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SEASONAL EFFECTS ON *IN VITRO* PRODUCED BOVINE EMBRYOS

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SUMMARY

The objective of this study was to examine the developmental ability of cattle oocytes after in vitro maturation (IVM) and fertilization (IVF) during two different seasons (spring and summer) .A total of 612 cumulus oocyte complexes (COCs) were isolated from bovine (cattle) ovaries collected at slaughterhouse. The oocytes were matured in vitro using TCM199 + 5mg/ml BSA supplemented with FSH, LH and E2 (1μl/ml medium) in 100 μl droplets under sterile paraffin oil in a humidified atmosphere of 5% CO_2 in air at 38.5° C for 24hrs . Sixteen-eighteen hrs after insemination with sperm prepared using the swim up method the oocytes were transferred into culture media of TCM199 +1mg /ml BSA and 1 μl/ml gentamycin under the same conditions of maturation for 8 days. Out of 287 oocytes collected during the spring, 269 (93.73%) were matured and of these 229 (85.13%) were fertilized. Meanwhile, during summer season out of 325 subjected to maturation, 252 (77.54%) were matured and from these 178 (70.63%) were fertilized. Significant differences (P<0.01) were found among the maturation and fertilization rates during the two different seasons. However, there was no significant difference between zygotes developed to morula (M) and blastocyst (Bl) stages in spring and summer (22.71 and 4.80 & 18.54 and 7.87 %, respectively) on day 8 post insemination .The results indicate that in vitro culture could be improve the developmental ability of in vitro matured and fertilized cattle oocytes through different seasons up to morula and blastocyst stages especially during the summer season in such a hot climates.

Keywords: *Bovine, oocytes, in vitro production, culture, development, season*

INTRODUCTION

Seasonality and heat stress may compromise oocyte quality, these can cause infertility, and some of this infertility may reflect damage to the developing oocytes. Indeed, there are two reports indicating that oocyte competence, as determined by developmental rate after in vitro fertilization (IVF) is lower in summer than in winter (Rocha *et al.*, 1998; Rutledge *et al.*, 1999). Al Katanani *et al.* (2002), suggested that oocyte competence in Holstein cattle located in a warm climate declines during summer. Such a result indicates that damage to the oocyte is one cause for reduced fertility during the summer in warm climates. Moreover, seasonal variation in oocyte competence can lead to reduced performance of procedures to produce *in vitro*-derived embryos by using oocytes recovered from slaughterhouse ovaries.

It is possible that heat stress can damage the oocyte during the period preceding antral follicle formation or that seasonal effects represent factors other than heat stress (Rocha *et al.*, 1998). Reduced developmental potential of oocytes after exposure to heat stress conditions may be due to alterations in nuclear or cytoplasmic maturation. Mouse oocytes exposed to maternal heat stress had a reduced ability to progress to metaphase II of meiosis (Baumgartner *et al.*, 1987).

There are several potential mechanisms by which heat stress could compromise oocytes. Heat stress has been reported to alter follicular development by reducing steroid hormone production (Wolfenson *et al.*, 1997; Wilson *et al.*, 1998) and these changes in follicular steroid concentration could disrupt oocyte growth. In addition, heat stress reduces growth of the dominant follicle (Badinga *et al.*, 1993) and causes incomplete dominance so that there is increased growth of subordinate follicles (Wolfenson *et al.*, 1995). Incomplete dominance could result in ovulation of an aged follicle; these follicles contain oocytes with reduced competence (Mihm *et al*; 1999).

The hyperthermia and heat stress in cows could also directly inhibit oocyte function. It can have deleterious effects on oocyte growth, protein synthesis or formation of transcripts required for subsequent embryonic development. In fact, cattle oocytes does not undergo increased synthesis of heat shock protein 70 in response to heat shock. Heat shock at 4°C during in vitro oocyte maturation decreased the ability of oocytes to become blastocysts following fertilization (Edwards and Hansen, 1997). Putney *et al.*, (1989) reported that heat stress of superovulated cows at the onset of estrus reduced fertility and subsequent embryonic development. The objective of the current study was to evaluate the effect of spring and summer on cattle oocyte competence for maturation, fertilization and the development of embryos *in vitro*.

MATERIALS AND METHODS

Oocyte collection and in vitro maturation (IVM):

Cattle ovaries were obtained from a slaughterhouse and transported to the laboratory at 24°C in transport medium (Dulbecco's phosphate buffered saline , D-PBS) within 3-5hrs .The ovaries were washed 3 times with 0.9%NaCl (saline) containing antibiotics (penicillin- G 400 IU/ml) and (streptomycin 500 µg/ml).

Cumulus oocyte complexes (COCs) were isolated from the follicles by slicing method as Echert and Neimann (1996) and placed into 35x10 mm petri dishes (Nunc, Roskilde, Denmark). The oocytes were recovered from the settled follicular fluid in dishes using a stereomicroscope. Only oocytes (Fig.1) with intact, compact cumulus oophorus and homogenous ooplasm were cultured. Selected oocytes were washed 3 times in CO_2 equilibrated washing medium (100µl droplets of TCM 199) (Sigma, St.Louis, MO, USA) +1mg/ml BSA Bovine serum albumin fraction V powder # A-3311, Sigma) under paraffin oil before the final in vitro maturation .The oocyte were assigned to 100µl droplets (10-20 oocytes/drop) of maturation medium TCM + 1mg/ml BSA supplemented with follicle stimulating hormone (FSH), leutinizing hormone (LH) and estradiol (1µl/ml medium) for maturation under sterile paraffin oil in a humidified atmosphere of 5% CO_2 in air at 38.5°C for 24hrs.

In vitro fertilization (IVF):

The media used for IVF were two modifications of tyrode's medium (Sp-TALP and Fert-TALP) as described by Parrish *et al.* (1988). The oocytes cultured for 24

hours were washed three times in CO_2-equilibrated Fert-TALP (100µl droplets) under sterile paraffin oil.

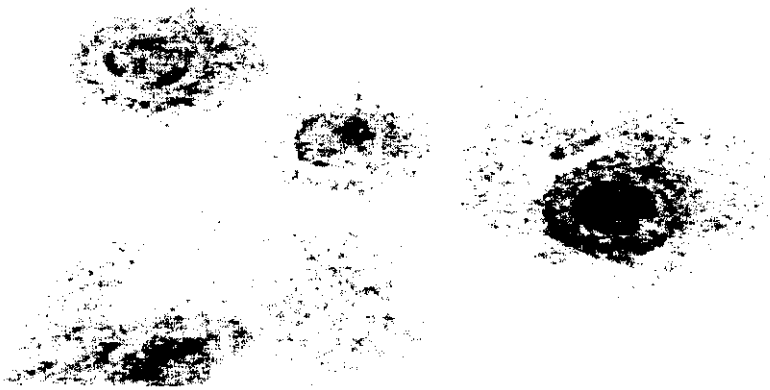

Figure 1. Bovine oocytes surrounded by compact cumulus cell complement subjected to maturation and fertilization in vitro

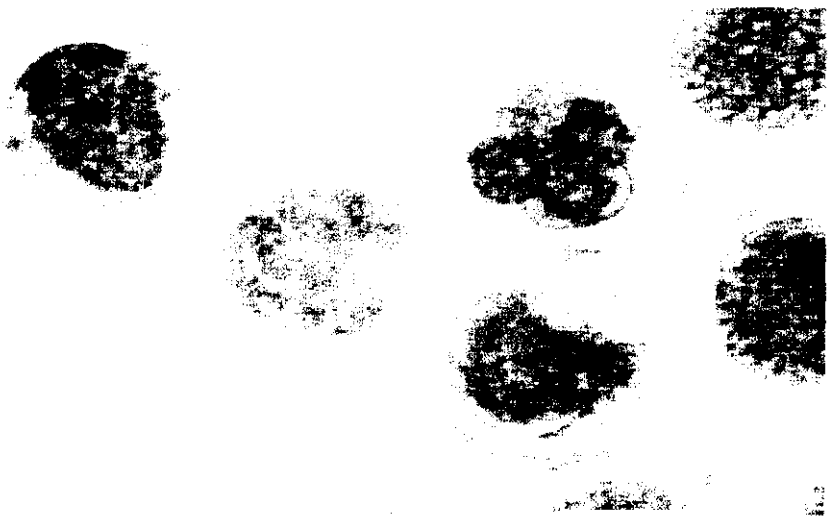

Figure 2. One, two, four and eight cell stages of bovine embryos cleaved on 24-48 hrs after IVF

Frozen cattle semen was used to fertilize the oocytes, 0.25 ml straws of frozen semen from a different bulls were thawed in water at 37° C for 1min and each straw was placed at the bottom of a 3ml tube with 1 ml of sperm-TALP, pH 7.2 containing BSA, Pyruvate, Gentamycin for swim-up separation of the motile fraction of semen. After 45 min of incubation (38.5°C, 5% CO_2 in air) the swim-up sperm fraction was aspirated, pulled, centrifuged (1200 rpm/10min) and the pellet was diluted in a fertilization medium (Fert-TALP containing BSA, Pyruvate and Gentamycin) at concentration of 1x106 sperm / ml, at the same time a mix of heparin (5.0µg/ml),

epinephrine (1 μM), penicillamine (20 μM) and hypotaurine (10 μM) was added .The conditions for the co-culture of sperm and oocytes were the same as those used for the maturation of the oocytes.

In vitro culture (IVC):

Sixteen-eighteen hrs after insemination, the fertilized oocytes (Fig.2) were washed and transferred into droplets (10-15/200μl drop) of TCM199+ 1mg/ml BSA and 1μl/ml gentamycin at 38.5°C in 5% CO_2 in air and cultured for 8 days .The embryos were scored for their development to morulae and blastocysts and examined by phase contrast microscopy and those showing compaction were classified as morulae (Fig.3) and those with a blastocole cavity were classified as blastocysts (Fig. 4).

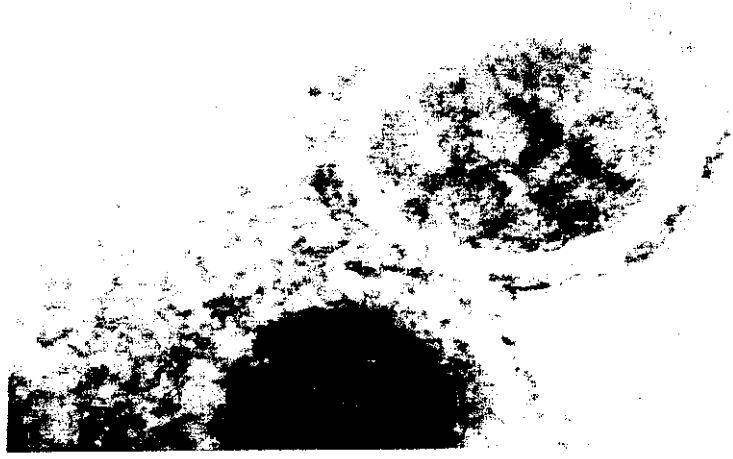

Figure 3. Early stage of morula on day 6 after IVF

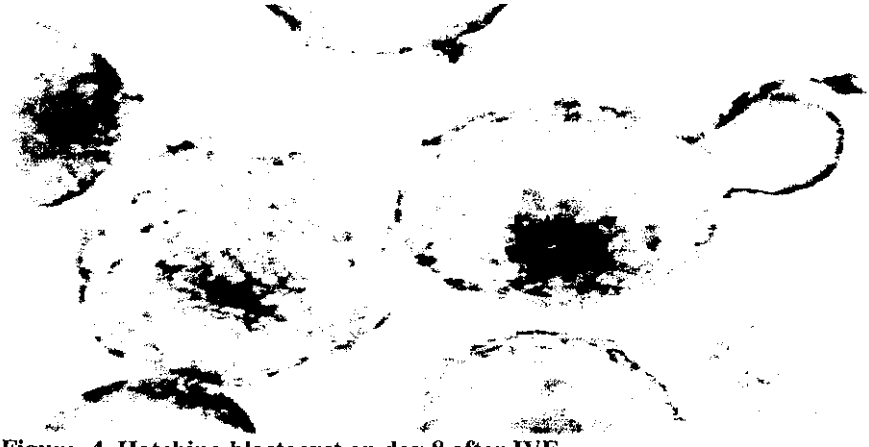

Figure 4. Hatching blastocyst on day 8 after IVF

Statistical analysis:

Data on the proportions of maturation, fertilization and developmental rate in vitro were calculated as a percentage in relation to the initial numbers of oocytes in each step before being analyzed by Z-test for the differences between two season (spring and summer) groups for each parameter tested. Five experiments (replicates) were conducted throughout each season. Each replicate represented oocytes collected on a particular day and subjected to maturation, fertilization and development *in vitro* up to hatching. Significance level was $^{**}P< 0.01$.

RESULTS AND DISCUSSION

Out of 60 ovaries (28 in spring and 32 in summer) sliced for oocyte recovery, 612 oocytes (Fig.1) were collected (287 and 325 for spring and summer, respectively) with average, 10 .25 and 10.16 of oocytes /ovary for spring and summer, respectively. That agrees with the result of Lonergan *et al.* (1992) and other scientists who found that the maximum number of oocytes obtained would be 10 oocytes. In the present study, a total of 287 oocytes subjected to maturation in vitro during spring, 269 were matured (93.73%). Meanwhile, out of 325 subjected to maturation during summer, 252 were matured (77.54%) (Table 1).

Significant difference ($P < 0.01$) was found between maturation *in vitro* during spring and summer seasons (Table 2), that coincide with Rocha *et al.* (1998) who reported that heat stress may compromise oocyte quality by damaging the developing oocyte and that is one cause for reduced fertility during the summer in warm climates. In addition, Putney *et al.* (1989), reported that heat stress reduced the developmental potential of oocytes possibly due to alterations in nuclear or cytoplasmic maturation. Given that heat stress has been reported to increase the number of small follicles (Trout *et al.*,1998), it is possible that oocytes recovered in summer came from follicles of average smaller size .The association between an oocyte's diameter and its ability to resume and complete meiotic division during in vitro maturation has been described in human and several domestic species including cattle, pigs, sheep and dogs .The results of Lechniak *et al.* (2002) study showed a correlation between oocyte size and the correctness of progress in meiotic maturation in vitro. Oocytes that follow a normal meiotic division were bigger than those showing various disturbances in their maturation processes it is mainly due to the failure of the first polar body extrusion (Plachot *et al.*, 1986). Similar chromosome configurations of the abnormal MI spreads (unpaired chromosomes, few bivalents) have also been observed by Ectors *et al.* (1995) in cattle oocytes. This may be one explanation for the previously reported findings on reduced developmental potential of embryos derived from small oocytes.

Table 1. Seasonal effect on bovine oocytes matured, fertilized and developed to morula or blastocyst (M / Bl) stages *in vitro*

SEASON	No. of exp.	No. of Ovaries	No. Of Oocytes	No. of Oocytes /Ovary	Oocytes Matured (%)	Oocytes Fertilized (%)	M (%)	BL (%)	TOTAL M/BL (%)
	1	4	28	7	27 (96.43)	24 (88.89)	7 (29.17)	1 (4.17)	8 (33.33)
	2	2	30	15	28 (93.33)	23 (82.14)	7 (30.43)	1 (4.35)	(34.78)
	3	4	47	11.75	40 (85.11)	30 (75.00)	9 (30.00)	1 (3.33)	(33.33)
Spring	4	8	112	14	108 (96.43)	93 (86.11)	18 (19.35)	4 (4.30)	22 (23.66)
	5	10	70	7	66 (94.26)	59 (89.39)	11 (18.64)	4 (6.78)	15 (25.42)
Total		**28**	**287**		**269 (93.73)**	**229 (85.13)**	**52 (22.71)**	**11 (4.80)**	**63 (27.51)**
	1	5	65	13	59 (90.77)	42 (71.19)	7 (16.67)	3 (7.14)	10 (23.81)
	2	6	64	10.67	57 (89.06)	40 (70.18)	6 (15.00)	3 (7.50)	9 (22.50)
	3	10	107	10.7	63 (58.88)	45 (71.43)	11 (24.44)	6 (13.33)	17 (37.78)
Summer	4	5	35	7	32 (91.43)	22 (68.75)	2 (9.09)	1 (4.55)	3 (13.64)
	5	6	54	9	41 (75.93)	29 (70.73)	7 (24.14)	1 (3.45)	8 (27.59)
Total		**32**	**325**		**252 (77.54)**	**178 (70.63)**	**33 (18.54)**	**14 (7.87)**	**47 (26.41)**

N.B. M = Morula Bl = Blastocyst

Table 2. Statistical differences between oocytes matured, fertilized and developed in vitro during two different seasons

PARAMETERS	SEASON					
	Spring		Summer			
	Number	%	Number	%	Value	Prob.
Oocytes matured	287	93.73	252	77.54	5.618	0.0000**
Ooctyes fertilized	269	85.13	178	70.63	3.999	0.0000**
Morula (M)	229	22.71	33	18.54	1.026	0.1524
Blastocyst (Bl)	229	4.80	14	7.87	1.276	0.1010
M / Bl	229	27.51	47	26.41	0.249	0.4016

** Significant (P<0.01) as determined by Z-test.

Significant difference (P<0.01, Table 2) was found among seasons concerning the fertilization rate, which was 85.13 and 70.63 % in spring and in summer, respectively (Table 1), that in agreement with Rocha *et al,* (1998) and Rutledge *et al.* (1999) who reported that oocyte competence, as determined by developmental rate after in vitro fertilization (IVF) is lower in summer than winter. Heat stress reduces growth of dominant follicle (Badinga *et al,.* 1993) and causes incomplete dominance so that there is increased growth of subordinate follicles (Wolfenson *et al.*,1995). Incomplete dominance could result in ovulation of aged follicles; such follicles contain oocytes with reduced competence for maturation and fertilization *in vitro*. Moreover, the study of Lechniak *et al.* (2002) revealed that after fertilization of the diploid oocytes (those showing various disturbances in their maturation process due to failure of the first polar body extrusion due to heat stress are significantly smaller than their haploid counterparts at the second metaphase stage MII) showed reduced developmental competence. These oocytes may be fertilized and give rise to triploid embryos that die before implantation. In human, diploid oocytes were occasionally penetrated but usually arrested early in development (Plachot *et al.,* 1986). Such cells displayed lower fertilizing ability (Nakaoka, *et al.,* 1998).

In contrast to maturation and fertilization ability in vitro the present study revealed that there was no difference in the developmental rate of embryos cleaved (Fig. 2) and developed up to morula and blastocyst stages during spring and summer (27.51 and 26.41%) (Table 1), which indicate that in vitro culture conditions could improve the developmental ability of cattle oocytes collected during different seasons and subjected to maturation, fertilization, and culture in vitro. In addition, the in vitro culture of fertilized oocytes may improve their ability for protein synthesis or formation of transcripts required for subsequent embryonic development where the ability of embryos themselves for transcription and protein synthesis start at the onset of fertilization (the transition from maternal control to embryonic expression itself). Hogan *et al.* (1986) reported that up to the mid-two cell stage (27 hrs post fertilization) the embryo appears to rely largely on protein and RNA synthesized during oogenesis. By the mid-two cell stage, many embryonic genes are switched on. Coincidentally much of the maternally inherited mRNA appears to be degraded rapidly. However, maternally coded proteins can persist beyond this time. In addition, Bensaude *et al.* (1983) determined the synthesis of 68 K and 70K heat-

shock proteins by G1 of 2-cell stage using the earliest expressed protein method. Moreover Bensaude and Morange (1983) detected the synthesis of 90K heat- shock protein at eight-cell and morula stages using the two-dimensional SDS polyacrylamide gel electrophoresis (PAGE) technique.

No difference also was seen in the developmental rate up to morula and blastocyst stages in the study of Rivera *et al.* (2002) by using oocytes recovered at a slaughterhouse throughout different seasons, the lack of seasonal variation may reflect a preponderance of cows that have superior thermoregulatory mechanisms (Finch, 1986). In a similar manner, no seasonal variation in IVF performance and subsequent embryonic development was found by Rocha *et al.* (1998). That's another possible explanation for the improvement of the developmental rate up to morula and blastocyst stages of IVM, IVF cattle oocytes in a hot climates.

Another factor affecting the development of embryos is that the addition of hormones (FSH or LH with E2) for in vitro maturation improved in vitro development of cattle embryos after IVF (Younis *et al.*, 1989). That agreed with our findings where addition of 1µl/ml medium of FSH, LH and estradiol for in vitro maturation improved the development of cattle embryos up to hatching. Similar findings has been reported by Fukui *et al.* (1982) and Fukushima and Fukui (1985).

In conclusion, the results indicate that oocyte competence for maturation and fertilization in vitro decline in summer than in spring season, which may due to heat stress leading to alterations in nuclear or cytoplasmic maturation. Another reason for low maturation and fertilization ability may refer to that small oocytes produced under heat stress conditions showing various disturbances in their maturation processes, it's possible also that heat stress can damage the oocytes during the period preceding antral follicle formation or that seasonal effects represent factors other than heat stress. Meanwhile, in vitro culture of IVM and IVF cattle oocytes recovered from slaughterhouse ovaries during summer season under the laboratory conditions, improved the developmental competence of the obtained embryos up to hatching.

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تأثير المواسم المختلفة على نمو وتطور أجنة الأبقار المنتجة معملياً

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تهدف هذه الدراسة إلى قياس قدرة بويضات الأبقار المأخوذة من مبايض الأبقار المذبوحة على النمو والحصول على أجنة (في مراحل الموريولا والبلاستوسيست) وذلك بعد إنضاجها وإخصابها تحت الظروف المعملية خلال موسمين مختلفين من السنة (الربيع والصيف).

استخدم في هذه الدراسة عدد ٦١٢ (٢٨٧ بويضة خلال فصل الربيع + ٣٢٥ خلال فصل الصيف) من بويضات الأبقار المأخوذة من مبايض الأبقار المذبوحة. هذه البويضات تم إنضاجها معملياً باستخدام البيئة الغذائية TCM199 مضافاً إليها البيومين السيرم البقري بمعدل ٥ ملجم/ مل وهرمونات الجونادوتروفين (FSH,LH and E2) بمعدل ١ ميكروليتر/مل من البيئة الغذائية وذلك تحت ظروف الإنضاج المعملي (5% CO2) عند درجة حرارة ٣٨,٥°م لمدة ٢٤ ساعة).

بعد ذلك تم إخصاب البويضات معملياً باستخدام أسْبرمات الأبقار المجهزة بطريقه (Swim up) وذلك لمدة ١٦-١٨ ساعة ، ثمَّ تم زراعة البويضات المخصبة معملياً لمدة ٨ أيام .

أظهرت النتائج المتحصل عليها أن من بين ٢٨٧ بويضة جمعت خلال فصل الربيع، ٢٦٩ (٩٣,٧٣%) نضجت معملياً وأن ٢٢٩ بويضة منهم (٨٥,١٣%) أخصبت معملياً بينما نجد أن من بين ٣٢٥ بويضة خضعت للإنضاج المعملي خلال فصل الصيف ٢٥٢ (٧٧,٥٤%) تم إنضاجها ومنهم ١٧٨ (٧٠,٦٣%) تم إخصابها معملياً.

أوضحت التحليلات الإحصائية وجود فروق معنوية بين القيم المتحصل عليها والتي تخص الإنضاج والإخصاب المعملي خلال فصلي الربيع والصيف حيث كانت القيم أعلي خلال فصل الربيع عنها خلال فصل الصيف بينما لا توجد فروق معنوية في القيم المتحصل عليها في معدل نمو الأجنة وتطورها إلى مراحل الموريويلا والبلاستوسيست حيث كانت نسبتها هي ٢٧,٥١%، ٢٦,٤١% خلال فصل الربيع والصيف على التوالي.

مما سبق يتضح أن بويضات الأبقار المنضجة والمخصبة معملياً خلال فصل الصيف قد تخطت مشكلة الانخفاض في معدل الإنضاج والإخصاب في المعمل وذلك بعد زراعتها معملياً مما أدى إلى التحسن في قدره الأجنة المخصبة على النمو وصولاً إلى مراحل الموريويلا والبلاستوسيست كغيرها التي تم إنضاجها وإخصابها في فصل الربيع وذلك يعزى إلى ضبط ظروف زراعة هذه الأجنة معملياً.