Evaluation of embryo production method on the quality of embryos produced by Nguni, Bonsmara, Boran beef cattle breeds.

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Abstract

The objective of this study was to compare the quality of embryos produced by *in vivo* and *in vitro* in Bonsmara, Nguni and Boran cattle. Hormonal treatment was used to produce embryos *in vivo* while oocytes retrieved by ovum pickup and ovary aspiration were used to produce *in vitro* embryos. Embryos produced by both methods in all the three breeds where evaluated for morphological structure based on the standards of the International Embryo Transfer Society. Data was analysed by ANOVA. No significant difference where observed among breeds in developmental competency of embryos produced *in vivo.* Development of blastocysts *in vivo* was significantly higher at day 8 in Bonsmara and Nguni compared to Boran. No significant difference observed between breeds at all development stages for the *in vitro* method. There were no significant breed differences in the number of blastocysts and blastocyst quality obtained by either the *in vivo* or *in vitro* method. Flushing and Ovum pickup produced a higher number of blastocysts compared to ovary aspiration. The number of Grade I embryos were significantly higher in flushing and OPU compared to ovary aspiration. In conclusion, the study showed that the *in vivo* method of embryo production is superior to the *in vitro* method in blastocyst development of Bonsmara, Boran and Nguni breeds.

Introduction

Livestock production is one of the leading agricultural sectors in South Africa, due to its contribution to the national economy (Scholtz and Theunissen, 2010). The growing demand for livestock products globally, prompted by an increase in the human population and urbanisation, necessitates an increase in the production of livestock products such as beef (Steinfeld et al., 2006; Otten and van der Weghe, 2011). There is a big need to improve livestock productivity in order to meet this demand, particularly in developing countries such as South Africa. Beef cattle production known to be the largest livestock farming sector in South Africa, and the non-commercial sector is one of the two sectors available in the production of beef. The non-commercial is dominated by farmers' in a communal and/ smallholder setup. The later described, mainly keep indigenous beef cattle breeds such as Bosmara, Nguni, Boran and other non-descriptive cattle breeds, which sustain their livelihoods through sales at informal market, during ceremonies and festive seasons (Mugwabana et al., 2018). Productivity in the smallholder sector is very low due to many reasons including poor management practices, low reproductive efficiency and failure to adoption new technologies (Mugwabana et al., 2018; Hadgu et al., 2020). The recent climatic and environmental challenges intensifies the problems faced by the smallholder sector, probing further difficulties for the sector to grow (Daly et al., 2020).

Most of the indigenous African cattle breeds are adapted to the local environmental challenges such as periodic droughts, seasonal dry periods, nutritional shortages, parasites, infectious diseases and high ambient temperatures (Mwai et al., 2015). Thus, making them suitable breed for the harsh South African environment, particularly in the smallholder sector, where they can survive with minimum feed supplementation or medication. These breeds are a viable alternative to imported breeds, under the challenging climatic conditions of Southern Africa (Mwai et al., 2015). Thereof, with the utilization of

locally adapted beef breed and the given opportunities for their improvement, smallholder farmers have the potential to participate in the beef market value chain by utilizing new developed technologies to meet the local beef demand.

In vitro and *in vivo* embryo production are advanced reproductive technology that have been proven to enhance reproductive potential and productivity in beef and dairy cattle industry in European cattle breeds. However, there is no data documenting the use of this technology and its efficiency in the smallholder beef cattle sector particulary in Southern Africa's adapted cattle breeds. In the past years, experiments on advanced embryo production technologies have generated a lot of information on the structure and embryo development with emphasis on cattle (Moore and Hasler, 2017). These technologies, currently, have varying degrees of efficiency, which necessitates intensive research. Previously, attention have focused on embryo recovery methods, however, subsequent steps leading to successful calving proved to be equally important (Greve and Callesen, 2005). Embryo quality is one of the main key drivers that has a direct effect on successful pregnancy after transfer. It is also evident in other studies that embryos with high quality have increased pregnancy rate (Rocha et al., 2016; Thompson, 2016). Culture environment such as *in vivo* or *in vitro*, the type of supplementation, the type of medium play a crucial role in determining the final embryo yield leading to pregnancy and subsequent birth of healthy calves (Lopes et al., 2020). However, different embryo production methods still need to be expanded to smallholder beef cattle farmers in order for it to have any impact. It is, therefore, important to first produce and assess the quality of embryos produced *in vivo* and *in vitro* prior to transfer to improve production. Thus, the study is aimed to evaluate the effectiveness of the two embryo production systems on the quality of embryos produced by Bonsmara, Nguni and Boran cattle as our model breeds.

Materials And Methods

Chemicals

All chemicals used in this study where purchased from Sigma-Adrich PTY LTD (South Africa) unless otherwise indicated.

Animals

All animals used in this study aged between 24–36 months and were kept at a quarantine area with access to water at all times. Bulls were fed on eragrostis grassad lib. A total of sixty (20 Nguni, 20 Bonsmara and 20 Boran) non-pregnant, healthy cycling cows were used as donors and evenly distributed to two embryo production methods, namely, ovum pick-up (*in vitro*) and embryo flushing (*in vivo*). Each donor cows was feeding on 7–10 kg lucerne, ad lib eragrostis grass and 1.5kg of Afgri® embryo concentrate per day.

Superovulation, AI and embryo flushing

A total of 30 donor cows (10 Nguni; 10 Bonsmara; 10 Boran) were superovulated according to the method described by Pontes et al. (2009) with slight modifications. Briefly, a controlled internal drug release

(CIDR®) (1.9g, Pfizer (Pty) Ltd, Sandton, Republic of South Africa) was placed into the vagina of each cow of the three different breeds on Day 0. An intramuscular injection of cloprostenol sodium (263µg, Estrumate®, Isando, and RSA) was administered to the cows after CIDR® removal on Day 8 of oestrous synchronization followed by intramuscular injection of estradiol benzoate (1g Pfizer (Pty) Ltd, Republic of South Africa) on Day 9. Heat was observed with the aid of heat mount detectors (Kamar®, RSA). Day 0 was repeated by inserting a new CIDR three days after heat observation. On day 4, two injections of follicle stimulating hormone (FSH), Folltropin-V® (20mg, Armidale, Australia) were administered at 12 h intervals initiated for 4 days on a decreasing dosage, plus two injections of prostaglandin 12 h apart on the last two days of Folltropin®. Then cows were inseminated twice (12 and 24 hours) after detection of standing oestrous with frozen/thawed semen from Nguni, Bonsmara and Boran bulls. Thereafter, embryo recovery was performed 7 days after AI, whereby an epidural anaesthesia was performed with a standard non-surgical technique to flush the uterine horns using a three way folley catheter. Retrieved embryos from the breeds studied were transferred into an embryo filter containing holding medium and evaluated using a stereo-microscope (Olympus SZ40, Olympus, Japan). Embryos were evaluated for embryo development (2–4 cells, 8-cell, Morula, Blastocyst).

Ovum pickup

Ovum pick up was performed as described by Petyim et al. (2000) with a minor modification. A total of 30 donor cows (10 Nguni' 10 Bonsmara and 10 Boran) were restrained in a crush pen then given an epidural injection on the head of the tail. Thereafter, the rectum was emptied and the vulva was cleaned thoroughly with 70% alcohol. Following cleaning, the transducer was advanced into the external of the cervix. Thereafter, ovaries were held through the rectum and positioned over the transducer face so that the targeted follicle is transected by the built in puncture line on the ultrasound monitor, which represented the projected needle path. When the targeted follicles were stabilized on the puncture line, the needle was inserted in the guide and advanced through the vaginal wall and into the follicle antrum. Follicular fluid was then aspirated using continuous negative pressure (about 95 mmHg) then transferred into the laboratory for oocytes searching under the stereo microscope.

Ovary aspiration

Ovaries were collected from a local abattoir, then transported to the laboratory in a pre-warmed (35°C) saline solution. Within two hours of collection, ovaries were washed twice with buffer saline for removal of excess blood contaminants. The oocytes were then retrieved by aspiration method using an 18 gauge hypodermic needle and 10ml syringe. Following aspiration, cumulus oocyte complexes (COCs) with three or more layers of cumulus cells were selected under a sterio microscope (Olimpus®) then washed three times in modified dulbecco phosphate buffered saline (mDPBS). Thereafter, oocytes were washed again three times in tissue culture medium (M199 + 10% FBS (foetus bovine serum) for preparation of *in vitro* maturation/fertilization/culture (IVMF).

IVMF and embryo production

In vitro maturation, fertilization and oocyte culture were carried out using procedures described by Huang et al. (2001) with slight modification. Briefly, the cumulus oocytes complexes (COCs) were matured for 24 h in TCM-199 (Gibco, Grand Island, NY) consisted of 10% FBS, 10 µg/ml Leutenizing Hormone, 1 µg/ml prostaglandin E_2 and 1 µg/ml FSH under humidified atmosphere of 5% CO_2 in air at 38.5°C. Following maturation, oocytes were fertilized in 70 µl drops of Bracket and Olifants Fertilization medium then co-incubated with frozen-thawed percoll gradient treated semen for 18 hrs at 38.5°C. After fertilization, presumptive zygotes were removed from IVF medium and the remaining cumulus cells were removed by vortex for a period of 1min 30sec. Presumptive zygotes were washed five times in 100 µl drops of synthetic oviductal fluid (SOF) supplemented with fatty acid free bovine serum albumin (BSA-FAF). Furthermore, presumptive zygotes were further cultured in synthetic oviductal fluid (SOF) supplemented with bovine serum albumin (BSA) and incubated at 38.5°C in 5% CO_2. The number of cleaved embryos was determined after 48 h of culture then further incubated until Day 8.

Embryo grading

Embryo grading was performed according to the methods of the international embryo transfer society, which is a “procedural guide and general information for the use of embryo transfer technology emphasizing sanitary procedures” (Stringfellow and givens, 2010). Briefly, Embryos were graded on different stages of development (2–4 cell, 8 cells, morula and blastocyst). Then, the blastocyst quality was classified as “Code 1, excellent or good; Code 2, fair; Code 3, poor; Code 4, dead or degenerating embryos.

Statistical analysis

Blastocyst formation data were analysed by ANOVA. Significant differences of the means were measured at 5% level. Means of the blastocyst formation were separated using fishers protected least significant difference (LSD) test. This test was only run if there was a significant difference following the ANOVA analysis.

Results

Embryo developmental stages

The developmental competency of embryos to blastocyst stage and expanded and hatched blastocysts produced *in vivo* did not differ significantly ($P > 0.05$) between the breeds (Table 1). However, on average, there were more expanded blastocysts in the Nguni breed and Boran breed *in vivo*. Blastocysts developed on day 8 *in vivo*, were significantly higher ($P < 0.05$) in Bonsmara and Nguni compared to Boran breed. No significant ($P < 0.05$) difference were observed between Bonsmara, Boran and Nguni on blastocyst stage under *in vitro* conditions (Table 1 and Fig. 1). After day 8, the Bonsmara breed revealed an average twice as much as blastocyst formation *in vitro* accompanied by slightly more hatched blastocysts ($P < 0.05$) than other breeds. The Nguni breed, showed slightly ($P < 0.05$) higher number of expanded blastocysts compared to those of other breeds.

Table 1
Developmental competency of *in vivo* and *in vitro* produced embryos in Bonsmara, Boran and Nguni cattle (Means ± SE)

Embryo Production	Breed	Blastocyst development (n)			
		Day 7	Day 8	Expanded	Hatched
In vivo	Bonsmara	13.0[a] ± 6.50	7.5[a] ± 1.91	7.8[a] ± 3.20	3.0[a] ± 1.82
	Boran	11.2[a] ± 8.26	4.5[b] ± 1.00	9.3[a] ± 8.80	2.3[a] ± 0.95
	Nguni	12.3[a] ± 6.89	7.5[a] ± 1.73	9.8[a] ± 5.41	2.2[a] ± 0.50
	P < 0.05	0.77	0.02	0.83	0.29
In vitro	Bonsmara	12.0[a] ± 7.06	6.5[a] ± 4.20	6.8[a] ± 6.23	1.8[a] ± 2.30
	Boran	10.8[a] ± 8.80	3.3[a] ± 1.51	7.8[a] ± 7.63	0.5[a] ± 1.00
	Nguni	14.0[a] ± 7.50	3.8[a] ± 1.25	9.5[a] ± 6.86	0.5[a] ± 0.57
	P < 0.05	0.76	0.58	0.63	0.55

[A,b]Values in the same column with different superscripts indicate significant differences between the treatment means (P < 0.05). *In vivo* (Flushed embryos) *in vitro* (OPU/Ovary aspiration) Blastocyst number (n).

Developmental competency of in vivo and in vitro produced embryos

Blastocyst quality was determined by visual assessment of embryo quality classification which is the subjective method. Blastocyst quality was classified as Grade I, II and III and is represented in Table 2, while the blastocyst quality (morphological grading) is presented in Fig. 2. There were no differences (P > 0.05) between the Bonsmara, Boran and Nguni cows in the number of blastocysts obtained following flushing (*In vivo*). All breeds showed, on average, a higher (P < 0.05) number of Grade I blastocysts *in vivo* than under *in vitro* conditions. Furthermore, the number of Grade II and Grade III blastocysts were significantly lower (P > 0.05) in all the breeds than those in grade I. Moreover, the number of Grade III embryos produced by *in vivo* method was significantly higher (P < 0.05) in Bonsmara cattle compared to Boranand Nguni cattle. There were no significant differences in the number of Grade I, Grade II and Grade

III blastocyst produced by the *in vitro* method. On average, all breeds produced higher (P < 0.05) number of Grade I embryos than Grade II or Grade III.

Table 2
Effect of breed on the quality of embryos produced *in vitro* and *in vivo* in Bonsmara, Boran and Nguni (Mean ± SE).

Method	Treatment		Morphological grading		
		Blastocyst (n)	Grade I	Grade II	Grade III
In vivo	Bonsmara	13.0 ± 6,48	9,8 ± 5,73	0,5 ± 0,57	$2,8^{a}$ ± 2,06
	Boran	11,3 ± 8,26	8,5 ± 8,50	1,5 ± 1,29	$0,0^{ab}$ ± 0,00
	Nguni	12,3 ± 6,89	8,5 ± 4,50	2,0 ± 2,44	$0,8^{b}$ ± 0,95
	P < 0,05	0,95	0,95	0,51	0,09
In vitro	Bonsmara	12,0 ± 7,07	4,5 ± 3,10	2,3 ± 0,95	2,5 ± 3,11
	Boran	10,8 ± 8,80	6,2 ± 5,79	3,0 ± 2,16	1,3 ± 1,50
	Nguni	14,0 ± 7,52	5,5 ± 2,64	4,8 ± 3,30	2,3 ± 1,70
	P < 0,05	0,85	0,83	0,41	0,64

^{A,b}Values in the same column with different superscripts indicate significant differences between the treatment means (P < 0.05). *In vivo* (Flushed embryos); *in vitro* (OPU/Ovary aspiration); Good (Grade I); Fair (Grade II); Poor (Grade III); Number (n)

Embryo flushing

Three different embryo retrieval methods on blastocyst formation and quality were also studied (Fig. 3). Significant (P < 0.05) differences in the number of embryos that developed to blastocyst were observed. Flushing and Ovum pickup method showed a significant (P < 0.05) higher number of blastocysts produced compared to ovary aspiration method, respectively. The number of morphologically good (Grade I) embryos were significantly high (P < 0.05) in both flushing and OPU compared to ovary aspiration. The number of Grade II embryos were significantly higher (P < 0.05) in embryos retrieved by the flushing method compared to that of aspiration but did not differ from those retrieved by OPU. In addition, flushing method produced less (P > 0.05) numbers of embryos compared to OPU and aspiration methods.

Discussion

Embryo evaluation is one of the critical phase that determine conception after embryo transfer. In our study, development of day 7 blastocyst to the hatching stage did not show any statistical difference ($P > 0.05$) in both the *in vivo* and *in vitro* methods. According to results obtained by Mahdavinezhad et al. (2019) the hatching rate of blastocyst produced *in vivo* was lower than their *in vitro* counterparts, which is in contrast with the results of this study. Even though a normal hatching process occurs in grade I (Excellent) the once developed *in vitro* still shows a hardened zona which negatively affect the hatching ability of the blastocyst (Velásquez et al., 2013). The effect of breed was evident in this study. For *in vivo* comparison between breeds, Nguni and Bonsmara resulted in higher ($P < 0.05$) number of day 8 blastocyst than the Boran breed, whereas, no difference ($p > 0.05$) were observed on the *in vitro* production system. Similar results where documented by Viana et al. (2010). Their study showed differences between breeds in embryo transcripts amount in *in vivo* production system and there were no differences observed in their *in vitro* counterparts. Although there was a lower variation in blastocyst development between the breed recorded in the current results, it is evident in the work of others that differences do exist in the reproductive performance between the breeds. However, there can be factors such as semen donor and embryo mortality during early development that also affect the ability of the embryo to develop and complete the gestation. (Abraham et al., 2012).

Blastocyst quality produced by different breeds was studied. No statistical ($p > 0.05$) difference in grade I – grade II embryos in all breeds studied. However, the Bonsmara breed resulted in higher ($p < 0.05$) rate of grade III embryos produced *in vivo* than Nguni breed. Incidences of obtaining higher rate of grade III embryos translate to less to zero chances of accomplishing pregnancy following transfer. In most cases, such embryos are discarded and are regarded as poor quality or abnormal embryos. This is agreement with other studies. Silva et al (2013) reported that embryo quality of Nellore (Bos Indicus) was better compared to Angus (Bos taurus) breeds. In our study, it did not really come as a surprise that the Bonsmara breed resulted in higher rate of fair quality (Grade III) embryos as it is a synthetic (Afrikanner x Herford) breed. It is suspected that heat stress might have been one of the factor contributing to the increased numbers of fair quality embryos in the later breed. Similarly, Satrapa et al., (2011) embryos of cross (Indicus x taurus) breeds which were found to be sensitive to heat than the bos indicus breeds.

Furthermore, significant ($P < 0.05$) differences were observed when three different oocytes retrieval methods were compared. In our study, blastocyst rate from OPU and flushing derived oocytes was significantly ($p < 0.05$) higher than those aspirated from abattoir ovaries. Oocytes retrieved from OPU and aspiration where handled and cultured in the same way. Prolonged ovary collection from the abattoir and temperature of the medium during transportation of ovaries could have contributed to poor oocytes development and subsequently affect blastocyst yield. Equally, Karadjole et al (2010) reported a higher proportion of blastocyst in OPU derived oocytes than the aspirated oocytes. In contrast, other studies reported a higher blastocyst rate from abattoir derived oocytes than the aspirated oocytes (Merton et al., 2008). The quality of oocytes is set to be lower in OPU due to factors such as the size of the needle and the vacuum pressure used during OPU. As a result, there is loss of cumulus cells that hinders oocytes

development to the blastocyst stage. Moreover, OPU and embryo flushing produced higher ($P < 0.05$) number of grade I and grade II blastocyst quality compared to Aspiration. A number of studies have reported a higher number of excellent-good quality embryos on OPU versus abattoir derived oocytes (Mapletoft et al 2015; Bó et al., 2019; de Oliveira Bezerra et al., 2019). Our study has however produced unsatisfactory results as there are still other factors such as heat, nutrition and hormonal treatments that affects the quality of embryos despite of the method used.

Conclusions

In conclusion, the study showed that the *in vivo* method of embryo production is superior to the *in vitro* method in blastocyst development of Bonsmara, Boran and Nguni breeds. Furthermore, there was no breed effect on embryo quality (grade I and grade II) in all breeds. Despite the increased numbers of grade III embryo produced by the Bonsmara, the breed still produce a considerable amount of morphologically good quality embryos within a pool of poorer ones. Based on the current findings, embryo production through *in vivo* embryo development and/or the utilization of ovum pickup technology could provide a fair opportunity to improve adapted beef cattle breeds and thereby increase beef cattle population to meet the population demand and income generation in poor rural populations. Therefore, these findings necessitates further research to improve embryo developmental rate as well as developing an adaptable method or protocol specifically for indigenous breeds.

Declarations

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethics approval

The experimental work was submitted and approved by the Agricultural Research Council-Animal Production ethics committee. Ref no. APIEC16/029

Consent to participate and consent for publication

All the authors have equally participated in this study and agreed to publish this work in this journal.

Data and material availability

The data are available from the corresponding author on request

Code availability

Not applicable

Author contribution

All authors designed the research work and prepared the manuscript daft; MHM designed the study, collected, analysed data and wrote the manuscript. CMP read, improved and approved the final manuscript. KM read, improved and approved the final manuscript. CBB read, improve and approved the final manuscript. JWN read improve and approved the final manuscript.

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Figures

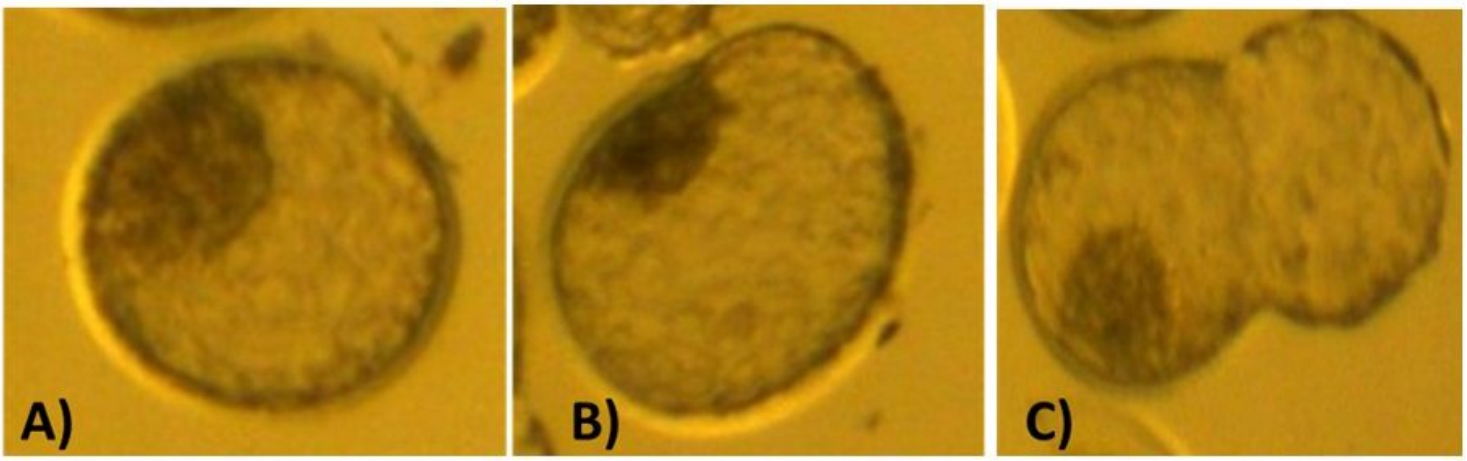

Figure 1

Embryo developmental stages: Blastocyst **(A),** Expanded blastocyst **(B)** and Hatched blastocyst **(C)**

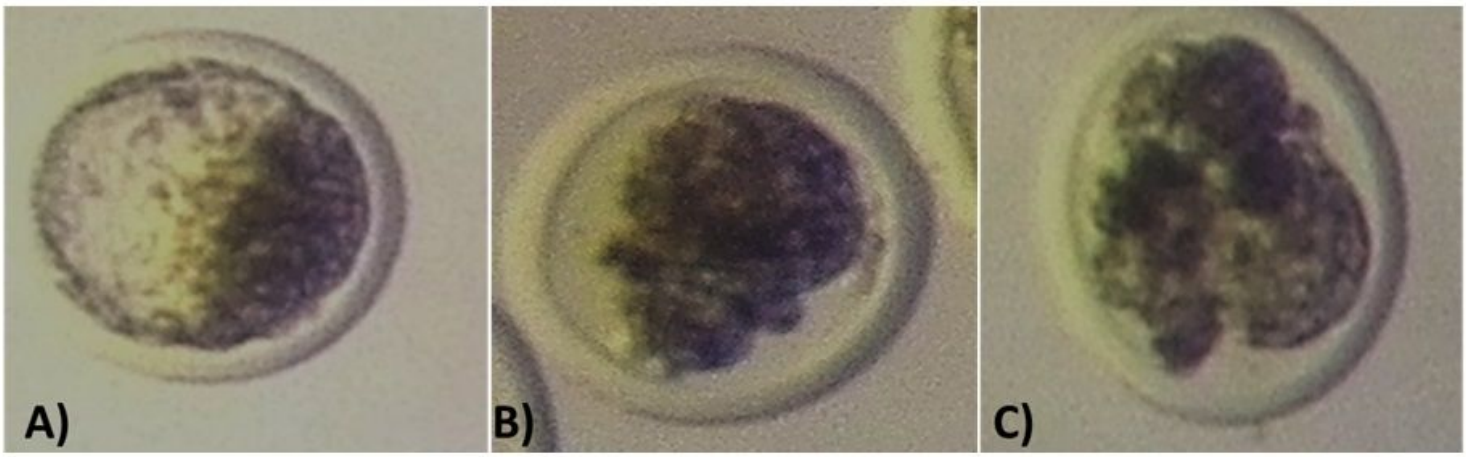

Figure 2

Embryo morphological grading Grade I Blastocyst (A), Grade II blastocyst (B) and Grade 3 blastocyst (C)

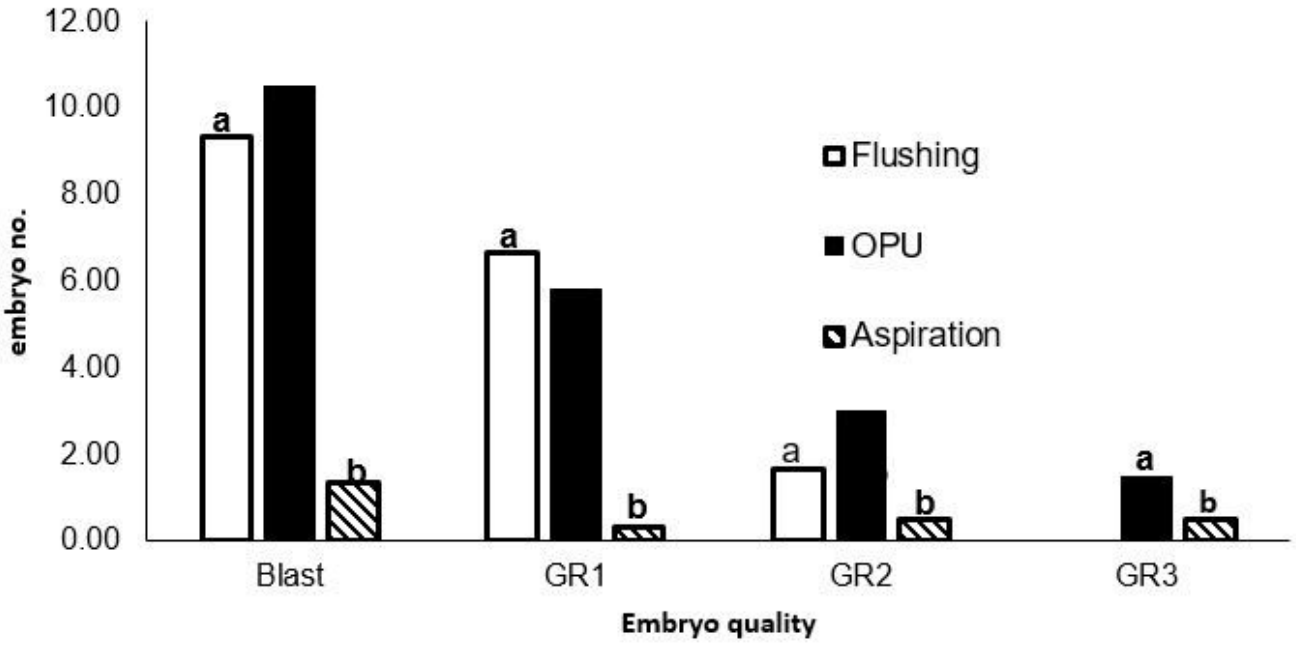

Figure 3

Effect of embryo flushing, ovum pick-up and aspiration of oocytes and embryo retrieval methods on embryo quality evaluated as Grade I (GR1), Grade II (GR2) and Grade III (GR3). The letters a and b represent significant differences between treatments ($P<0.05$)