with inadequate serum albumin concentrations. Oocytes (n = 1024) were recovered from donors with inadequate (≤35.9 g/L), or adequate serum albumin concentrations (≥36.0 g/L). Four hundred and sixty oocytes originated from cows with inadequate serum albumin and 564 from cows with adequate serum albumin. Oocytes of these cohorts were randomly allocated to a control and supplemented fetal bovine serum *in vitro* embryo culture protocol. Multiple linear, logistic and Poisson regression analyses were performed to estimate the effects of

different covariates on linear, binary and count data respectively. Mixed effects Poisson regression was performed for the number of oocytes that developed into blastocysts by the seventh day of culture. Adequate serum albumin concentration of donor cows independently resulted in 46% increased blastocyst formation in the control protocol (P = 0.02). Although fetal bovine serum supplementation of the culture protocol did not affect blastocyst formation in oocytes originating from cows with inadequate serum albumin, it independently reduced blastocyst formation by 30% in oocytes originating from cows with adequate serum albumin (P = 0.02). Other independent predictors of blastocyst outcome included higher serum urea nitrogen, lower beta (β)-hydroxybutyric acid concentrations and lower fat classification of donor cows. It is concluded that adequate serum albumin of donor cows is a significant predictor of developmental competence of oocytes, and that *in vitro* supplementation of fetal bovine serum does not improve developmental competence of oocytes and can lead to negative blastocyst outcomes. Further research is required to determine optimal protein supplementation for oocytes originating from inadequately nourished cows.

Keywords:

Bovine

Albumin

Follicular fluid

Oocyte

Developmental competence

1. Introduction

Balanced nutrition is an essential requirement for maintenance of normal body condition and it directly affects fertility in cattle. Nutrition has specific effects on ovarian follicular growth by acting at several different levels of the hypothalamic-pituitary-gonadal axis where ovarian function and embryo production are directly and indirectly controlled by systemic effects of growth factors [1]. Recognised detrimental effects of undernutrition in cattle include delayed puberty, irregular oestrous cycles, lowered conception rates and reduced birth weights [2]. Cattle breeds in southern African countries differ in their ability to adapt to poor nutritional conditions experienced during severe drought periods. During these periods, low levels of dietary energy and protein intake result in the breakdown of fundamental body reserves.

Successful oogenesis prepares the oogonia in ovarian follicles filled with protein rich follicular fluid; at least 40 serum proteins have been identified in bovine follicular fluid [3]. The most abundant plasma protein found in the blood of mammals is serum albumin and reduced concentrations indicates reduced amino acid synthesis from diets, poor liver health or protein losing pathological conditions. After synthesis, serum albumin is transferred into plasma as a non-glycosylated protein where it may reach high concentrations of up to 40 g/L. Serum albumin plays a significant part in maintaining the redox potential in extracellular fluids while regulating the osmotic pressure of the plasma in the vascular system (oncotic pressure) [4, 5]. Due to its ability to bind a diverse range of small molecules using specific binding sites, serum albumin acts as transport agent for various endogenous and exogenous molecules such as fatty acids, hormones, nutrients, drugs and metabolites.

Albumin forms part of a family of structurally un-related acute phase proteins that are activated, intensified and managed as part of an animal's reaction to both acute and chronic inflammation [6]. Serum albumin concentrations may increase (positive acute phase reaction) or decrease (negative acute phase reaction) during inflammation. Reduced serum albumin concentrations of older animals are generally accompanied with increased globulin and reduced total serum protein concentrations. Both diet and inflammatory based responses can be indicated by globulin concentrations as determined by measuring the difference between total protein and albumin concentrations in blood. Remarkably, the downregulation of the hepatic synthesis of albumin, commonly considered to be a negative acute phase protein, increases reserves of free amino acids to ensure gluconeogenesis for an increased energy source [7, 8]. This means that albumin may not only serve as indicator of nutrition, but also acts as a binding protein that regulates innate immune reactions, thus also serving as a nonspecific defence system and as indicator of inflammation.

Strydom et al. (2008) reported serum albumin concentrations ranging from 18.1 g/L to 35.9 g/L in undernourished cattle and 31.3 g/L to 41.1 g/L in adequately nourished animals and showed that albumin is the best predictor of malnourishment in cattle in South Africa [9].

Changes in steroid, gonadotrophin and growth factor concentrations in follicular fluid are associated with oocyte quality and it is therefore likely that the delivery of metabolites in follicular fluid, transported by albumin may influence oocyte quality. Some studies however suggest that reduced oocyte and embryo quality are the main factors responsible for decreased reproductive performance of cows rather than it being a sole result of disruption in gonadotrophin secretion [10, 11]. The uterus of viviparous animals has to adapt significantly

to provide a suitable environment for the attachment and growth of an embryo and it has been determined that most luminal uterine proteins originate from blood serum albumin [12].

No previous reports were found on the potential role of albumin as possible predictor of oocyte competence in cattle. Changes in the physiological condition and concentrations of metabolites, gonadotrophins, steroids, growth factors, electrolytes and variations in nutrition of cows affect the condition of the follicles (size, growth, atresia), the features of follicular fluid (FF) and also subsequent oocyte quality and viability [13-15]. Factors like ketosis, heat stress, malnourishment and movement between different environments leads to poor physiological conditions that may have detrimental effects on fertility by reducing ovulation rates, the number of follicles and viability of oocytes [16, 17].

Many studies reported on effects of variation in the nutritional status (diets) of cattle and many of these reports focussed on effects of nutritional imbalances and negative energy balances on follicular fluid, oocyte growth and fertility of cattle. Increased dietary energy and high concentrations of easily degradable protein and urea nitrogen increase concentrations of blood urea and ammonia. Direct associations between oocyte quality and blood urea nitrogen (BUN) concentrations in the ovarian follicular environment and also increased ammonia concentrations in both plasma and FF significantly alter follicular growth while embryo quality decreases [2, 13]. Comprehensive reviews of these effects have been documented [18-20].

A close association between protein compositions of follicular fluid and serum were reported where it was found that variations in individual protein concentrations in serum were also present in follicular samples [3]. However, although follicular fluid relative to serum were found to be similar in all follicles, the ratios decreased for proteins with increased molecular weight and the mean albumin (molecular weight 68 kDa) concentrations varied between 97% and 141% of serum albumin in follicular fluid. Higher concentrations of larger proteins in the follicular fluid were associated with larger follicles leading to the assumption that follicular fluid forms the physiological, biochemical and metabolic environment for the nuclear and cytoplasmic maturation of oocytes and the ovulation process [3]. It could alternatively be concluded that the differentiation processes, accompanied by composite morphological, biochemical and molecular alterations of various components of the follicle such as oocytes and cells (granulosa, theca) are dependent on normal follicular growth.

The objectives of this study were to determine if serum albumin concentrations of donor cows could predict the developmental competence of oocytes by taking the nutritional status of donor cows into consideration, and if *in vitro* embryo production media can be optimised by

supplementation with additional fetal bovine serum for oocytes derived from donor cows with inadequate (<35.9 g/L) concentrations of serum albumin.

2. Materials and methods

2.1. Reagents and media

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich (Johannesburg, South Africa). Mediums used for IVF culture procedures were based on protocols used by the Department of Veterinary Medicine and Animal Reproduction, Federico II University, Naples, Italy [21, 22]. In short, the aspiration medium was TCM 199 supplemented with 25 mM HEPES, 2 mM sodium bicarbonate, 2 mM sodium pyruvate, 1 mM L-glutamine, 10 µL/mL amphotericin B (H199) supplemented with 2% fetal bovine serum (FBS, LTC Tech, Johannesburg, South Africa) and 95.6 SI/mL heparin. The *in vitro* maturation (IVM) medium was TCM 199, 0.5 µg/mL FSH, 5 µg/mL LH, 0.8 mM L-glutamine and 50 µg/mL gentamycin. The *in vitro* fertilization (IVF) medium was Tyrode's modified medium [23] without glucose and bovine serum albumin (BSA), supplemented with 5.3 SI/mL heparin, 30 µM Penicillamine, 15 µM hypo taurine and 1 µM epinephrine. The *in vitro* culture (IVC) medium consisted of Synthetic Oviduct Fluid (SOF) medium [24] with 30 µL/mL essential amino acids and 10 µL/mL non-essential amino acids. Each medium was supplemented with FBS according to experimental cohorts described in paragraphs 2.3 and 2.5 below.

2.2. Blood sampling and analyses

Blood samples were collected in 10 mL tubes (BD Vacutainer® SST™ II Advance, Becton Dickinson, USA) from coccygeal veins of cows immediately following captive-bolt stunning at a local abattoir. Samples were centrifuged (300 X g, 20 min), kept cool and serum aliquoted as follow:

1st aliquot: 100 µL immediately analysed for total protein and albumin concentration (Cobas Integra 400 Plus, Roche, South Africa), and

2nd aliquot: remainder stored at -80^{0}C for later analyses of beta (ß)-hydroxybutyric acid (BHBA), serum urea nitrogen (SUN) and serum bilirubin concentrations using same equipment. An aliquot of 500 µL of follicular fluid from each cow's supernatant was frozen at -80°C for later analysis of albumin, BHBA, SUN and bilirubin concentrations. Samples yielding outlier results were analysed a second time, following which data of samples yielding

a second result that differed by more than 5% of the initial result were removed, alternatively the initial value was used.

2.3. Oocyte recovery and experimental design

Cows received in the abattoir were from a variety of beef farms unknown to the research team. The breed of each cow sampled was recorded by a knowlegable person familiar with local cattle breeds. Following blood collection, ovaries were recovered and transported in pairs to the laboratory in physiological saline at 32 - 37°C. Cumulus oocyte complexes (COCs) were aspirated from follicles of 3 - 8mm in diameter and only those with multi-layered cumulus cells and uniform cytoplasm were selected, washed twice in aspiration media, once in pre-equilibrated IVM media before they were pooled into three groups based on clinical serum albumin concentration values from each donor cow:

Group 1: COCs from cows with <35.9 g/L blood serum albumin concentration (inadequate serum albumin),

Group 2: COCs from cows with ≥36.0 g/L blood serum albumin concentration (adequate serum albumin).

These groups were subdivided into two experimental cohorts: one exposed to the normal IVM protocol containing 15% FBS and the other with 20% FBS followed by 5% FBS for the normal IVC protocol and 10% for the other respectively.

Due to importation restrictions during the trial period the researchers were unable to obtain serum albumin and therefor used FBS, that contains albumin as its major protein component, as alternative supplement.

2.4. In vitro maturation and fertilization

Equal groups of 14 to 25 COCs were matured in 380 µL pre-equilibrated IVM medium overlaid with mineral oil (Delfran, South Africa) in four well plates (NUNC®, AEC Amersham, South Africa) for 22 – 24 h at 39°C, and 5.5% CO_2, 20% O_2, balance nitrogen and saturated humidity (95%). *In vitro* matured COCs were washed and transferred into 270 µL of IVF medium overlaid with mineral oil. Frozen sperm from an Ayrshire bull previously tested for IVF were thawed at 37°C for 30 s and separated on a differential gradient (BoviPure®, ALVAK, South Africa) by centrifugation (10 min at 150 X g). The pellet was reconstituted in 2 ml of IVF media and centrifuged at 150 X g for 10 min where after it was diluted with IVF medium and added in insemination wells at a concentration of 1 x 10^6 motile spermatozoa/ml.

2.5. In vitro culture (IVC)

Gametes were co-incubated for 20 – 22 h at 39°C, and 5.5% CO_2, 20% O_2, balance nitrogen and saturated humidity (95%), after which presumptive zygotes were denuded by vortex for 1 min in 350 µL of TCM-HEPES containing 5% FBS in a 2 ml vial (Eppendorf®, Lasec, South Africa), washed twice in same medium and transferred to IVC medium. Zygotes were randomly distributed into 380 µL of IVC medium. Experimental cohorts exposed to the normal protocol were cultured in IVC media containing 5% FBS, whereas IVC media used for cohorts exposed to the high serum protocol was supplemented with an additional 5% FBS, so that the media contained 10% FBS. Zygotes were placed and cultured in a modular chamber (Billups-Rothenberg®, USA), gassed for four to five min with TRIMIX gas (Afrox, South Africa) consisting of 5% CO_2, 6% O_2, balanced nitrogen at 39°C and saturated humidity (95%). Modular chambers were kept in aa incubator (Thermo Forma Steri-cycle CO_2®, Labotec, South Africa). Evaluation of blastocysts commenced on Day 7 and eight (Day 0 = IVF day), based on recommendations of the International Embryo Transfer Society (IETS) [25].

2.6. Statistical analysis

Sample size calculation was based on outcomes on Day 7 of the *in vitro* embryo production system, where differences between blastocyst rates of cohorts were expected. For each group of COCs pooled by serum albumin concentration of the donor cow, within each IVC/IVM repeat, weighted average values for carcass data and serum and follicular fluid chemistry data were calculated. The weighted average values were derived from individual cow data based on the proportional contribution of oocytes to the pool by every cow, using the following formula:

Weighted average value

= Individual cow value$_{\text{Cow 1}}$ x Proportion of oocytes in the pool$_{\text{Cow 1}}$

+ Individual cow value$_{\text{Cow1+n}}$ x Proportion of oocytes in the pool$_{\text{Cow1+n}}$

Data were entered into two spreadsheets in Microsoft Excel®: one data set for individual cow information up to just prior to *in vitro* cohort pooling, and a second data set for IVM/IVC data including the weighted average values. Data were analyzed with transferred into NSCC 2007 (Kaysville, UT, USA) and STATA 14 (StataCorp, Texas, USA) for statistical analyses. Proportions (such as cleavage and blastocyst rates) were compared between experimental cohorts using the Fisher exact test. Normality of data was determined by the kurtosis normality test. Mean values were compared between experimental cohorts for normally distributed data using Student's t-test and medians were compared for skewed data using the Wilcoxon rank-

sum test. Correlations were determined using the Pearson's product moment and were observed in scatterplots. Statistical significance was assumed when $P \leq 0.05$. Mixed effects multiple linear regression models were constructed for continuous outcomes with the slaughter date or the laboratory run as a random effect. All potential predictor variables were initially included into the models, and were removed one by one from the model based on the highest Wald P-value until all predictors left in the model had Wald P-values ≤ 0.10. At this point all excluded variables were again included one by one to test for confounding. Confounding was considered when a predictor changed the coefficient of another predictor by ≥15%, in which case both predictors were kept in the model.

Follicular fluid was diluted during the aspiration process in order to ensure that all oocytes were recovered out of the aspiration line, and because this dilution was different for each cow, a dilution factor of follicular fluid (for each cow) was modelled into the mixed effects linear regression model of follicular fluid albumin. The dilution factor was calculated as follows: (serum urea nitrogen – follicular fluid urea nitrogen) / follicular fluid nitrogen. Serum and follicular fluid urea nitrogen concentrations were used to calculate the diluting factors because of the strong correlation between urea nitrogen concentrations in serum and follicular fluid, being similar to previous findings [10].

Mixed effects Poisson regression models were constructed for the number of degenerate oocytes aspirated from each cow using the total number of oocytes cultured as exposure variable, and also for the number of zygotes that developed into blastocysts per experimental cohort using the number of oocytes that were cultured as exposure variable. Interaction between the IVM/IVC protocol used (supplemented with fetal bovine serum or not) and the experimental group according to serum albumin concentrations of the cows from which the oocytes originated was assessed within the mixed effects Poisson regression model of the number of oocytes that developed into blastocysts. Independent variables considered in multiple regression models included breed, age, carcass weight, fat grading, number of oocytes harvested and numbers of different qualities of oocytes. Individual concentrations of serum albumin, total serum protein, BHBA, SUN and bilirubin of cows as well as numbers, proportions or the weighted average of different qualities per cohort of oocytes in the *in vitro* embryo production system were also evaluated.

3. Results

3.1 Cow data

The 65 cows used in this trial had a mean age, based on dentition [26], of 7.9 years (SD 3.8) ranging from one to thirteen years, and were represented by Bonsmara (n = 21), Brahman (n = 16), Nguni (n = 3) and mixed breeds (n = 25). Cows with adequate serum albumin concentrations (≥36.0 g/L) were younger (P = 0.02) than those with inadequate serum albumin (≤35.9 g/L) (Table 1). Cows with adequate serum albumin had higher follicular fluid albumin concentrations (P = 0.02) but lower total serum protein and calculated serum globulin concentrations than cows with inadequate serum albumin. None of the other variables measured per cow differed between the serum albumin concentration groups (Table 1).

Table 1: Cow data per experimental group

Group	**Inadequate serum albumin concentration (≤35.9 g/L)**	**Adequate serum albumin concentration (≥36.0 g/L)**
N	28	37
Pregnant (n)	10	15
Age (years)*	8.5[a] (7 – 13)	7[b] (5 – 10)
Carcass weight (kg)**	245.5 (228.0;263.0)	257.9 (240.2;275.5)
Fat classification**	1.93 (1.55;2.31)	2.16 (1.78;2.54)
Total number oocytes harvested*	24.1 (18.6 - 29.5)	26.5 (21.4 - 31.6)
Number viable oocytes harvested*	18.1 (13.8 – 22.4)	19.4 (15.9 – 22.8)
Beta(ß)-hydroxybutyric acid (mmol/L)**	0.31 (0.28;0.34)	0.36 (0.31;0.41)
Follicular fluid beta(ß)-hydroxybutyric acid (mmol/L)**	0.26 (0.24;0.29)	0.27 (0.24;0.30)
Serum bilirubin (µmol/L)**	3.98 (2.91;5.05)	4.35 (3.33;5.37)
Follicular fluid bilirubin (µmol/L)**	3.34 (2.60;4.07)	3.41 (2.73;4.09)
Serum urea nitrogen (mmol/L)*	5.13 (4.31;5.94)	5.68 (5.09;6.28)
Follicular fluid urea nitrogen (mmol/L)**	4.03 (3.37;4.69)	4.19 (3.69;4.70)
N	27	37
Total serum protein (g/L)**	82.7[a] (78.9;86.6)	78.3[b] (76.4;80.3)
Serum albumin (g/L) **	31.8[a] (30.1;33.4)	39.6[b] (38.7;40.5)
N	26	35
Follicular fluid albumin (g/L)**	24.5[a] (22.5;26.6)	28.0[b] (25.9;30.1)

* Median (IQR)

** Mean (95% CI)

(g/L = gram per liter; kg = kilogram; mmol/L = millimole per liter; µmol/L= micromole per liter; IQR = interquartile range; CI = confidence interval)

The mean numbers of oocytes harvested per cow (25.5, 95% CI 21.8; 29.1) and viable oocytes per cow (18.8, 95% CI 16.2; 21.4) were similar for cows with adequate and inadequate serum albumin concentrations (Table 1).

Carcass weight and classification (fat classification) did not differ for cows with adequate and inadequate serum albumin concentrations although the values were numerically lower for cows with inadequate serum albumin concentration (Table 1).

Serum urea nitrogen concentrations of Brahman cows were higher ($P < 0.05$) than those of the Bonsmara cows (mean, 95% CI 6.31, 5.14; 7.48 vs 4.63, 3.96; 5.31 mmol/L), but no other breed differences were detectable (data not presented).

The highest correlation between serum and follicular fluid concentrations of the same metabolite existed for urea nitrogen, followed by BHBA and bilirubin (Table 2, Figures 1 and 2). Serum albumin concentrations did not correlate with follicular fluid albumin concentrations in cows with an albumin concentration of ≥36.0 g/L ($P = 0.61$), however serum albumin concentrations correlated negatively with follicular fluid albumin concentrations for cows with an inadequate serum albumin concentration (≤35.9 g/L) (Pearson correlation coefficient -0.46, $P = 0.02$).

Table 2: Pearson correlation matrix of serum and follicular fluid metabolites

Total serum protein (TSP) (g/L)	**TSP**							
Serum albumin (SAlb) (g/L)	-0,13	**SAlb**						
Follicular fluid albumin (FFAlb) (g/L)	-0,15	0,14	**FFAlb**					
Beta(ß)-hydroxybutyric acid (BHBA) (mmol/L)	-0,22	0,19	-0,12	**BHBA**				
Follicular fluid Beta(ß)-hydroxybutyric acid (FFBHBA) (mmol/L)	-0,11	0,00	***0,42****	***0,58****	**FFBHBA**			
Serum urea nitrogen (SUN) (mmol/L)	0,01	0,17	0,18	-0,16	-0,07	**SUN**		
Follicular fluid urea nitrogen (FFUN) (mmol/L)	0,02	0,02	***0,51****	*-0,29*	0,09	***0,86****	**FFUN**	
Serum Bilirubin (SBill) (µmol/L)	0,05	0,13	-0,12	***0,34****	0,25	***0,35****	***0,39****	**SBill**
Follicular fluid Bilirubin (FFBill) (µmol/L)	-0,04	0,03	***0,39****	0,07	***0,37****	-0,07	0,10	***0,49****

**Coefficients in bold italics are significant ($P \leq 0.05$)*

Coefficients in black boxes highlight the same metabolite in serum and follicular fluid

(g/L = gram per liter; mmol/L = millimole per liter; µmol/L= micromole per liter; *P* = probability)

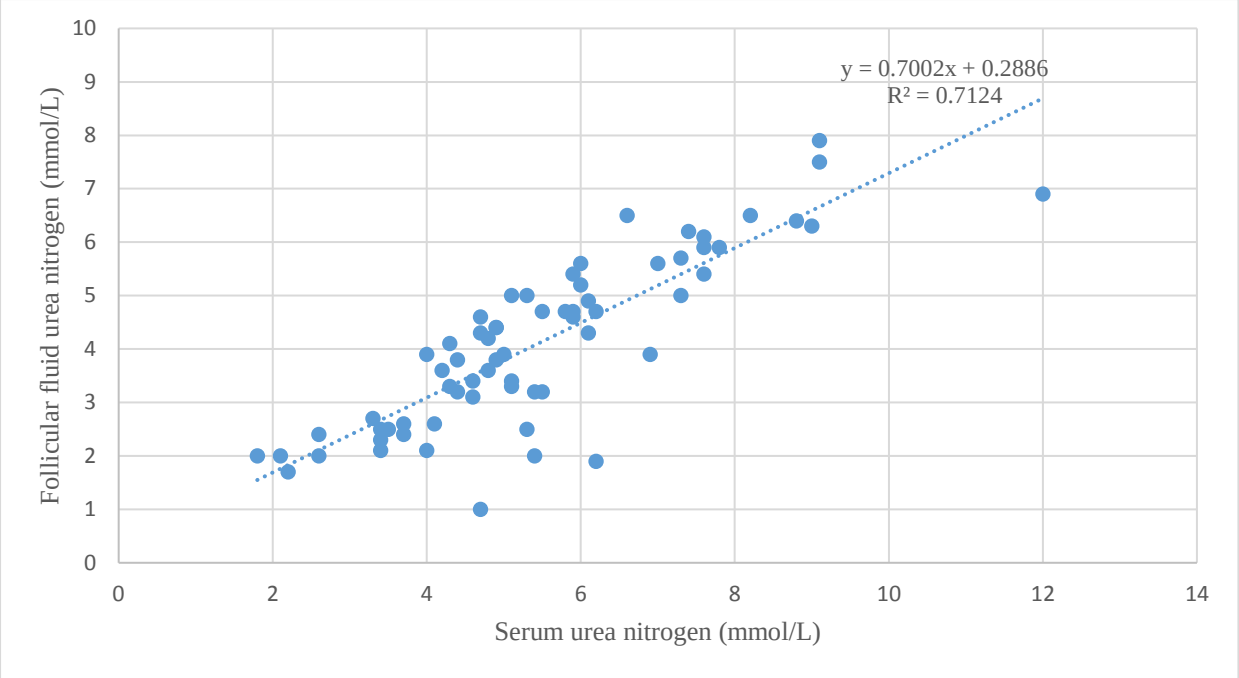

Figure 1: Scatterplot of serum and follicular fluid urea nitrogen concentration per cow (mmol/L = millimole per liter)

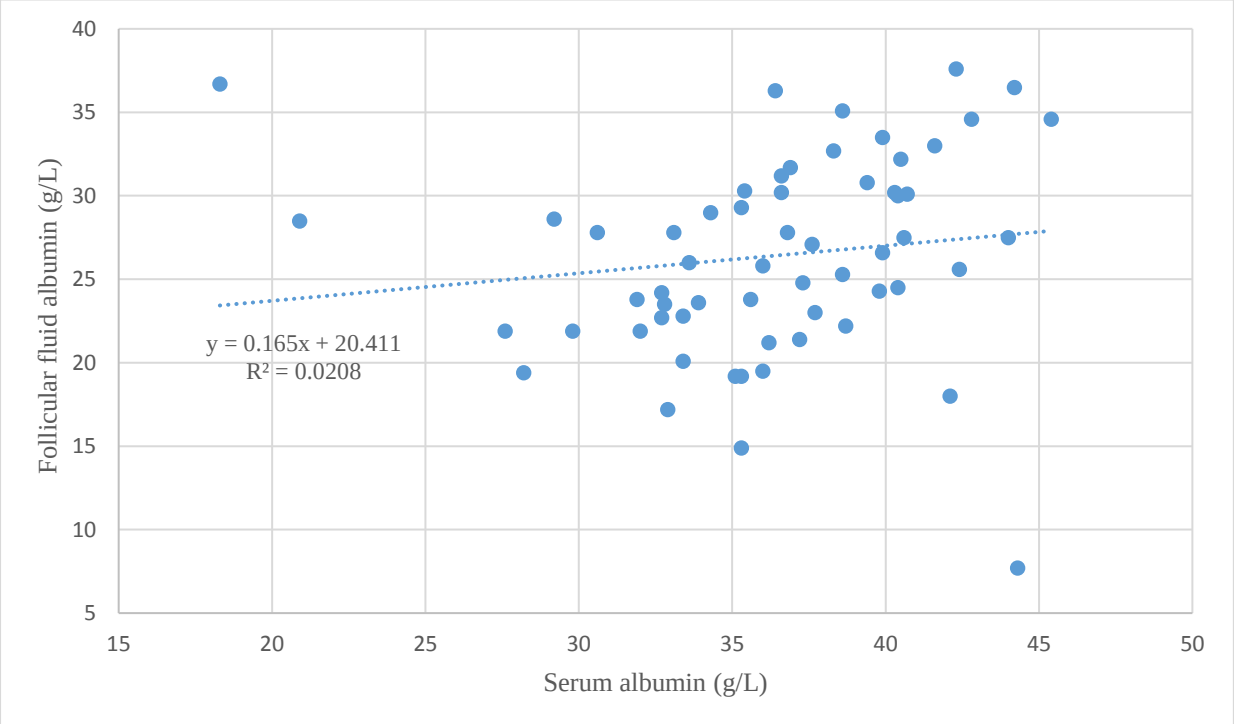

Figure 2: Scatterplot of serum and follicular fluid albumin per cow (g/L = gram per liter)

In serum, only bilirubin concentration correlated with BHBA and urea nitrogen concentrations. In follicular fluid, all metabolites measured correlated with each other, with the

exception of urea nitrogen in follicular fluid that did not correlate with follicular fluid concentrations of bilirubin or BHBA (Table 2).

Breed (Brahman vs mixed), pregnancy status (not pregnant), younger age as defined by carcass classification category and two ovaries harvested (relative to only one) were independently associated with an increasing number of oocytes aspirated per cow after adjusting for the random effect of the day on which ovaries were harvested (Table 3).

Table 3: Mixed effects linear regression model of the total number of oocytes aspirated per cow

Predictor	**Concentration**	**Coefficient(ß)**	**95% CI**		***P***
Breed	Mixed	1.00	-	-	-
	Bonsmara	5.86	-1.46	13.19	0.12
	Nguni	6.38	-4.12	16.98	0.24
	Brahman	7.78	0.66	14.90	0.03
Pregnancy Status	Not pregnant	1.00	-	-	-
	Pregnant	-5.09	-10.89	0.70	0.09
Carcass classification (age)	AB (≤ 2 years)	1.00	-	-	-
	B (2.5 - 4 years)	-2.02	-15.86	11.82	0.78
	C (≥5 years)	-8.85	-18.94	1.23	0.09
Number of ovaries	One	1.00	-	-	-
	Two	22.55	7.42	37.69	<0.01
Random effects, estimate (SE); 95% CI					
Slaughter date		2.77×10^{-8} (3.90×10^{-7}); 2.81×10^{-20}, 2.73×10^{4}			

(β = beta; CI = confidence interval; *P* = probability; SE = standard error)

Breed (Bonsmara vs mixed) and an increasing age (years) were independently associated with the proportion of degenerate oocytes aspirated after adjusting for the random effect of the day on which ovaries were harvested (Table 4).

Table 4: Mixed effects Poisson regression model of the total number of degenerate oocytes aspirated per cow

Predictor	**Concentration**	**IRR**	**95% CI**		***P***
Breed	Mixed	1.00	-	-	-
	Brahman	1.01	0.69	1.47	0.97
	Bonsmara	1.48	0.97	2.25	0.07
	Nguni	1.57	0.84	2.95	0.16
Age (years)		1.04	1.00	1.10	0.07
Total number of oocytes harvested		Exposure variable			
Random effects, estimate (SE); 95% CI					
Slaughter date	0.03 (0.01); 0.02, 0.05				

(IRR = internal rate of return; CI = confidence interval; *P* = probability; SE = standard error)

Younger age (years), increasing follicular fluid BHBA and increasing serum albumin concentrations were independently associated with an increasing concentration of albumin in follicular fluid after adjusting for the dilution factor of serum and follicular fluid urea nitrogen and the random effect of the slaughter date (Table 5).

Table 5: Mixed effects linear regression model of follicular fluid albumin concentration (g/L) per cow

Predictor		**Coefficient(ß)**	**95% CI**		***P***
Age (years)		-0.58	-0.84	-0.32	<0.01
Serum albumin (g/L)		0.29	0.13	0.44	<0.01
Follicular fluid beta(ß)-hydroxybutyric acid (mmol/L)		14.65	5.04	24.26	<0.01
Dilution factor	Serum urea nitrogen (mmol/L)	-4.31	-5.24	-3.39	<0.01
	Follicular fluid urea nitrogen (mmol/L)	6.91	5.73	8.09	<0.01
Random effects, estimate (SE); 95% CI					
Slaughter date		0.58 (1.01); 0.02, 17.55			

(g/L = gram per liter; β = beta; CI = confidence interval; *P* = probability; mmol/L = millimole per liter; SE = standard error)

3.2 In-vitro culture data

A total number of 1024 oocytes were cultured during six laboratory runs in 56 droplets. Of these, 460 oocytes originated from cows with inadequate serum albumin concentrations (≤35.9 g/L) and 564 from cows with adequate serum albumin (≥36.0 g/L). The number of oocytes per droplet ranged from 10 - 27, and the mean number of oocytes per droplet was similar for the different experimental groups (Table 6). A higher proportion of oocytes cleaved in the group with inadequate serum albumin than in the group with adequate serum albumin ($P < 0.01$, Table 6), but the proportion of oocytes that cleaved was not affected by the IVM/IVC protocol used ($P > 0.05$, Table 6).

A higher proportion of oocytes originating from cows with adequate serum albumin reached the blastocyst stage by Day 7 in the control IVM/IVC protocol when compared to the supplemented protocol ($P < 0.01$, Table 6). The proportion of oocytes that reached the blastocyst stage by Day 7 in the control protocol was higher for oocytes originating from cows with adequate serum albumin concentrations compared to oocytes originating from cows with inadequate serum albumin concentrations ($P = 0.05$), but supplementing the IVM/IVC media with fetal bovine serum did not significantly affect the proportion of oocytes originating from cows with inadequate serum albumin concentrations (Table 6). For cows with adequate serum albumin concentration (≥36.0 g/L) the supplemented IVM/IVC

protocol was independently associated with a lower proportion of blastocysts. Serum albumin concentration ≥36.0 g/L was independently associated with a higher proportion of blastocysts when the standard IVM/IVC protocol was used. Other predictors that were independently associated with an increasing proportion of blastocysts were an increasing weighted average blood urea nitrogen concentration and decreasing weighted average follicular fluid beta (ß)-hydroxybutyric acid and fat grading after adjusting for the random effect of the laboratory run (Table 7). Breed was not associated with developmental competence of oocytes.

Table 6: *In vitro* culture data per experimental group and treatment

	Adequate serum albumin concentration		**Inadequate serum albumin concentration**	
IVM/IVC protocol	Control	Supplemented FBS	Control	Supplemented FBS
n (Droplets)	15	15	13	13
Oocytes per droplet *	18.60[a] (15.26;21.94)	18.60[a] (15.20;22.00)	17.69[a] (15.49;19.89)	17.69[a] (15.62;19.76)
Oocytes cultured**	284	280	230	230
	564		460	
Cleaved oocytes**	179 (0.63)[a]	181 (0.65)[a]	163 (0.71)[b]	168 (0.73)[b]
	360 (0.64)[a]		331 (0.72)[b]	
Blastocysts**	103 (0.36)[a]	72 (0.26)[b]	65 (0.28)[b]	70 (0.30)[a,b]
	175 (0.31)[a]		135 (0.29)[a]	

* Mean (95% CI)

** Count (Proportion)

[a,c]Means and proportions with different superscripts within rows differ significantly ($P \leq 0.05$)
(IVM = *in vitro* maturation; IVC = *in vitro* culture; n = number; CI = confidence interval)

Table 7: Mixed effects Poisson regression model of the number of blastocysts obtained per culture well

Predictor	Concentration	IRR (Count ratio)	95% CI		*P*
IVM/IVC protocol	Supplemented vs Control (cows with serum albumin ≤35.9 g/L)	1.08	0.77	1.51	0.67
	Supplemented vs Control (cows with serum albumin ≥36.0 g/L)	0.70	0.52	0.94	0.02
Serum albumin concentrations of cows	≥36.0 vs ≤35.9g/L (Supplemented IVM/IVC protocol)	0.94	0.67	1.33	0.76
	≥36.0 vs ≤35.9g/L (Control IVM/IVC protocol)	1.46	1.06	2.02	0.02
Weighted average blood urea nitrogen (mmol/L)		1.52	1.31	1.77	<0.01
Weighted average follicular fluid beta(ß)-hydroxybutyric acid (mmol/L)		8.82×10^{-4}	1.88×10^{-5}	4.14×10^{-2}	<0.01
Weighted average fat grading (0 – 5)		0.63	0.47	0.85	<0.01
Number of oocytes cultured		Exposure variable			
Random effects, estimate (SE)					
Laboratory run		7.93×10^{-12} (4.70×10^{-3})			<0.01

(IRR = internal rate of return; CI = confidence interval**;** *P* = probability; IVM = *in vitro* maturation; IVC = *in vitro* culture; g/L = gram per liter; mmol/L = millimole per liter**;** SE = standard error)

4. Discussion

The ultimate test to evaluate the developmental competence of an oocyte lies in its ability to resume meiosis, be fertilised and develop into a blastocyst that will result in the production of normal, healthy offspring [27, 28]. The objectives of this study were to determine if serum albumin concentrations of donor cows independently predict the developmental competence of oocytes, and if *in vitro* embryo production media can be optimised according to donor cow albumin concentration by additional supplementation with fetal bovine serum.

Results of this study indicate that total serum protein and calculated serum globulin concentrations of cows with adequate serum albumin were lower than in cows with inadequate serum albumin. This might be explained by considering that cows with adequate serum albumin in the study were younger than those with inadequate serum albumin and it may indicate that inadequate serum albumin concentration did not act as a proxy for malnourishment in this study as was expected, but may in fact have occurred due to an increase in the globulin fraction [9]. This is a significant finding indicating that the association of inadequate serum albumin with poor developmental competence of oocytes is likely to be as a result of a direct effect of albumin, rather than albumin acting as a proxy for malnourishment. Keeping the negative acute phase characteristic of serum albumin in mind, this study was not designed to rule out the possibility of inflammation being a confounder of the effect of low serum albumin

on the developmental competence of oocytes. Further studies should use other negative acute phase proteins as markers of inflammation to rule out confounding effects.

Several studies reported higher recovery rates of viable oocytes and embryo yields from *Bos indicus* breeds than from *Bos taurus* breeds and it would seem that the effects of breed on oocyte quality and quantity became more apparent when environmental influence such as heat stress were taken into consideration [29-32]. Specific information on the quality and viability of recovered Bonsmara oocytes are not well documented in the literature. Bonsmara is a composite breed consisting of ⅝ indigenous Afrikaner (*Bos taurus africanis*) and ⅜ exotic Shorthorn or Hereford (*Bos taurus*) breeds [33, 34]. The significant association between the numbers of oocytes harvested from Brahman cows in relation to other breeds (Table 3) in the current study might be as a result of the *Bos indicus* characteristics that generally provide improved oocyte recovery rates and quality, but these physiological differences between breeds still lack substantial explanation.

In the current study, pregnant cows were independently associated with a decreasing number of oocytes aspirated per cow after adjusting for the random effect of the day on which ovaries were harvested (Table 3). These results correlate with findings of a number of studies where significantly reduced follicle and oocyte numbers were described in pregnant cows [35-37].

In the mixed effects Poisson regression model, age as determined by dentition [26], was an independent predictor of the proportion of degenerate oocytes harvested after adjusting for breed. In the model shown in Table 6 none of the serum metabolites predicted the proportion of degenerate oocytes, indicating that there is another age related factor that has this effect. An interesting report in 2013 found differences in gene expression profiles of oocytes and embryos between aged and young cows and the researchers indicated that oocytes from aged cows showed protein and maternal age related mitochondrial dysfunction [38]. They further suggested that this abnormality is a result of increased oxidative stress and differences in the mitochondrial genome and further reported that the activation of the SIRT1 gene in oocytes may be a possible countermeasure against ageing events in oocytes obtained from aged cows.

In the present study, follicular fluid of individually aspirated follicles was pooled and then diluted by rinsing the aspiration line per cow prior to metabolite concentration analysis. This explains differences in concentrations of BHBA, urea and total protein obtained by Leroy et al. (2004) where fluid from individual follicles was analysed without dilution. The aim of the current study was to follow oocytes from cow to blastocyst and the focus was not on the

follicular fluid; it was therefore more important to the researcher to obtain all possible oocytes from each cow than to have undiluted follicular fluid. The extent of flushing the aspiration line was different for each cow and depended on the size of the follicles and ease of the aspiration process. Despite this, similar correlations for urea nitrogen, (Figure 1) BHBA and total protein concentrations between blood serum and follicular fluid were found to those previously reported in mares and dairy cows [10, 39]. The correlation between serum and follicular fluid urea nitrogen was particularly high and significant as seen in the R-squared value (Figure 1, Table 4) indicating that the variation in serum urea nitrogen explained 71% of the variation in follicular fluid urea nitrogen. This has been reported before, indicating a passive diffusion of urea from body fluids to follicular fluid [10, 39, 40]. We therefore used urea nitrogen concentrations in serum and follicular fluid as "correction factor" for the dilution of follicular fluid due to the aspiration and flushing process (Table 5).

However, in the case of follicular fluid albumin level the R-squared value indicates that only 2% of the variation can be explained by serum albumin level (Figure 2). This finding is in contradiction to previous reports [3, 41, 42] and could be related to the fact that follicles are ovarian avascular compartments, separated from the perifollicular stroma by a follicular wall that is referred to as a "blood-follicle barrier" [41, 43, 44]. Furthermore, one should also consider that albumin is a protein (molecular weight 66 - 68 kDa); we hypothesize that smaller molecules might filter through the "blood-follicle barrier" easier than the larger molecules such as proteins, hence the reduced concentrations of albumin in the follicular fluid at first with subsequent higher concentrations as permeability increases with development of the follicle. Except for the tight junction-permeability barrier of endothelial cells in micro-vessels that surrounds the follicle, the ovarian follicles are further surrounded by a network of capillaries in the theca interna and the basement membrane [33], and oocytes and cumulus cells may have a regulatory function on metabolites in follicular fluid to maintain oocyte quality [45, 46]. In the current study this may have resulted in the phenomenon that at lower serum albumin concentrations ($\leq$ 36.0 g/L), the concentrations of albumin in follicular fluid appeared to be maintained more constantly, in fact the correlation between serum and follicular fluid albumin became negative as serum albumin concentrations decreased below 39.0 g/L (Figure 2). This poorly understood complexity of mechanisms involved in the physical transfer of molecules at the site of the "blood-follicle barrier" needs further investigation.

Albumin significantly controls the ionized concentrations of important metals, it serves as a major circulatory protein responsible for the transport of Ca^{2+} and Mg^{2+}, is the principle

carrier of hydrophobic and normally insoluble fatty acids and it inactivates toxic lipophilic metabolites such as bilirubin [4, 47]. It further acts as transport agent for a wide range of smaller molecules such as fatty acids, metabolites, hormones, drugs, nutrients, minerals, gonadotrophins, steroids, growth factors and ions which are essential building blocks for oocyte maturation and growth [48, 49].

To the knowledge of the authors no previous studies focused on the role that serum albumin might play as predictor of oocyte developmental competence in cattle. In an interesting study a significant elevated oxidized state in human follicular fluid that contained degenerate oocytes were found compared to follicular fluid containing normal oocytes and at the time of oocyte retrieval, lower concentrations of albumin were present in the follicular fluid in relation to concentrations in serum. Furthermore, the redox state of serum was shifted towards the oxidative state with increasing age and endometriosis and it was hypothesised that albumin in follicular fluid might have an inherit duty in safeguarding oocytes from oxidative damage [48]. Based on these reports, it can be confirmed that factors other than metabolites in follicular fluid that are in close association with aging, may also affect the proportion of degenerate oocytes harvested in cows.

In the present study a higher proportion of oocytes originating from cows with adequate serum albumin cleaved when compared to cows with inadequate serum albumin concentrations ($P < 0.01$, Table 6). These results are in agreement with a previous report that indicated reduced *in vitro* maturation, cleavage and blastocyst yields from cows that experienced negative energy balance potentially as a result of malnourishment [50]. However, within the current study's IMV/IVC serum based albumin supplemented experimental groups, no differences in cleavage rates were recorded between the control and supplemented protocols. This means that oocytes from cows with inadequate albumin concentrations and malnourishment have a reduced cleavage potential compared to those cows with adequate albumin concentrations and that the supplementation of the culture media *in vitro* with fetal bovine serum does not improve cleavage rates. The current culture protocol did not include Day 2 cleavage evaluation and it is therefore uncertain what impact parthenogenesis (oocyte development without fertilization by spermatozoa) had on the cleavage outcomes [51].

Serum albumin has been described as a product of protein anabolism and indicated as a positive predictor of growth and maintenance of energy utilization, supporting the notion that the interplay between protein and energy rather than the effect of excess protein alone, is responsible for reduced fertility outcomes in cattle [20, 52]. According to our results,

decreasing follicular fluid BHBA (representing positive energy balance) and increasing serum albumin concentrations can be associated with bovine oocyte quality and viability whereas other predictors that were independently associated with an increasing proportion of blastocysts were an increasing weighted average blood urea nitrogen and fat grading (Table 7).

We found that a higher proportion of oocytes originating from cows with adequate serum albumin reached blastocyst stage by Day 7 in the control IVM/IVC protocol when compared to the supplemented protocol ($P \leq 0.05$, Table 6). Previous studies confirmed the ideal concentrations of protein in IVM/IVC media [53] and it is well documented that excessive accumulation of lipids in early bovine embryos is caused by the excessive presence of serum in culture media [54, 55]. In the current study it was anticipated that for cows with inadequate serum albumin, additional supplementation of the IVC/IVM media with FBS would improve the developmental competence of oocytes. Interestingly, supplementing the IVM/IVC media with additional FBS, that contains albumin as its major protein component, did not significantly affect the proportion of blastocysts from oocytes originating from cows with inadequate serum albumin concentrations (Table 6) as was the case for oocytes from cows with adequate serum albumin. This warrants further investigation of the effects of finer adjustments to the protein content of IVC/IVM media on the developmental competence of oocytes from cows with different concentrations of serum albumin.

During the course of this study, the researcher acknowledged the importance of the vast complexity of processes and factors involved that influence oocyte quality, oocyte competence and fertility in cattle. The current study aimed at evaluating one small aspect, namely the role of serum albumin as predictor of oocyte competence and could not address the magnitude of other reported factors involved nor could it report on the role of other FBS components such as other proteins, fatty acids and growth factors and their roles as potential predictors in the developmental competence of oocytes.

5. Conclusions

It is concluded that adequate serum albumin of donor cows (≥36.0 g/L) is a significant independent predictor of *in vitro* developmental competence of oocytes. It is also concluded that further studies to investigate optimisation of *in vitro* embryo production protocols in accordance with the serum albumin level of oocyte donors are warranted.

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References

[1] Armstrong D. Effects of maternal age on oocyte developmental competence. Theriogenology 2001;55:1303-22.

[2] Armstrong D, McEvoy T, Baxter G, Robinson J, Hogg C, Woad K, et al. Effect of dietary energy and protein on bovine follicular dynamics and embryo production in vitro: associations with the ovarian insulin-like growth factor system. Biol Reprod 2001;64:1624-32.

[3] Andersen MM, Kroll J, Byskov A, Faber M. Protein composition in the fluid of individual bovine follicles. J Reprod Fertil 1976;48:109-18.

[4] Majorek KA, Porebski PJ, Dayal A, Zimmerman MD, Jablonska K, Stewart AJ, et al. Structural and immunologic characterization of bovine, horse, and rabbit serum albumins. Mol Immunol 2012;52:174-82.

[5] Hamerly T, Heinemann J, Tokmina-Lukaszewska M, Lusczek ER, Mulier KE, Beilman GJ, et al. Bovine serum albumin as a molecular sensor for the discrimination of complex metabolite samples. Anal Chim Acta 2014;818:61-6.

[6] Andrews AH, Blowey RW, Boyd H, Eddy RG. Bovine Medicine: Diseases and Husbandry of Cattle. 2nd ed. Blackwell Science; 2008.

[7] Shamay A, Homans R, Fuerman Y, Levin I, Barash H, Silanikove N, et al. Expression of albumin in nonhepatic tissues and its synthesis by the bovine mammary gland. J Dairy Sci 2005;88:569-76.

[8] Ceciliani F, Ceron J, Eckersall P, Sauerwein H. Acute phase proteins in ruminants. Proteomics 2012;75:4207-31.

[9] Strydom S, Agenas S, Heath M, Phillips C, Rautenbach G, Thompson P. Evaluation of biochemical and ultrasonographic measurements as indicators of undernutrition in cattle. Onderstepoort J Vet Res 2008;75:207.

[10] Leroy J, Vanholder T, Delanghe J, Opsomer G, Van Soom A, Bols P, et al. Metabolite and ionic composition of follicular fluid from different-sized follicles and their relationship to serum concentrations in dairy cows. Anim Reprod Sci 2004;80:201-11.

[11] Rhoads M, Rhoads R, Gilbert R, Toole R, Butler W. Detrimental effects of high plasma urea nitrogen levels on viability of embryos from lactating dairy cows. Anim Reprod Sci 2006;91:1-10.

[12] Fischer B, Beier HM. Uterine environment in early pregnancy. In: Sreenan JM, Diskin MG, editors. Embryonic Mortality in Farm Animals. Netherlands: Springer; 1986. p. 93-108.

[13] Iwata H, Inoue J, Kimura K, Kuge T, Kuwayama T, Monji Y. Comparison between the characteristics of follicular fluid and the developmental competence of bovine oocytes. Anim Reprod Sci 2006;91:215-23.

[14] Vassena R, Mapletoft RJ, Allodi S, Singh J, Adams GP. Morphology and developmental competence of bovine oocytes relative to follicular status. Theriogenology 2003;60:923-32.

[15] Ginther O, Rakesh H, Hoffman M. Blood flow to follicles and CL during development of the periovulatory follicular wave in heifers. Theriogenology 2014;82:304-11.

[16] Rhind S, McMillen S, McKelvey W, Rodriguez-Herrejon F, McNeilly A. Effect of the body condition of ewes on the secretion of LH and FSH and the pituitary response to gonadotrophin-releasing hormone. J Endocrinol 1989;120:497-502.

[17] Dominguez M. Effects of body condition, reproductive status and breed on follicular population and oocyte quality in cows. Theriogenology 1995;43:1405-18.

[18] Leroy J, Opsomer G, Van Soom A, Goovaerts I, Bols P. Reduced fertility in High-yielding dairy cows: Are the oocyte and embryo in danger? Part I The importance of negative energy balance and altered corpus

luteum function to the reduction of oocyte and embryo quality in High-yielding dairy cows. Reprod Domest Anim 2008;43:612-22.

[19] Leroy J, Van Soom A, Opsomer G, Goovaerts I, Bols P. Reduced Fertility in High-yielding Dairy Cows: Are the Oocyte and Embryo in Danger? Part II Mechanisms Linking Nutrition and Reduced Oocyte and Embryo Quality in High-yielding Dairy Cows. Reprod Domest Anim 2008;43:623-32.

[20] Leroy J, Vanholder T, Van Knegsel A, Garcia-Ispierto I, Bols P. Nutrient prioritization in dairy cows early postpartum: mismatch between metabolism and fertility? Reprod Domest Anim 2008;43:96-103.

[21] Zullo G, Albero G, Neglia G, De Canditiis C, Bifulco G, Campanile G, et al. L-ergothioneine supplementation during culture improves quality of bovine in vitro–produced embryos. Theriogenology 2016;85:688-97.

[22] Rusciano G, De Canditiis C, Zito G, Rubessa M, Roca MS, Carotenuto R, et al. Raman-microscopy investigation of vitrification-induced structural damages in mature bovine oocytes. PloS One 2017;12:e0177677.

[23] Parrish J, Susko-Parrish J, Leibfried-Rutledge M, Critser E, Eyestone W, First N. Bovine in vitro fertilization with frozen-thawed semen. Theriogenology 1986;25:591-600.

[24] Tervit HR, Whittingham DG, Rowson LE. Successful culture in vitro of sheep and cattle ova. J Reprod Fertil 1972;30:493-7.

[25] Stringfellow DA, Givens MD. Manual of the International Embryo Transfer Society: a procedural guide and general information for the use of embryo transfer technology emphasizing sanitary procedures. 4th ed: Savory, III. International Embryo Transfer Society 2010.

[26] How To Estimate Cattle Age By Their Teeth. http://www.cowboyway.com/HowTo/CowAgeTeeth.htm: Amazon, USA; 1999.

[27] Lonergan P, Rizos D, Ward F, Boland MP. Factors influencing oocyte and embryo quality in cattle. Reprod Nutr Dev 2001;41:427-37.

[28] Sirard M-A, Richard F, Blondin P, Robert C. Contribution of the oocyte to embryo quality. Theriogenology 2006;65:126-36.

[29] Pontes J, Silva K, Basso A, Ferreira C, Santos G, Sanches B, et al. 179 Comparison of oocyte and embryo production among Bos Taurus, Bos Indicus, and Indicus-Taurus donor cows. Reprod Fertil Dev 2010;22:248.

[30] Camargo LSdA, Viana JHM, Sá WFd, Ferreira AdM, Ramos AdA, Vale Filho V. Factors influencing in vitro embryo production. Anim Reprod 2006;3:19-28.

[31] Sales J, Iguma L, Batista R, Quintão C, Gama M, Freitas C, et al. Effects of a high-energy diet on oocyte quality and in vitro embryo production in Bos indicus and Bos taurus cows. J Dairy Sci 2015;98:3086-99.

[32] Rocha A, Randel R, Broussard J, Lim J, Blair R, Roussel J, et al. High environmental temperature and humidity decrease oocyte quality in Bostaurus but not in Bostaurus cows. Theriogenology 1998;49:657-65.

[33] Bonsma J. Livestock production--a global approach. Cape Town, South Africa: Tafelburg Publishers Ltd.; 1980.

[34] Bosman L, van Marle-Köster E, van der Westhuizen R, Visser C, Berry D. Population structure of the South African Bonsmara beef breed using high density single nucleotide polymorphism genotypes. Livest Sci 2017;197:102-5.

[35] Eikelmann E, Frank K, Schindler L, Niemann H. Factors Affecting Embryo Production-Repeated ultrasound-guided follicular aspiration in pregnant heifers and cows. Theriogenology 2000;53:351-.

[36] Meintjes M, Bellow M, Broussard J, Paul J, Godke R. Transvaginal aspiration of bovine oocytes from hormone-treated pregnant beef cattle for IVF. Theriogenology 1993;39:266.

[37] Aller J, Mucci NC, Kaiser GG, Callejas SS, Alberio RH. Effect of repeated eCG treatments and ovum pick-up on ovarian response and oocyte recovery during early pregnancy in suckling beef cows. Anim Reprod Sci 2012;133:10-5.

[38] Takeo S, Kawahara-Miki R, Goto H, Cao F, Kimura K, Monji Y, et al. Age-associated changes in gene expression and developmental competence of bovine oocytes, and a possible countermeasure against age-associated events. Mol Reprod Dev 2013;80:508-21.

[39] Collins A, Palmer E, Bezard J, Burke J, Duchamp G, Buckley T. A comparison of the biochemical composition of equine follicular fluid and serum at four different stages of the follicular cycle. Equine Vet J 1997;29:12-6.

[40] Valckx S, De Pauw I, De Neubourg D, Inion I, Berth M, Fransen E, et al. BMI-related metabolic composition of the follicular fluid of women undergoing assisted reproductive treatment and the consequences for oocyte and embryo quality. Hum Reprod 2012;27:3531-9.

[41] Shalgi R, Kraicer P, Rimon A, Pinto M, Soferman N. Proteins of human follicular fluid: the blood-follicle barrier. Fertil Steril 1973;24:429-34.

[42] McGaughey R. A comparison of the fluids from small and large ovarian follicles of the pig. Biol Reprod 1975;13:147-53.

[43] Okuda Y, Okamura H, Kanzaki H, Takenaka A, Morimoto K, Nishimura T. An ultrastructural study of capillary permeability of rabbit ovarian follicles during ovulation using carbon tracer (author's transl). Nihon Sanka Fujinka Gakkai Zasshi 1980;32:859-67.

[44] Bagavandoss P, Midgley Jr A, Wicha M. Developmental changes in the ovarian follicular basal lamina detected by immunofluorescence and electron microscopy. J Histochem Cytochem 1983;31:633-40.

[45] Taft RA, Denegre JM, Pendola FL, Eppig JJ. Identification of genes encoding mouse oocyte secretory and transmembrane proteins by a signal sequence trap. Biol Reprod 2002;67:953-60.

[46] Santos P, Chaveiro A, Simoes N, Moreira da Silva F. Bovine oocyte quality in relation to ultrastructural characteristics of zona pellucida, polyspermic penetration and developmental competence. Reprod Domest Anim 2008;43:685-9.

[47] Tshuma T, Holm DE, Fosgate GT, Lourens DC. Pre-breeding blood urea nitrogen concentration and reproductive performance of Bonsmara heifers within different management systems. Trop Anim Health Prod 2014;46:1023-30.

[48] Otsuki J, Nagai Y, Matsuyama Y, Terada T, Era S. The influence of the redox state of follicular fluid albumin on the viability of aspirated human oocytes. Syst Biol Reprod Med 2012;58:149-53.

[49] Russell KE, Roussel AJ. Evaluation of the ruminant serum chemistry profile. Vet Clin North Am Food Anim Pract 2007;23:403-26.

[50] Leroy J, Vanholder T, Opsomer G, Van Soom A, Kruif Ad. The In Vitro Development of Bovine Oocytes after Maturation in Glucose and β-Hydroxybutyrate Concentrations Associated with Negative Energy Balance in Dairy Cows. Reprod Domest Anim 2006;41:119-23.

[51] Brevini T, Pennarossa G, Vanelli A, Maffei S, Gandolfi F. Parthenogenesis in non-rodent species: developmental competence and differentiation plasticity. Theriogenology 2012;77:766-72.

[52] Papadopoulos S, Lonergan P, Gath V, Quinn K, Evans A, O'Callaghan D, et al. Effect of diet quantity and urea supplementation on oocyte and embryo quality in sheep. Theriogenology 2001;55:1059-69.

[53] Van Langendonckt A, Donnay I, Schuurbiers N, Auquier P, Carolan C, Massip A, et al. Effects of supplementation with fetal calf serum on development of bovine embryos in synthetic oviduct fluid medium. J Reprod Fertil 1997;109:87-93.

[54] Ferguson E, Leese H. Triglyceride content of bovine oocytes and early embryos. J Reprod Fertil 1999;116:373-8.

[55] Abe H, Yamashita S, Itoh T, Satoh T, Hoshi H. Ultrastructure of bovine embryos developed from in vitro–matured and–fertilized oocytes: Comparative morphological evaluation of embryos cultured either in serum-free medium or in serum-supplemented medium. Mol Reprod Dev 1999;53:325-35.