EFFECTS OF CRlaa AND TCM-199 MEDIA ON IN-VITRO EMBRYONIC DEVELOPMENT UNTIL BLASTOCYST STAGE IN CATTLE*

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ÖZET

Sığırlarda Crlaa ve TCM-199 vasatlarının blastosist aşamasına kadarki in-vitro embriyonik gelişim üzerine etkileri.

Sığır oositlerinin in-vitro maturasyonu ve fertilizasyonu (IVM / IVF) araştırma ya da transfer için çok sayıda embriyonun üretilmesi açısından önemli tekniklerdir. Bu metotların kullanılmasıyla, gelişimlerinin farklı dönemlerinde olan embriyolar elde edilebilir. Ancak, blastosist verimi şu an mevcut olan tekniklerle hala düşük düzeydedir. Çünkü, bu tekniklerin verimliliği henüz yeterli değildir, kültür için gelişme şartları iyi bir şekilde anlaşılamamıştır ve sığır embriyolarının in-vitro şartlarda, blastosist aşamasına kadar gelişmelerini sağlayacak gereksinimleri hakkında daha çok bilgi için birçok çalışma sürdürülmektedir. Bu çalışmanın amacı, sığır embriyoları için en yaygın biçimde kullanılan kültür vasatlarından, CRlaa ve TCM-199 vasatlarının IVM / IVF yoluyla elde edilmiş sığır embriyolarının, 1-hücre aşamasından blastosist aşamasına kadarki gelişimleri üzerindeki etkilerinin karşılaştırılmasıdır. CRlaa serum içermeyen basit bir vasattır. Fakat 4. günde % 10 oranında fötal buzağı serumu (FCS) vasat içerisine eklenmektedir.

Fertilizasyondan sonra, embriyolar 1. günden 9. güne kadar kontrol edildiler ve her gelişme dönemindeki yüzdelik oranları kaydedildi. CRlaa vasatı ile TCM-199 vasatı karşılaştırıldığında; sonuç, embriyoların CRlaa vasatı içerisinde daha yüksek bir oranda morula ve blastosiste gelişme yeteneğine sahip olduklarını göstermiştir. Buna rağmen, morula ve blastosiste gelişme oranı (%25) hala düşüktür. Verimin böylesine düşük olmasının nedenlerinin açıklanması ve sığır embriyolarının gelişimi için optimum kültür vasatının bulunması için daha çok araştırma yapılması gerekmektedir.

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Anahtar Kelimeler: Sığır, in-vitro maturasyon, in-vitro fertilizasyon, in-vitro kültür, CR1aa, TCM-199.

SUMMARY

In-vitro maturation and fertilization (IVM / IVF) of bovine oocytes are important techniques used for mass-production of embryos either for research or transfer. Using these methods, embryos at different developmental stage can be obtained. However, the blastocyst yield is still low while using the current available techniques because the efficiency of these techniques are not yet optimal. Developmental conditions for culture have not been well established, and a lot of research is under way to learn more about the requirements of bovine embryos to develop to the blastocyst stage in-vitro. The objective of this study was to compare development of IVM / IVF embryos from the 1-cell stage to the blastocyst stage by using two different media (CRlaa and TCM-199), the most commonly used embryo culture media for bovine embryos. CRlaa is a simple serum-free medium, while 10 % fetal calf serum (FCS) is added to CRlaa on day 4.

Embryos were examined once a day from day 1 up to day 9 after fertilization, and the percentage of each developmental stage was recorded. The results showed that embryos have a greater ability to develop to the morula and blastocyst stage when CRlaa-medium is used and compared to TCM-199. However, the overall percentage of development to morula and blastocyst was still low (25%). More research is nedeed to explain why the yield is so low, and to find the optimal culture medium for development of bovine embryos.

Key Words: Bovine, in-vitro maturation, in-vitro fertilization, in-vitro culture, CR1aa, TCM-199.

INTRODUCTION

In-vitro maturation and fertilization (IVM / IVF) and in-vitro embryonic development have evolved from being simply research objectives to being useful and important tools used by laboratories worldwide. During the past decade, many live calves have been obtained in cattle. The first reported bovine pregnancy, resulting in the first IVF calf called Virgil, was produced in the laboratory of First[1], where this study was done. These techniques offer many practical aspects such as treatment of infertility, mass production of embryos for both commercial and research purposes, and overcoming oviductal obstruction, endometrial diseases, infundibular adhesions, and non-functional cervix[2]. This system does not only apply to bovine embryos, but to other domesticated species, endangered species, primates and even human[3]. These techniques were also of great impact on development of other techniques such as gene injection into pronuclear eggs, resulting in the production of transgenic animals, cloning of embryos or animals. Finally, biological events such as the cell cycle, meiosis control, follicular or sperm maturation, syngamy, early embryonic development and interaction with maternal tissues in-vitro can now be studied easily by using this system[4].

For oocytes to be fertilized in-vitro, however, they have to be able to develop competently in order to produce viable embryos. Development competence is defined as the ability of a fertilized oocyte to undergo complete development to term, although it is also equated to ability to form advanced stage of preimplantation embryos[1]. The culture system must mimic the final events of oogenesis that occur in the oocyte during the preovulatory period in selected follicules[3]. In the past 10 years or so, much progress has been made in this area, and it is astonishing to see production of viable embryos when there is such little understanding of the events that take place in-vivo. However, the number of embryos that develop to the morula and blastocyst stages are still low. Inadequate culture systems are responsible for this low yield. Bovine embryos cultured in-vitro to the one-two-or four-cell stages rarely develop past the 9- to 16-cell stage. This phenomenon is known as "the developmental block", and it occurs in late 4-8 cell stage embryos[5].

Acquiring competent oocytes and preimplantation embryos to the required developmental stage in plentiful numbers at a commercially acceptable price can only be achieved if a developmental medium can be made that contains all the necessary ingredients. This study was designed to compare the effects of two different developmental media, namely CRlaa and TCM-199 on embryos over a 9 day period, ie development from the 1-cell to the blastocyst stage.

MATERIALS and METHODS

Ovaries were obtained from the slaughterhouse and transported in saline (0.9 % NaCl; 30 ± 2°C). They were then washed with tap water at 30°C. Aspiration of the follicular contents was then carried out using a stereomicroscope in 100 mm plates in a warm room (30 ± 2°C). Oocytes were first washed 3 times in TL-HEPES[6], and then placed in maturation plates (10 oocytes / 50 µl drop TCM-199 medium under mineral oil).

A Percoll gradient was used to separate motile sperm. Two ml of 90 % Percoll was pipetted to the bottom of a 15 ml conical tube, and 2 ml of 45 % Percoll were carefully placed on top. Frozen sperm was first thawed at 35°C for 1 minute, and then layered onto the Percoll gradient. The tube containing the Percoll was then centrifuged at 700 X g for 15 minutes at room temperature. The pellet containing live sperm was recovered, and sperm concentration was determined using a hemocytometer. Sperm was then diluted to 50 X 10^6 spermatozoits / ml in BGMI sperm medium[7]. Fertilization of oocytes was carried out as described by Parrish et al., 1988[8]. Briefly, after 20-24 hours incubation in maturation media at 39°C and 5 % CO_2, the oocytes were washed twice in TL-HEPES and then transferred to 44 µl fertilization drops under mineral oil. Ten oocytes were added to each drop. Fertilization medium is modified Tyrode's based medium containing 0.2 mM Na-pyruvate, 6 mg/ml fatty acid free-bovine serum albumin (BSA-FAF) and 25 µg/ml gentamycin. Oocytes were cultured until the addition of spermatozoa. Then, when sperm is ready, 2 µl of sperm suspension was added into each fertilization drop at a final concentration of 1.0 X 10^6 sperm cells/ml, 2 µl of PHE

(20 μM penicillamine, 10 μM hypotaurine and I μM epinephrine) and 2 μl of 2 μg/ml heparin were added. Oocytes and sperm were co-cultured for 24 hours[8].

At 24 hours after insemination, the cumulus cells were removed by placing the presumptive zygotes in an 1.5 ml eppendorf tube, and vortexing at high speed for 3 minutes. Embryos were then randomly divided into 2 groups. The first group was placed in 50 μl CRlaa developmental medium[9,10], while the second group was placed in TCM-199 developmental medium, which contained 10% FCS. At this time, CRlaa did not contain FCS, but it was added on day 4. Fertilization day was considered as day 0. After transfer to each medium, embryos were counted each day thereafter and classified to their cleavage stages.

The experiment was replicated three times, and each time approximately 200 oocytes were used. Table I shows the numbers of oocytes and presumptive zygotes used for each trial. During vortexing procedure, some presumptive zygotes were lost.

Table: I

Numbers of Oocytes and Presumptive Zygotes Used For Each Trial

	CRlaa		TCM-199	
	Number of matured and fertilized oocytes	Number of presumptive zygotes after vortexing	Number of matured and fertilized oocytes	Number of presumptive zygotes after vortexing
1. trial	100	95	100	98
2. trial	110	102	110	105
3. trial	105	100	105	97
Total	315	297	315	300

RESULTS

The objective of this study was to compare the developmental ability of embryos in two different media, CRlaa vs TCM-199. As shown in Figure I and Table II, embryos developed in CRlaa had a greater ability to develop to different stages, beginning with cleavage to the 4-cell stage up to the blastocyst stage. The fertilization percentage was approximately 75 % and cleavage to 2-cell stage was comparable between CRlaa and TCM-199 in this study (The percentages of 2-cell stage embryos are 17.50 %, 10.43 %, and 7.43 % in CRlaa and 28.00 %, 18.66 %, and 20.66 % in TCM-199 on days 5,7, and 9, respectively). Development to 8-16-cell and morula-blastocyst stages was higher for CRlaa medium when compared to TCM-199 (The percentages of 8-16-cell stage embryos are 47.47 %, 35.01 %, and 24.66 % in CR1aa and 20.00 %, 26.00 %, and 20.66 % in TCM-199 on days 5,7, and 9, respectively; and the percentages of morula-blastocyst stage embryos are 13.80 %, and 26.35 % in CRlaa and 3.33 %, and 8.66 % in TCM-199 on days 7, and 9, respectively. There was no development to morula-blastocyst stage in both media on day 5). Figure I shows a graph of the average data obtained from this study. Table II shows the numbers and percentages of embryonic stages for each trial and the average data obtained from all trials.

Tablo: II

The numbers, and percentages of embryos for each trial according to developmental stages and average numbers and percentages of embryos obtained from all trials according to the developmental stages

Embryonic Stages	CRlaa (day 5)				TCM-199 (day 5)			
	1.trial	2.trial	3.trial	total	1.trial	2.trial	3.trial	total
Unfertilized	25 (26.3%)	27 (26.4%)	26 (26.4%)	78 (26.26%)	25 (25.5%)	25 (23.8%)	22 (22.6%)	72 (24.00%)
2-cell	16 (16.8%)	19 (18.6%)	17 (17.0%)	52 (17.50%)	28 (28.5%)	29 (27.6%)	27 (27.8%)	84 (28.00%)
4-cell	9 (9.4%)	8 (7.8%)	9 (9.0%)	26 (8.75%)	26 (26.5%)	30 (28.5%)	28 (28.8%)	84 (28.00%)
8-16-cell	45 (47.3%)	48 (47.0%)	48 (48.0%)	141 (47.47%)	19 (19.3%)	21 (20.0%)	20 (20.6%)	60 (20.00%)
Morula-blastocyst	0	0	0	0	0	0	0	0
Total	95	102	100	297	98	105	97	300

Embryonic Stages	CRlaa (day 7)				TCM-199 (day 7)			
	1.trial	2.trial	3.trial	total	1.trial	2.trial	3.trial	total
Unfertilized	23 (24.2%)	26 (25.4%)	25 (25.4%)	74 (24.91%)	23 (23.4%)	24 (22.8%)	20 (20.6%)	67 (22.33%)
2-cell	10 (10.5%)	11 (10.7%)	11 (10.7%)	31 (10.43%)	19 (19.3%)	20 (19.0%)	17 (17.5%)	56 (18.66%)
4-cell	15 (15.7%)	16 (15.6%)	16 (15.6%)	47 (15.82%)	28 (28.5%)	31 (29.5%)	30 (30.9%)	89 (29.66%)
8-16-cell	33 (34.7%)	36 (35.2%)	36 (35.2%)	104 (35.01%)	25 (25.5%)	27 (25.7%)	26 (26.8%)	78 (26.00%)
Morula-blastocyst	14 (14.7%)	13 (12.7%)	13 (12.7%)	41 (13.80%)	3 (3.0%)	3 (2.8%)	4 (4.1%)	10 (3.33%)
Total	95	102	100	297	98	105	97	300

Embryonic Stages	CRlaa (day 9)				TCM-199 (day 9)			
	1.trial	2.trial	3.trial*	total	1.trial	2.trial	3.trial	total
Unfertilized	25 (26.3%)	27 (26.4%)	26 (26.2%)	78 (26.35%)	24 (24.4%)	26 (24.7%)	25 (25.7%)	75 (25.00%)
2-cell	7 (7.3%)	8 (7.8%)	7 (7.0%)	22 (7.43%)	21 (21.4%)	22 (20.9%)	19 (19.5%)	62 (20.66%)
4-cell	14 (14.7%)	15 (14.7%)	16 (16.1%)	45 (15.20%)	25 (25.5%)	26 (24.7%)	24 (24.7%)	75 (25.00%)
8-16-cell	23 (24.2%)	24 (23.5%)	26 (26.2%)	73 (24.66%)	21 (21.4%)	21 (20.0%)	20 (20.6%)	62 (20.66%)
Morula-blastocyst	26 (27.3%)	28 (27.4%)	24 (24.2%)	78 (26.35%)	7 (7.1%)	10 (9.5%)	9 (9.2%)	26 (8.66%)
Total	95	102	99	296	98	105	97	300

* One embryo was lost in the third trial

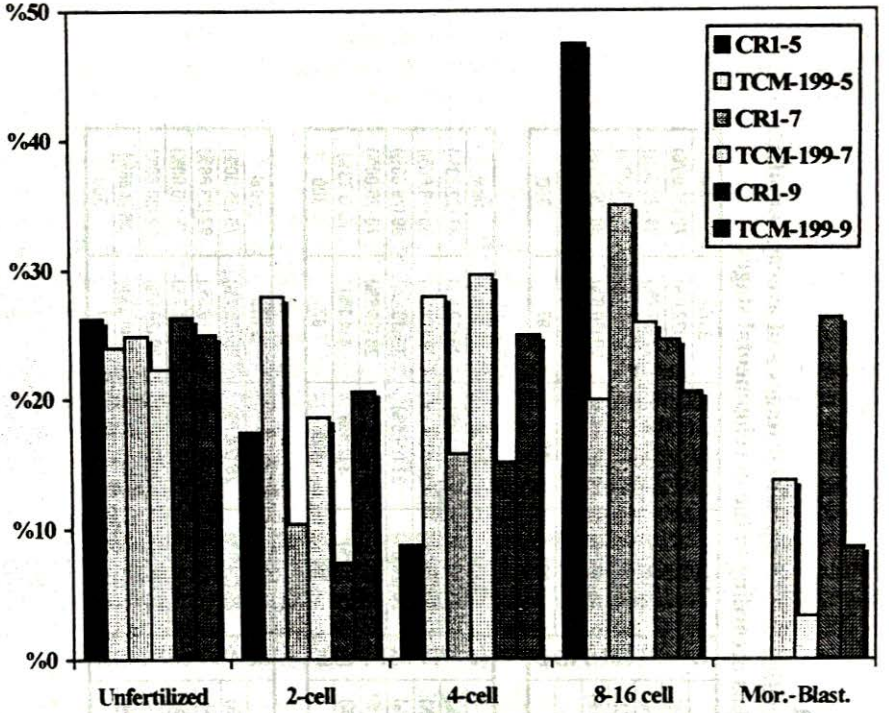

Figure: 1

A Graph of Data Showing Percentage of Embryonic Stages Obtained From Days 5, 7 and 9.

DISCUSSION

The objective of this trial was to compare the blastocyst formation capability between CRlaa and TCM-199 media. This study suggested that the CRlaa medium has a higher rate of blastocyst formation, suggesting that CR1aa has a greater capacity for embryo growth and survival.

The purpose of an in-vitro medium is to supply the fertilized oocyte an ideal environment in which it can develop to the blastocyst stage. The "perfect" medium would have to contain all the necessary nutrients, sufficent hormones, and the right rate of gaseous exchange to support cleavage of the oocyte.

The compositions of early culture media were based on serum with pyruvate, lactate, glucose, and antibiotics to retard bacterial growth[11]. The early media, however, led to inconsistent results. This is possibly due to the vast biochemical control within a developing embryo. Growth factors and fluctuating hormone levels must also be considered beyond basic embryo nourishment. There is also speculation that cleavage produces metabolic changes within the embryo that could probably be detrimental to an embryo developing in-vitro. A single medium may simply be insufficient to meet all the changing requirements within an embryo,

despite the fact that a medium could be optimal for a cell through the 4-cell stage. This theory is supported by Brison and Leese's research[12] in 1991. They found that early embryos (one to eight-cell) use pyruvate for metabolic needs, while the 16-cell embryos use glucose for cellular fuel. While early media contain both glucose and pyruvate, there are many recent studies that suggest that glucose can be detrimental to early embryonic development[11].

Another obstacle in media research is the "developmental block" which apparently occurs at various early cleavage times within different species[11]. Early cleavage is dependent on proteins and mRNA stored within the oocyte. In early in-vitro trials (in the 1960's), it was conventional that embryos cultured from primary oocytes could rarely be expected to progress beyond the 8-cell stage. However, embryos that were cultured from a 16-cell stage had an excellent chance of being matured to compaction and beyond. It is now known that this was due to the developmental cell block. The block consists of a drastic change in embryo protein synthesis which is the inner signal that ends maternal control of cleavage and begins embryonic control of growth. In the bovine embryo, the switch from embryological control to maternal control has been shown to occur in the late 4-cell to early 8-cell stage. At this stage, a large number of embryos exhibit an inability to adjust to media control of maturation, and remain "blocked" at the 8-cell stage. Once this block has been initiated, the embryo cannot be salvaged and dies[11].

Several compounds and compositions have been tested to promote development past cell blockage. Barnes and Eyestone's research[13] in 1990 suggested that a large factor in the developmental block was due to inadequate energy metabolism at the time of the 4 to 8-cell cleavege, and that glucose was a major inhibitory compound. In 1989, Chatot et al.[14] showed that in mouse embryos, this problem could be overcome by glucose-free media. Other factors that may decrease blastocyst formation rates are physical factors besides media. These include light exposure, temperature, air quality and composition, and general handling of the embryo[11].

This trial suggested that CRlaa exhibited a greater capacity over TCM-199 to carry a fertilized oocyte to the 16-cell stage as seen on day 5, normal development time for 16-cell stage, and upper stages as well. In order to determine why CRlaa is a more capable medium, we must compare the components of both media and consider embryological differences (that are beyond the control) that may be responsible for a certain oocyte's capacity for in-vitro development.

CRlaa contains the basic Minimum Essential Medium, which is a blend of amino acids, vitamins, inorganic salts, ribonucleosides, and deoxyribonucleosides. Another major component of CRlaa is the Basal Medium Eagle, which is a supplemental mixture of additional amino acids, vitamins, and inorganic salts. CRlaa also contains NaCl, KCl, $NaHCO_3$, Na-pyruvate (embryological "food"), glutamine (a more desirable morula "food" than glucose), and gentamycin (for bacterial growth inhibition)[9,10].

TCM-199, while providing a variety of amino acids and vitamins, may actually contain some additives which may prove detrimental to an embryo. Hypoxantine is present in TCM-199 at levels of 6-30 µl/ml. It has been proven to be a significant factor in encouragement of blocking development[11]. However, this

effect can be minimized if some cumulus cells are allowed to remain attached to the fertilized oocyte. It was also shown that a much higher percentage of zygotes progressed past the 8-cell stage in TCM-199 when the embryos were not stripped of all cumulus cells[11].

Also, the standard TCM-199 is supplemented with bovine blood serum and pyruvate to serve as metabolic fuel for the embryo.

When compared to CRlaa, it is apparent that TCM-199 has a smaller number of components. The Tech-line culture reference catalog of 1994 lists the base components of TCM-199, called Medium 199, as containing 23 amino acids and 17 vitamins. It is not listed as the Minimum Essential Medium, a major component of CRlaa, which has a mixture of 28 amino acids, 12 vitamins, but also contains ribonucleosides and deoxyribonucleosides. While we cannot accredit CRlaa's greater capacity to the number of various amino acids or vitamins, it could be suggested that the ribonucleosides and deoxyribonucleosides play an important role in aiding cleavage.

This trial could have been affected by several factors. First, the oocytes were aspirated, washed, and generally handled for a more extended period of time than was probably ideal. There is a possibility that during washing, the primary oocytes were exposed to heat, light, and physical contact due to technical inexperience at that time.

In conclusion, it is a formidable task to attempt to optimize a medium that will support early life outside the womb. We are removing many biochemical reactions typical of an animal in early pregnancy and trying to compensate for these reactions with a simple medium.

Obviously, improvements in media have been made, but still a medium is required that will provide consistent results for all cattle. Oocytes undergo many changes. Thus, when we prepare a medium, we have to consider these factors. Manipulations of amino acids, vitamins, hormones, and sera could be used to further improve cell cleavage. However, perhaps media should not be the only thing improved. Oocyte handling techniques, sperm preparation, and growth environments should be explored too.

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