UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS RURAIS
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA VETERINÁRIA

ÁCIDO ASCÓRBICO NA PRODUÇÃO *IN VITRO* DE EMBRIÕES BOVINOS

TESE DE DOUTORADO

Sandra Elisa Pozzobon

Santa Maria, RS, Brasil

2008

ÁCIDO ASCÓRBICO NA PRODUÇÃO *IN VITRO* DE EMBRIÕES BOVINOS

por

Sandra Elisa Pozzobon

Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Medicina Veterinária, Área de Concentração em Fisiopatologia da Reprodução, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Doutor em Medicina Veterinária**.

Orientador: Profª. Mara Iolanda Batistella Rubin

Santa Maria, RS, Brasil

2008

Universidade Federal de Santa Maria
Centro de Ciências Rurais
Programa de Pós-Graduação em Medicina Veterinária

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ÁCIDO ASCÓRBICO NA PRODUÇÃO *IN VITRO* DE EMBRIÕES BOVINOS

elaborada por
Sandra Elisa Pozzobon

como requisito parcial para obtenção do grau de

Doutor em Medicina Veterinária

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Santa Maria, 17 de novembro de 2008.

DEDICO

À minha família:
meus pais Sadi e Eudes,
meus irmãos Misso, Jairo e Silvia,
pelo apoio, carinho e exemplo.
Ao Quinho,
pela paciência, dedicação e amor.

AGRADECIMENTOS

Agradeço à Deus, fonte de toda sabedoria, por estar sempre presente.

À minha família de coração: Paulo, Silvia, Guto, Lorenzo e Ana, pelo incentivo constante, confiança e otimismo. Aos meus sobrinhos, João Paulo e Ana Luisa, pela alegria e energia que transmitem, à vó Cecília e aos cunhados Rafael e Tanizia, pelo apoio.

Aos meus orientadores, Dra. Mara Iolanda Batistella Rubin e Dr. Carlos Antonio Mondino Silva, pela orientação, amizade e oportunidades.

À Dra. Mari Lourdes Bernardi, pelas valiosas contribuições e auxílio incondicional nesta pesquisa. Ao Prof. José Henrique Souza da Silva pelas análises estatísticas e atenção dispensada.

Aos professores Dr. Flávio D. De La Corte e Dra. Karin Erica Brass, pelos ensinamentos ao longo do curso.

À Dra. Dominguita L. Graça, Dra. Agueda P. C. de Vargas, Dr. Claudio S. L. de Barros, pelo exemplo profissional, conselhos de amizade e carinho com que sempre me receberam.

Aos amigos de longa data, Dr. Fábio Gallas. Leivas e Dra. Daniela Brum pela amizade e ensinamentos.

Ao amigo Amauri Reis, pelo incentivo e apoio nesta etapa.

Aos grandes amigos: Fabrício, Luiz Fernando, Rodrigo, Ricardo, Carol, Vivian, Andressa e Jordana, pelos ensinamentos e companheirismo. Aos colegas do Embryolab, em especial o Gilson, Juliano, Robinson, Stéfano, Ricardo, Daiane e Mariana, pela colaboração direta durante a realização deste trabalho e agradável convivência.

À Universidade Federal de Santa Maria e ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Instituições públicas de qualidade e de excelência na formação profissional.

À Pecplan/ABS, ao Frigorífico Silva e Brenda Impex, cujos suportes viabilizaram a execução deste trabalho.

A todos que contribuíram para a realização desta etapa, meus sinceros agradecimentos.

“É melhor tentar e falhar,
que preocupar-se e ver a vida passar;
é melhor tentar, ainda que em vão,
que sentar-se fazendo nada até o final.
Eu prefiro na chuva caminhar,
que em dias tristes em casa me esconder.
Prefiro ser feliz, embora louco,
que em conformidade viver ..."

(Martin Luther King)

RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria

ÁCIDO ASCÓRBICO NA PRODUÇÃO *IN VITRO* DE EMBRIÕES BOVINOS

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Santa Maria, 17 de novembro de 2008.

Embriões bovinos cultivados *in vitro* são susceptíveis ao estresse oxidativo causado pela alta concentração de oxigênio, que contribui para a formação de radicais livres. Por isso, sistemas antioxidantes de defesa são necessários para neutralizar as espécies reativas de oxigênio e seus efeitos prejudiciais. Este estudo teve como objetivo avaliar a adição de diferentes concentrações (100, 250 e 500µM) do antioxidante ácido ascórbico (AA) aos meios de maturação (MIV, 9 repetições) ou cultivo (CIV, 8 repetições) *in vitro* de embriões bovinos (Capítulo 1). A concentração que propiciou maior taxa de desenvolvimento embrionário e melhor qualidade dos embriões foi adicionada aos meios de maturação e/ou cultivo *in vitro* em diferentes atmosferas gasosas (Capítulo 2, 10 repetições). Meios sem antioxidante serviram como grupos-controle. Complexos *cumulus*-oócitos obtidos de ovários de frigorífico foram distribuídos aleatoriamente em grupos e maturados em TCM-199 adicionado de pFSH, bLH e 10% de soro de vaca em estro (SVE), por 22 a 24h, em estufa a 39℃ e 5% CO_2 em ar e umidade saturada. Para a produção *in vitro* foram utilizados 20-40 oócitos/grupo em 400µL de meio em placa de quatro poços (Cap. 1) e 13-32 oócitos/grupo em gotas de 200µL de meio sob óleo mineral (Cap. 2). A fecundação *in vitro* (D0) foi conduzida com sêmen congelado *Bos taurus taurus* selecionado por *Swim-up* (Cap. 1) e gradiente de Percoll (Cap. 2), com $1x10^6$ espermatozóides/mL em TALP-Fert com heparina e PHE, por 18 a 22h, nas mesmas condições da MIV. Os prováveis zigotos foram cultivados *in vitro* em meio SOFaaci com 5% de SVE sob óleo mineral, no sistema de bolsas gaseificadas (*bag system*) a 5% CO_2, 5% O_2 e 90% N_2 e umidade saturada (Cap. 1 e 2), ou em estufa a 5% CO_2 em ar e umidade

saturada (20% O_2 em ar; Cap. 2). Para analisar a porcentagem de embriões no D2, D7 e D9 utilizou-se o delineamento blocos ao acaso, tendo como critério de bloqueio o dia da coleta (Cap. 1). No Cap. 2, para analisar a taxa de clivagem no D2 foram considerados os efeitos da adição do AA na maturação, adição do AA no cultivo e a interação entre os dois fatores. Os dados do desenvolvimento embrionário no D7 e D9 foram analisados após transformação arcoseno raiz quadrada e foram considerados os efeitos da presença do AA na maturação, presença do AA no cultivo, a atmosfera gasosa e a interação entre esses fatores, considerando o dia de realização da rotina no modelo de análise. Para contagem de células dos blastocistos os dados foram analisados após transformação logarítmica de base 10, sendo utilizadas 8 repetições (Cap. 1) e 10 repetições (Cap. 2), com nível de significância de 5%. No Capítulo 1, a taxa de clivagem e o desenvolvimento embrionário no D7 foram similares (P>0,05) entre as diferentes concentrações de AA adicionadas na MIV ou CIV. Entretanto, a adição de 100µM de AA no CIV apresentou tendência (P<0,07) de maior produção de blastocistos (32,3%) quando comparado ao grupo com 500µM (19,7%). A produção de blastocistos no D9 aumentou significativamente quando 100µM de AA foi adicionado na MIV (P<0,05) comparado ao grupo-controle (38,7 vs 29,0%), ou no CIV (P<0,01) comparado com a concentração de 500µM (28,1 vs 14,1%). O número de células por blastocisto foi similar (P>0,05) entre as diferentes concentrações de AA e o grupo-controle. No Capítulo 2, não houve efeito da interação (P>0,05) entre os fatores estudados (AA na MIV, AA no CIV e atmosfera de cultivo) sobre o D2, D7 e D9. A taxa de clivagem e o desenvolvimento embrionário no D7 e D9 não diferiram (P>0,05) entre os grupos com e sem antioxidante, independentemente da atmosfera gasosa. No entanto, maior número de células foi observado nos blastocistos eclodidos (P<0,05) quando o AA foi incluído no meio de cultivo. Os resultados indicam que a concentração de 100µM de ácido ascórbico pode ser utilizada durante a MIV ou CIV para incrementar a produção embrionária, especialmente no D9, além de melhorar a qualidade dos blastocistos eclodidos, independentemente da atmosfera gasosa de cultivo.

Palavras-chave: maturação *in vitro*; cultivo *in vitro*; ácido ascórbico; oxigênio; embrião; bovino

ABSTRACT

Doctoral Thesis in Veterinary Medicine
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria, Santa Maria, RS, Brazil

ASCORBIC ACID IN THE *IN VITRO* PRODUCTION OF BOVINE EMBYOS

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ADVISER: MARA IOLANDA BATISTELLA RUBIN
Santa Maria November, 17th 2008.

In vitro cultured bovine embryos are susceptible to oxidative stress caused by high oxygen concentration, that contributes to free radicals formation. Therefore, antioxidant defense systems are needed to neutralize the reactive oxygen species and their harmful effects. The aim of this study was to evaluate the addition of different concentrations (100, 250 and 500µM) of ascorbic acid (AA) antioxidant to *in vitro* maturation (IVM, 9 replications) or culture (IVC, 8 replications) media of bovine embryos (Chapter 1). The concentration that provided greatest embryonic development rate and better embryo quality was added to *in vitro* maturation and/or culture media at different gaseous atmospheres (Chapter 2, 10 replications). Media without antioxidant served as control-groups. *Cumulus*-oocyte complexes obtained from bovine ovaries at the slaughterhouse were randomly distributed in groups and matured in TCM-199 with addition of pFSH, bLH and 10% estrus cow serum (ECS), for 22 to 24h, in an incubator at 39℃ and 5% CO_2 in air and saturated humidity. The *in vitro* production was performed using 20-40 oocytes/group in 400µL of medium in 4-well dishes (Chap. 1) and 13-32 oocytes/group in 200µL drops of medium under mineral oil (Chap. 2). The *in vitro* fertilization (D0) was performed with *Bos taurus taurus* frozen semen selected by *Swim-up* (Chap. 1) and Percoll gradient (Chap. 2) with $1x10^6$ spermatozoa/mL in TALP-Fert with heparin and PHE, for 18 to 22h, at the same IVM conditions. The presumptive zygotes were *in vitro* cultured in SOFaaci medium with 5% ECS under mineral oil, in the b*ag system* at 5% CO_2, 5% O_2 and 90% N_2 and saturated humidity (Chap. 1 and 2), or in an incubator at 5% CO_2 in air and saturated humidity (20% O_2 in air; Chap 2). To analyze the percentage

of embryos on D2, D7 and D9 a randomized block delineation was used, having as blockade criteria the collection day (Chap. 1). In Chap. 2, the cleavage rate on D2 was analyzed considering the effects of AA addition on maturation and culture and the interaction between the two factors. The embryonic development data on D7 and D9 were analyzed after arc sine square root transformation and considered the effects of AA presence on maturation and culture, the gaseous atmosphere and the interaction among these factors, taking into account the routine day in the analyses model. Blastocyst cell number was obtained after base 10 logarithmic transformation of data, using 8 (Chap. 1) and 10 replications (Chap. 2), with a significance level of 5%. In Chapter 1, the cleavage rate and embryonic development on D7 were similar ($P>0.05$) among the different AA concentrations added to IVM or IVC. However, the 100µM AA addition to IVC tended ($P<0.07$) to show higher blastocyst production (32.3%) when compared to the 500µM group (19.7%). The blastocyst production on D9 increased significantly when 100µM AA were added to IVM ($P<0.05$) compared to control-group (38.7 vs 29.0%), or on IVC ($P<0.01$) compared to 500µM concentration (28.1 vs 14.1%). The blastocyst cell number was similar ($P>0.05$) among the different AA concentrations and the control-group. In Chapter 2, there was no effect of interaction ($P>0.05$) among the factors studied (AA on IVM, AA on IVC and atmosphere of culture) on D2, D7 and D9. Cleavage rate and embryonic development on D7 and D9 did not differ ($P>0.05$) between groups with and without antioxidant addition, independently of the gaseous atmosphere. However, a greater cell number was observed in hatched blastocysts ($P<0.05$) when the AA was included in the culture medium. The results indicate that 100µM ascorbic acid concentration can be used during IVM or IVC to increase embryo production, especially on D9, and improve the quality of hatched blastocysts, independently of the culture gaseous atmosphere.

Key-words: *in vitro* maturation; *in vitro* culture; ascorbic acid; oxygen; embryo; bovine

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SUMÁRIO

1 INTRODUÇÃO

A maioria das células cultivadas *in vitro* são expostas ao risco de alterações causadas pelos radicais livres de oxigênio. Nos embriões, algumas lesões podem ocorrer pela falta de sistemas neutralizantes e pela exposição à alta concentração de oxigênio (UMAOKA et al., 1992). Sabe-se que a concentração de oxigênio no lúmen do trato reprodutivo da fêmea é aproximadamente 1/3 (3-9%) daquela encontrada sob condições convencionais de cultivo *in vitro* (MASTRIOANNI JR; JONES, 1965). O cultivo *in vitro* (CIV) de embriões com alta concentração de oxigênio (20%) produz mais radicais livres (FOWLER; CALLINGHAM, 1978) que embriões cultivados sob 5%, ou 7% de oxigênio (NASR-ESFAHANI et al., 1990a; LIU; FOOTE, 1995a). Os efeitos prejudiciais dos radicais livres derivados do oxigênio no embrião durante o CIV já foram demonstrados em bovinos (OLSON; SEIDEL, 2000; ALI et al., 2003).

Mesmo sob condições basais, o metabolismo aeróbico gera espécies reativas de oxigênio (ROS). Estas são formadas durante os passos intermediários da redução do oxigênio: o radical ânion superóxido (O_2^--), o peróxido de hidrogênio (H_2O_2) e o radical hidroxil (OH-), correspondem à redução por um, dois e três elétrons, respectivamente (GUÉRIN et al., 2001). A formação de ROS é um processo fisiológico que ocorre na célula quando existe desvio de elétrons para o oxigênio durante as reações de transferência de elétrons na cadeia respiratória mitocondrial e em outros sistemas de transferência de elétrons intracelulares (GUILLE; JOENJE, 1991; HO et al., 1996). Esses radicais causam estresse oxidativo nos gametas e embriões (GUÉRIN et al., 2001). Guérin et al. (2001) consideram ainda que o estresse oxidativo tem origem no desenvolvimento embrionário imperfeito, enquanto para Ali et al. (2003) parece provável que embriões cultivados *in vitro* são expostos ao estresse oxidativo porque seus mecanismos de defesa são insuficientes para proteger sua delicada estrutura celular.

As espécies reativas de oxigênio podem se originar do metabolismo do embrião via diversos mecanismos enzimáticos e/ou do ambiente onde este se encontra. A contribuição relativa de cada fonte parece diferente dependendo da

espécie, do estágio de desenvolvimento e das condições de cultivo. Diversos fatores exógenos e condições de cultivo podem aumentar a produção de ROS pelos embriões (GUÉRIN et al., 2001). Quando gerados *in vitro*, as ROS reagem com as proteínas celulares e lipídios resultando na inativação das enzimas e peroxidação da membrana lipídica, respectivamente (HALLIWELL; GUTTERIDGE, 1989; 1995; IWATA et al., 1998); causam alterações do ácido desoxirribonucleico (ALI et al., 2003) e ácido ribonucleico; bem como induzem a disfunção mitocondrial e inibem a fusão espermatozóide-oócito (AITKEN et al., 1993). A apoptose também pode ser induzida quando a concentração de oxigênio for supra-fisiológica (VAN SOOM et al., 2002); e também pode alterar muitos tipos de moléculas celulares e induzir retardo e bloqueio do desenvolvimento (GUÉRIN et al., 2001). O balanço entre a produção de ROS e a neutralização é um importante fator para a aquisição da habilidade fecundante *in vitro* (DE LAMIRANDE et al., 1997).

Diversos mecanismos de defesa contra as ROS estão presentes em ambos: embriões e ambiente uterino, e estes tem ação complementar. *In vivo*, oócitos e embriões estão protegidos externamente contra o estresse oxidativo pelos neutralizadores de oxigênio presentes nos fluidos folicular e tubárico, compreendendo principalmente antioxidantes não-enzimáticos tais como hipotaurina, taurina e ácido ascórbico; enquanto a proteção interna compreende principalmente enzimas antioxidantes: superóxido dismutase (SOD), glutationa peroxidase e gama-glutamilcisteina sintetase. Transcrições codificadas por estas enzimas estão presentes no oócito, embrião e tuba uterina e há indicação de sua importância quando estocados durante a maturação do oócito de maneira a permitir o desenvolvimento do embrião. O dano oxidativo pode resultar da super produção de ROS e/ou da diminuição da liberação dos mecanismos neutralizadores das ROS. Três estratégias evitam o estresse oxidativo: a prevenção contra formação de ROS, interceptação (por antioxidantes) e reparo (GUÉRIN et al., 2001).

A manipulação de gametas e embriões favorece a geração de ROS e pode explicar parcialmente a taxa reduzida de embriões viáveis em muitas espécies, pois diminuem as defesas antioxidantes (GUÉRIN et al., 2001). Entretanto, já foi relatado que em concentrações fisiológicas, as ROS participam no processo de desenvolvimento celular e que o cultivo *in vitro* resulta em maiores concentrações de oxigênio que no ambiente *in vivo*, levando ao aumento dos níveis de ROS (LUVONI et al., 1996). A adição de componentes antioxidantes aos sistemas de CIV de

embriões estimula o desenvolvimento (NASR-ESFAHANI et al., 1990b; NODA et al., 1991; NASR-ESFAHANI et al., 1992; PAYNE et al., 1992; UMAOKA et al., 1992; ORSI; LEESE, 2001) e também resulta na subseqüente sobrevivência à criopreservação (TARIN; TROUNSON, 1993).

Os antioxidantes são definidos como “qualquer substância que, quando presente em baixa concentração comparada aos substratos oxidáveis, significativamente atrasa ou inibe aquele substrato” (HALLIWELL; GUTTERIDGE, 1989). Esta definição inclui componentes de natureza enzimática e não enzimática (GUÉRIN et al., 2001). O ácido ascórbico, ascorbato ou vitamina C é um importante antioxidante não enzimático e hidrofílico, capaz de neutralizar peróxidos de hidrogênio, ânions superóxido, radicais livres hidroxil e oxigênio individual (HALLIWELL; GUTTERIDGE, 1989). Adicionalmente, é possível que sua presença em grandes quantidades no fluido folicular (PASZKOWSKI; CLARKE, 1999), induza a liberação de taurina e hipotaurina no líquido tubárico no momento da ovulação (GUÉRIN et al., 2001), indicando que este pode atuar fisiologicamente como um antioxidante no desenvolvimento de oócitos e embriões (PASZKOWSKI; CLARKE, 1999).

Como os fatores envolvidos na obtenção de embriões produzidos *in vitro* de excelente qualidade são inúmeros, é importante que haja uma evolução no sistema de produção *in vitro*. Portanto, modificações do ambiente de cultivo do embrião, tanto pela redução do oxigênio atmosférico (BETTERBED; WRIGHT, 1985; PABON et al., 1989; NAKAO; NAKATSUJI, 1990; UMAOKA et al., 1992; EPPIG; WIGGLESWORTH, 1995; LIU; FOOTE, 1995b; BERNARDI et al., 1996), ou pela adição de substâncias para reduzir os níveis de radicais livres são requeridos para o estabelecimento de um sistema de cultivo adequado (NODA et al., 1991; UMAOKA et al., 1992; LI et al., 1993; LIU; FOOTE, 1995b; DE MATOS et al., 1995; LUVONI et al., 1996).

A escassez de informações sobre o efeito do ascorbato na produção *in vitro* de embriões na espécie bovina indica a necessidade de pesquisas para melhor entendimento do papel do referido antioxidante.

Este estudo teve como objetivo avaliar diferentes concentrações de ácido ascórbico no meio de maturação e no cultivo *in vitro* de embriões bovinos (Capítulo 1). Dentre as concentrações testadas, a que propiciou maior produção e melhor qualidade de blastocistos foi utilizada na maturação e/ou cultivo *in vitro* de embriões

em dois sistemas de gaseificação: estufa a 5% CO_2 em ar (20% de oxigênio em ar) ou a 5% CO_2, 5% O_2 e 90% N_2 no sistema de bolsas gaseificadas (5% de oxigênio) (Capítulo 2).

2 CAPÍTULO 1

In vitro production of bovine embryos in medium with ascorbic acid

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Artigo submetido ao periódico **Animal Reproduction Science** em Outubro/2008

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Abstract

The effect of ascorbic acid antioxidant (100, 250 and 500µM) on *in vitro* maturation (IVM) or culture (IVC) media of bovine embryos was evaluated to assess development and embryo quality. Media without antioxidant were used as control-groups. *Cumulus*-oocyte complexes from qualities 1 and 2 obtained from bovine ovaries collected at the slaughterhouse were randomly distributed (20-40/group) and matured in 400µL TCM-199 added of pFSH, bLH and 10% estrus cow serum (ECS) in an incubator for 22 to 24h, at 39ºC, with 5% CO_2 in air and saturated humidity. *In vitro* fertilization was performed with frozen semen from *Bos taurus taurus* selected by Swim-up with $1x10^6$ spermatozoa/mL in 400µL TALP-Fert with heparin and PHE for 18 to 22h, at the same IVM conditions. The embryo *in vitro* culture was performed in 400µL SOFaaci with 5% ECS under mineral oil, in an incubator at 5% CO_2, 5% O_2 and 90% N_2 at 39ºC and saturated humidity, for 9 days. No difference was observed ($P>0.05$) in the cleavage rate among groups with and without ascorbic acid during IVM or IVC. There was no difference ($P>0.05$) in the embryo development rate on D7 (D0 = fertilization day) among the various ascorbic acid concentrations added to the oocytes during IVM. The addition of 100µM ascorbic acid during IVC showed a tendency ($P<0.07$) of higher blastocyst production on D7 (32%) than 500µM (20%). Blastocyst production on D9 increased significantly when 100µM ascorbic acid was added on IVM ($P<0.05$) compared to the control-group (39 vs 29%); or on IVC ($P<0.01$) compared to the 500µM concentration (28 vs 14%). The average cell number of blastocysts was similar ($P>0.05$) among groups with and without ascorbic acid. The results suggest that 100µM ascorbic acid can be used during IVM or IVC on *in vitro* bovine embryo production procedures.

Key words: *In vitro* maturation; *In vitro* culture; Antioxidants; Ascorbic acid; Bovine.

1. Introduction

Oxidative damages occur in cells during *in vivo* and *in vitro* development, due to free radicals exposure generated by exogenous agents (e.g., radiation, chemicals, hyperoxia), as well as endogenous processes such as physiological cellular metabolism. Under extreme oxidative conditions, or when the antioxidant protective mechanisms of cells are compromised, cellular injury and death may occur (Olson

and Seidel, 2000). Mammalian embryos are susceptible to damage caused by reactive oxygen species (ROS), and they increase the production of oxygen free radicals when cultured *in vitro* (Luvoni et al., 1996; Olson and Seidel, 2000).

The metabolism of oxygen inevitably results into ROS formation. Depending on the site of formation and the tension of oxygen, several reactive oxygen species are produced, such as superoxide anions (O_2^--), hydroxyl radicals (OH^-) and hydrogen peroxide (H_2O_2), or several unstable oxidized lipids (Blondin et al., 1997). *In vitro* research has displayed the effects of ROS in reproduction; some of those are detrimental ones, such as spermatozoa motility reduction and axonemal protein phosphorylation (De Lamirande and Gagnon, 1992), *in vitro* two-cell block of mouse embryos (Nasr-Esfahani et al., 1990; Noda et al., 1991), and decrease on embryonic development (Pabon et al., 1989). For Blondin et al. (1997) there are evidences that ROS might be beneficial at some steps of reproduction for allowing the successful gamete interaction. Undeniably, *in vitro* culture results in higher oxygen concentrations and ROS production than *in vivo* environments (Luvoni et al., 1996), which affects early development of mouse embryos (Umaoka et al., 1992), hamster (McKiernan and Bavister, 1990) and bovine (Nagaro et al., 1994), and might partially explain the low rate of viable embryos obtained in many species. Thus, *in vitro* culture systems constitute limited conditions for embryo development and the oxidative stress is unavoidable (Guérin et al., 2001).

Living organisms have natural protection known as ROS scavengers (antioxidants) to counteract the negative effects of reactive oxygen species. Different cellular compartments contain different antioxidants. Intracellularly, there is the superoxide dismutase (SOD), catalase and glutathione peroxidase (Meister, 1983). Associated to the membranes alpha-tocopherol and carotenoids were identified, and extracellularly, there is the ascorbic acid (Gutteridge and Halliwell, 1988), as well as many proteins. The addition of such antioxidants *in vitro* has increased the sperm motility and improved the performance of spermatozoa in the zona-binding test, as well as increased the percentage of embryo production (Blondin et al., 1997). Therefore, to protect oocytes and embryos from oxidative stress during *in vitro* culture, several antioxidants can be used in the culture media (Ali et al., 2003), and among them, the ascorbic acid.

The objective of this study was to determine the effect of different ascorbic acid concentrations to *in vitro* maturation (IVM) and culture (IVC) media, on the subsequent bovine embryonic development and quality.

2. Materials and methods

The experiment was performed from nov/2006 to may/2007, in the Laboratory of Embryology (EMBRYOLAB) of the Federal University of Santa Maria (UFSM), in Santa Maria – RS. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated.

2.1. Collection of the ovaries and obtention of the cumulus-oocyte complexes (COC)

Ovaries were collected from cows at the slaughterhouse 22 Km far from the laboratory and transported in 0.9% saline solution at 30ºC in a thermos flask. In the laboratory, the ovaries were washed initially in alcohol 70ºGL warmed to 30ºC and twice in 0.9% saline solution pre-warmed at the same temperature. The follicles with 2 to 8mm diameter were punched and aspirated with the aid of a vacuum pump with 25mmHg pressure. After ten minutes, the follicular fluid sediment was transferred to a 100x15mm petri dish to identify the COC under stereomicroscope. *Cumulus*-oocyte complexes quality 1 and 2, selected according to the morphological aspect (De Loos et al., 1989), were randomly distributed (20-40/ group) and washed in centrifuged follicular fluid and in modified culture medium TCM-199 (Gibco BRL. Grand Island, NY, USA) with 5.95mg/mL HEPES (25mM), 0.025mg/mL sodium pyruvate and 10% estrus cow serum (ECS).

2.2. In vitro maturation (IVM) of COC

The IVM was carried out in 4-well culture dishes (Nunc®) in 400μL TCM-199 Earle's salts medium, with 2.2mg/mL sodium bicarbonate, 0.025mg/mL sodium pyruvate, 10% ECS, 0.5μg/mL porcine follicle-stimulating hormone (pFSH) and 0.05mg/mL bovine luteinizing hormone (bLH). The COC remained 22 to 24 hours in an incubator at 39ºC, with 5% CO_2 in air and saturated humidity.

2.3. In vitro fertilization (IVF) of COC

The matured COC were transferred to 400μL TALP-Fert (Parrish et al., 1986) medium, with 6mg/mL bovine serum albumin (BSA), 0.11mg/mL sodium pyruvate, supplemented with 50μg/mL heparin and 10μg/mL penicillamine, hypotaurine and epinephrine (PHE).

For the fertilization frozen semen from only one *Bos taurus taurus* bull was used. The 0.5mL straws were thawed for 10 seconds in the air and 20 seconds in a water bath at 37℃, evaluating the progressive motility and the spermatic vigor. As sperm separation method Swim-up (Parrish et al., 1986) in TALP-Sperm medium containing 6mg/mL BSA and 0.22mg/mL sodium pyruvate was used. After one hour, 800μL of the intermediate layer containing the mobile spermatozoa was centrifuged (600x g, 10min.). From the formed pellet (200μL) 10μL were removed for determination of the sperm cell concentration in Neubauer chamber (dilution 1:20).

The COC were inseminated with a final concentration of $1x10^6$ spermatozoa/mL. The co-culture (spermatozoa and COC) was carried out in an incubator for 18 to 22 hours, at 39℃ in an atmosphere of 5% CO_2 in air and saturated humidity.

2.4. In vitro culture (IVC) of embryos

The presumptive zygotes were transferred to tubes with 400μL modified TCM-199 medium with 5.95mg/mL HEPES (25mM), 0.025mg/mL sodium pyruvate and 10% ECS and submitted to mechanical agitation by vortex for 90 seconds in order to denude the *Cumulus oophorus* cells. The zygotes were adequately washed in TCM-HEPES medium and transferred to 400μL synthetic oviductal fluid (SOFaaci) medium added of amino acids, citrate and inositol (Holm et al., 1999) containing 5% ECS and 0.025mg/mL sodium pyruvate. In this medium, in 4-well dishes (Nunc®) under mineral oil, all the structures remained in a culture incubator in the bag system at 5% CO_2, 5% O_2 and 90% N_2 at 39℃ and saturated humidity, for 9 days.

2.5. Experimental design

In *Experiment 1*, ascorbic acid (*L-Threoascorbic acid*, AA 4544) at 100μM

(n=333, group 1), 250µM (n=335, group 2) and 500µM (n=326, group 3) concentrations were added to *in vitro* maturation medium, while in *Experiment 2*, the culture medium was supplemented with the respective concentrations (n=381, group 1; n=364, group 2 and n=340, group 3). Media without ascorbic acid addition were used as control-groups (C), in experiments 1 (n=321) and 2 (n=319).

2.6. Evaluations

The *in vitro* embryonic development rates evaluated were the following: cleavage on the second day (D2); early blastocysts, blastocysts, expanded blastocysts, hatching blastocysts and hatched blastocysts on day 7 (D7); and, expanded blastocysts, hatching blastocysts and hatched blastocysts on day 9 (D9) of culture, considering the fertilization day as day zero (D0), according to the classification recommended by the International Embryo Transfer Society (IETS; Robertson and Nelson, 1998).

On D9, the hatching and hatched blastocysts were fixed in 2% paraformaldehyde and loaded in 0.5mL straws for cell count. The embryos were stained with Hoechst (33342) in the final concentration of 10µg/mL PBS. The nuclei visualization and counting were made in epifluorescence microscope equipped with excitation filter (365mm) and emission filter (410nm).

2.7. Statistical analysis

To analyze the percentage of cleavage embryos on D2 and embryonic development on D7 and D9 randomized block delineation were used, with 9 replications in experiment 1 and 8 replications in experiment 2, having as blockade criteria the collection day. For blastocysts cell number counting, 8 replications in both experiment 1 and experiment 2 were applied, and the data analyzed after logarithmic transformation of base 10. In the experiment 2, the cell number was analyzed only in hatched blastocysts due to the low number of hatching blastocysts ($n \leq 5$) in two of the tested groups. The data were analyzed by GLM procedure of SAS statistical package, version 8.01 (2001), presented as the average of each group and compared by Tukey test, with 5% of significance level.

3. Results

3.1. Experiment 1

The cleavage rate and the embryonic development until the seventh day of 1.315 COC *in vitro* produced were similar (P>0.05) among 100, 250 and 500µM of ascorbic acid concentrations in the *in vitro* maturation (IVM) and the control-group (Fig. 1). There was a greater production of blastocysts on day 9 (P<0.05) when 100µM of ascorbic acid was added to the IVM medium, compared to the control (without antioxidant). The hatching or hatched blastocysts cell number (Table 1) did not differ (P>0.05) among groups of COC treated with 100, 250 and 500µM of ascorbic acid and the COC control-group.

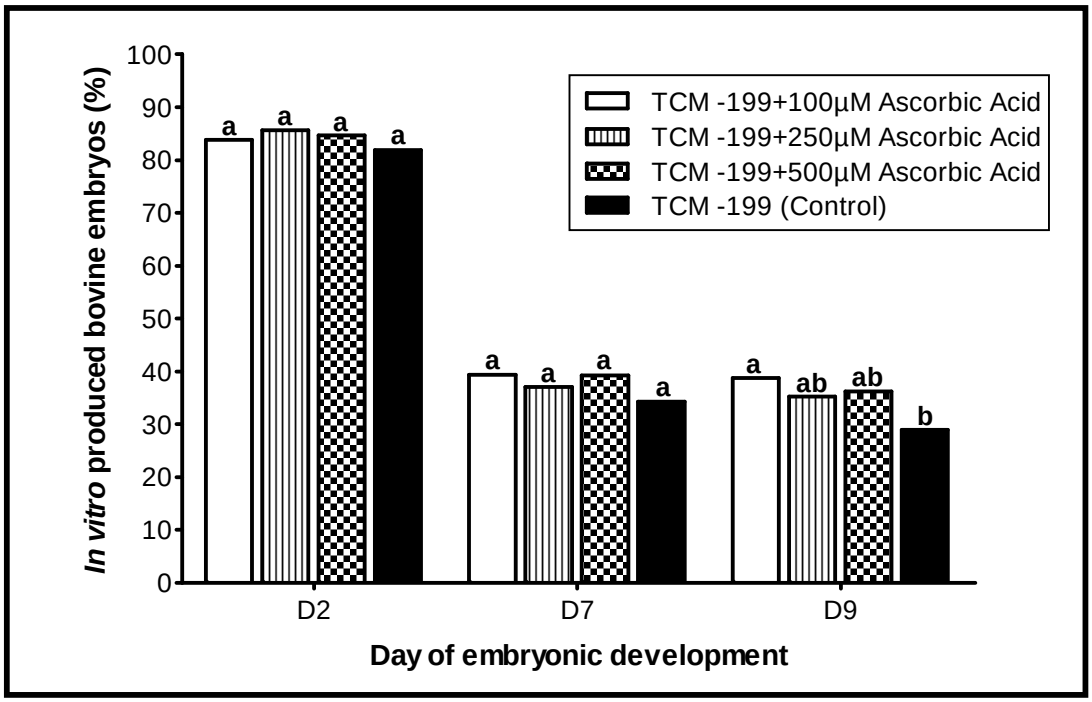

Fig. 1. Cleavage rate (D2) and embryonic development (D7 and D9) of bovine *cumulus*-oocyte complexes *in vitro* matured in TCM-199 medium, with or without ascorbic acid addition (P<0.05).

Table 1

Average cell number of hatching and hatched blastocysts visualized by Hoechst fluorescent staining obtained from bovine *cumulus*-oocyte complexes *in vitro* matured, in TCM-199 medium, with or without ascorbic acid addition

Embryo stage	TCM-199 + Ascorbic Acid			TCM-199 (control)
	100µM	250µM	500µM	
Hatched blastocysts	158.67 (81-264) n=48	170.22 (70-287) n=45	156.88 (68-297) n=52	159.02 (61-287) n=42
Hatching blastocysts	116.82 (61-279) n=11	140.56 (79-201) n=9	105.00 (58-151) n=14	116.78 (83-195) n=9

There was no difference among the groups (P>0.05).

3.2. Experiment 2

The cleavage rate of 1.404 COC *in vitro* cultured did not differ (P>0.05) when 100, 250 or 500µM of ascorbic acid were added to the culture medium (IVC) and compared to the control-group (Fig. 2). With the addition of 100µM of ascorbic acid in the SOFaaci medium there was a tendency on D7 (P<0.07) and greater blastocyst production on D9 (P<0.01) compared to the cultured embryos in the presence of 500µM of this antioxidant. There was no difference among the groups (P>0.05) on the hatched blastocyst cell number (Table 2).

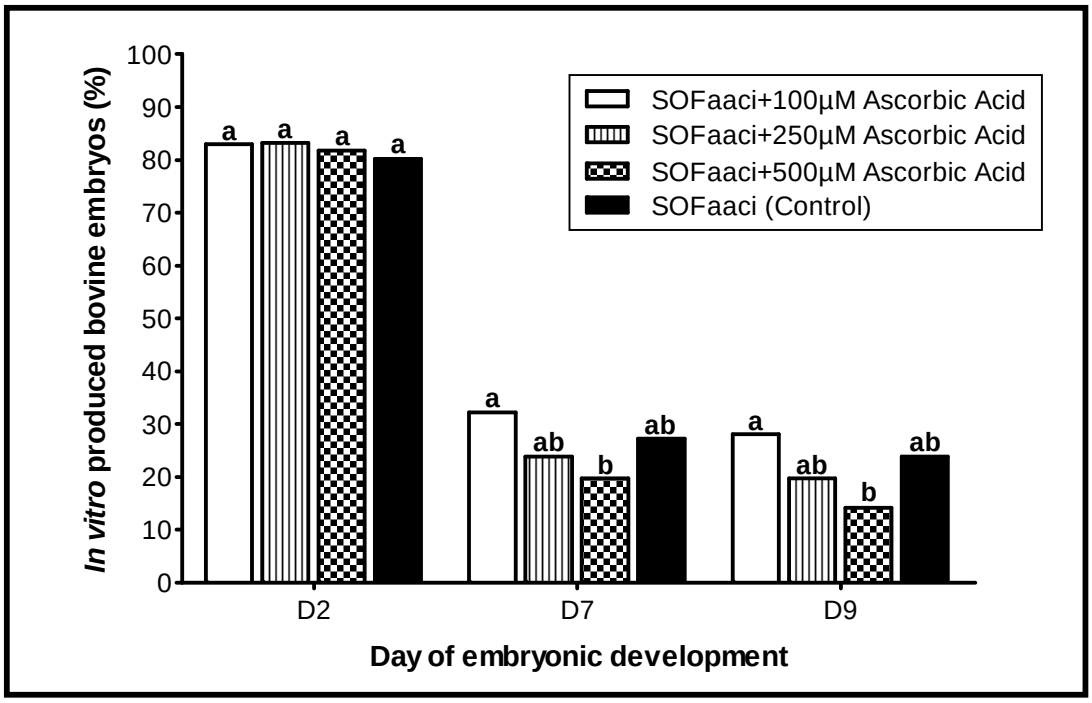

Fig. 2. Cleavage rate (D2) and embryonic development (D7 and D9) of bovine *cumulus*-oocyte complexes *in vitro* cultured in SOFaaci medium, with or without ascorbic acid addition (D7: P<0.07; D9: P<0.01).

Table 2

Average cell number of hatched blastocysts visualized by Hoechst fluorescent staining obtained from bovine *cumulus*-oocyte complexes *in vitro* cultured, in SOFaaci medium, with or without ascorbic acid addition

Embryo stage	SOFaaci + Ascorbic Acid			SOFaaci (control)
	100µM	250µM	500µM	
Hatched blastocysts	124.69 (54-211) n=36	105.94 (77-157) n=16	134.75 (66-249) n=8	138.44 (84-246) n=27

There was no difference among the groups (P>0.05).

4. Discussion

The importance of protecting pre-implantation embryos from oxidative damage *in vitro* is widely recognized and for this, the oxygen concentration in gaseous environment must be reduced (Iwata et al., 1998; Guérin et al., 2001), to have a radical buffering system produced by co-culture cells (Guérin et al., 2001), or adding

supplements to the culture medium (Iwata et al., 1998; Guérin et al., 2001). The redox potential of culture medium is very important probably by limiting the peroxidation process, which allows the embryo development. Compounds such as ascorbate, cysteine and glutathione (GSH) contribute to maintaining this redox potential (Guérin et al., 2001). There are numerous reports about the beneficial effects of low oxygen concentration in mouse (Pabon et al., 1989; Umaoka et al., 1992; Eppig and Wigglesworth, 1995), sheep (Betterbed and Wright, 1985; Bernardi et al., 1996) bovine (Nakao and Nakatsuji, 1990; Liu and Foote, 1995) and the antioxidant supplementation on embryo development *in vitro* (Noda et al., 1991; Umaoka et al., 1992; Li et al., 1993).

In experiment 1, different concentrations of ascorbic acid supplemented to *in vitro* maturation under an atmosphere of 20% O_2 results in 84% of cleavage rate, without any difference to control-group without antioxidant. This similarity among the groups in the cleavage rate (average of 82%) also remained when the different concentrations of this antioxidant were present in the *in vitro* period culture (experiment 2) under 5% O_2 in the bag system. This suggests that the “more anaerobic” metabolism of the early embryo minimizes the generation of reactive oxygen species by the mitochondria, and thus reduces the risk of oxidative stress, not evidencing the effect of the antioxidant.

In porcine, the exposition to the alpha-tocopherol on IVM, more active form of vitamin E, or L-ascorbic acid (vitamin C) promotes the development of denuded oocytes from metaphase I (MI) to metaphase II (MII) and prevents *Cumulus oophorus* cell DNA fragmentation at certain levels, especially with 10µM of alpha-tocopherol or with 250µM L-ascorbic acid (Tao et al., 2004). *Cumulus oophorus* cells protect the porcine oocytes from oxidative stress by elevating the intracellular glutathione (GSH) content (Tatemoto et al., 2000); whereas in denuded oocytes the GSH content markedly decreases (Tao et al., 2004). In fact, the GSH has already been described in 1976 (Meister and Tate) for an important action in protecting mammalian oocytes against oxidative damage. The antioxidant addition, alpha-tocopherol and ascorbic acid increases the levels of GSH in denuded oocytes, but cannot improve the further development when added to COC, due to the existence of the surrounding *Cumulus oophorus* cells (Tao et al., 2004). These comments probably explain the fact that no effect of the addition of 100, 250 or 500µM of ascorbic acid has been observed in the maturation on the cleavage rate and embryonic development until D7, in this study.

The antioxidants alpha-tocopherol and/or ascorbate when added to *in vitro* maturation medium, do not modify the nuclear maturation rates in bovine oocytes (Dalvit et al., 2005), neither in rats (Takami et al., 1999). The presence of alpha-tocopherol (Dalvit et al., 2005), ascorbate (Blondin et al., 1997; Dalvit et al., 2005), alpha-mercaptoethanol and superoxide dismutase (Blondin et al., 1997) on IVM, do not exert immediate effect on cytoplasmic maturation of bovine oocytes, because does not modify the *in vitro* fertilization (IVF) percentage. When the maturation medium is supplemented with alpha-tocopherol and ascorbic acid, the percentage of blastocysts *in vitro* produced diminishes when compared to the control (without antioxidants), in the ascorbic acid presence (Blondin et al., 1997; Dalvit et al., 2005), or alpha-tocopherol (Dalvit et al., 2005) separately. However, the combination of both natural antioxidants is related with cytoplasmic and/or membrane events occurring during maturation, which become evident in more advanced stages of embryo development (Dalvit et al., 2005).

In humans, the concentration of ascorbate in body fluids varies from 30 to 150µmol/l (Halliwell and Gutteridge, 1989) and in follicular fluid it is 1/68 of the serum concentration (Paszkowski and Clarke, 1999). The largest production of blastocysts on D9 with 100µM of ascorbic acid both in the IVM medium, compared to the control, as in the IVC medium, compared to the 500µM concentration, shows the beneficial effect of this ascorbic acid concentration. The negative effect of ascorbic acid used throughout the period of culture is possibly associated to the high concentration (500µM). This value is very above of present in the body fluids. When this high concentration was present during the *in vitro* culture, the effect can be observed by compromising the development in the later stage, in the D9 case.

The instability of ascorbic acid under various oxidative conditions, such as exposure to neutral pH, light and heavy metals, which results in rapid degradation (Yamamoto et al., 1990), might explain its beneficial or harmful effects, which depends on the concentration. The ascorbic acid 2-O-alpha-glucoside (AA-2G), a stable ascorbate derivative, is characterized by its high stability toward oxidative degradation and its nonreducibility (Muto et al., 1990; Yamamoto et al., 1990). The use of 250µM of AA-2G in the maturation medium of porcine oocytes can potentiate the cellular protection of oocytes against oxidative stress, through the continuous supply of ascorbic acid, which scavenges not only intracellular but also extracellular ROS. However, higher concentrations of AA-2G (500 or 750µM) showed no effect on

the induction of oocyte maturation and male pronucleus formation, even in the presence of greatly higher levels of ascorbic acid in the oocytes, revealing that the accumulation of ascorbic acid beyond the optimum concentration ranges may have deleterious effects on maturation events occurring in both, nucleus and cytoplasm (Tatemoto et al., 2001).

When bovine oocytes are matured *in vitro* in the presence of 10, 100 or 500µM of ascorbic acid, the developmental competence of the oocytes after IVF does not improve, as observed by Blondin et al. (1997) evaluating the percentage of ≥64 cells stage embryos. Olson and Seidel (2000), using chemically defined *in vitro* culture medium, report that the presence of 100µM of ascorbic acid in medium with vitamin E or vitamin E+EDTA results in different cell numbers per blastocyst when compared to the medium containing vitamin E alone, or to the control (56 and 58 vs 84 and 75, respectively). In the present study, the fact that the ascorbic acid has not been combined with other antioxidant might be associated with the absence of effect in the cell number at the blastocysts stage (D9).

Contradictory results of antioxidant use in the *in vitro* production of bovine embryos might be due to different types and doses of antioxidants, to the stages when they are added, and to the fact of being used separately or in association with other antioxidants.

5. Conclusion

Based on the results of the present study, the 100µM concentration of ascorbic acid was not harmful to the maturation and to the embryonic development, increasing the blastocysts production on D9.

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3 CAPÍTULO 2

Ascorbic acid during the *in vitro* maturation and/or culture of bovine embryos: effect under different oxygen tensions

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Artigo submetido ao periódico **Reproduction**

em Outubro/2008

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Abstract

Among the factors that affect *in vitro* embryonic development, oxygen tension is considered to be of great influence. To avoid the oxidative stress caused by reactive oxygen species, antioxidants have been used during oocyte and embryo culture. In this experiment, the *in vitro* production and quality of bovine embryos *in vitro* matured and/or cultured in the presence or absence of ascorbic acid (AA) were evaluated in two culture systems with different oxygen tension. *Cumulus*-oocyte complexes were matured with or without 100μM AA in TCM-199 with 10% estrus cow serum (ECS), pFSH and bLH, for 22-24h in an incubator at 39ºC and 5% CO_2 in air and saturated humidity. The sperm was selected by Percoll gradient and the insemination (D0) performed with $1x10^6$ spermatozoa/mL in TALP-Fert with heparin and PHE, for 18-22h. The presumptive zygotes were *in vitro* cultured in the presence or absence of 100μM AA in SOFaaci medium containing 5% ECS, in an incubator at 5% CO_2 in air (20% oxygen in air) or in bag system at 5% CO_2, 5% O_2 and 90% N_2 (5% oxygen), both at 39ºC and saturated humidity. Cleavage rate and embryonic development on D7 and D9 were not affected by the presence of ascorbic acid during maturation or culture ($P>0.05$). However, the cell number of hatched blastocysts was higher ($P<0.05$) when ascorbic acid was included in the culture medium. The results show that 100μM of ascorbic acid does not improve the blastocyst production, both in high (20% in air) or low (5%) oxygen tension, but it improves the quality of blastocysts.

Introduction

Over the last decades, the research showed that the development potential of embryos *in vitro* is basically dependent on the quality of oocytes from which it originates (Lonergan *et al*. 2003; Rodriguez & Farin 2004). However, there is also evidence that the culture environment can affect the embryos quality (Lonergan *et al*. 1999; Van Soom *et al*. 2002; Yuan *et al*. 2003).

Many factors can influence the culture environment such as medium composition, protein supplementation, total number of embryos cultured in determined volume and gaseous atmosphere (Carolan *et al*. 1996; Khurana & Niemann 2000). Among these factors, the oxidative stress induced by greater oxygen

concentration has received special attention in the last years (Ali *et al*. 2003; Fatehi *et al*. 2005; Corrêa *et al*. 2008).

Higher concentrations of oxygen during the culture have been considered detrimental to embryo development, probably due to accumulation of reactive oxygen species – ROS (Luvoni *et al*. 1996). The ROS are produced during the aerobic metabolism, even under basal conditions. Oocytes and embryos also produce endogenous ROS through many enzymatic actions and metabolic pathways (Nasr-Esfahani & Johnson 1991; Guérin *et al*. 2001) and at physiological concentrations they take part into many cellular processes. But when the ROS production overwhelms the antioxidant capacity, oxidative stress occurs modifying the cellular functions and compromising the cell survival (Droge 2002). Therefore, the ROS must be continuously inactivated in order to keep only the small amount necessary to physiological cellular functions (Hossein *et al*. 2007).

Mechanisms of protection against ROS exist in *in vivo* systems and include enzymes such as superoxide dismutase, catalase and glutathione peroxidase (Gardiner & Reed 1995; Lapointe *et al*. 1998; Guérin *et al*. 2001); as well as low molecular weight antioxidants, such as L-ascorbic acid, uric acid, glutathione and tocopherols (McEvoy *et al*. 2000), which act as ROS scavengers. The follicular and oviductal fluids are rich in oxygen scavengers which protect against the oxidative damage (Gardiner & Reed 1995; Lapointe *et al*. 1998). However, when oocytes and embryos are *in vitro* cultured, they are deprived from these natural defense systems and, therefore, it is crucial to protect them against oxidative stress adding antioxidants into culture medium to optimize embryonic production (Wang *et al*. 2002; Hossein *et al*. 2007).

The susceptibility of embryos to ROS varies according with the developmental stage (Ali *et al*. 2003) and, consequently, the need of antioxidant supplements into culture media may not be the same for the different stages. This work was performed with the objective to verify the effects of 100μM ascorbic acid on *in vitro* maturation and/or culture, in two gaseous atmospheres, on the production and quality of bovine embryos.

Materials and methods

The experiment was carried out from August to November 2007, in the

Laboratory of Embryology (EMBRYOLAB) of the Federal University of Santa Maria (UFSM), in Santa Maria – RS. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated.

Collection of the ovaries and obtention of the cumulus-oocyte complexes (COC)

Ovaries were collected from cows at the slaughterhouse, far 22 km from the laboratory and transported in 0.9% (w/v) saline solution at 30ºC, in a thermos flask. In the laboratory, the ovaries were washed in alcohol 70ºGL warmed to 30ºC and twice in 0.9% (w/v) saline solution pre-warmed at 30ºC. With the aid of a vacuum pump with 25mmHg pressure follicles from 2 to 8mm diameter were punched and aspirated. After ten minutes, the follicular fluid sediment was transferred to a 100x15mm petri dish to identify the COC under stereomicroscope. *Cumulus*-oocyte complexes quality 1 and 2 selected according to the morphological aspect (De Loos *et al*. 1989) were randomly distributed in groups (13-32/group), washed in centrifuged follicular fluid and in modified culture medium TCM-199 (Gibco BRL, Grand Island, NY, USA) with 5.95mg/mL HEPES (25mM), 0.025mg/mL sodium pyruvate and 10% (v/v) estrus cow serum (ECS, Embryolab, Santa Maria, RS, Brazil).

In vitro maturation (IVM) of COC

The IVM of 1.674 COC was carried out in petri dishes in drops of 200µL TCM-199 Earle's salts medium, containing 2.2mg/mL sodium bicarbonate, 0.025mg/mL sodium pyruvate, 10% (v/v) ECS, 0.5µg/mL porcine follicle-stimulating hormone (pFSH, Bioniche, Belleville, Ontario, Canada) and 0.05mg/mL bovine luteinizing hormone (bLH, Bioniche, Belleville, Ontario, Canada), under mineral oil. The COC remained 22 to 24 hours in an incubator at 39ºC, with 5% CO_2 in air and saturated humidity. Half of the oocyte groups were matured in the presence of 100µM of ascorbic acid (*L-Threoascorbic acid*, AA 4544) and the other half were matured in groups without antioxidant.

In vitro fertilization (IVF) of COC

The matured COC were transferred to drops of 200μL TALP-Fert (Parrish *et al*. 1986) medium with 6mg/mL bovine serum albumin (BSA), 0.11mg/mL sodium pyruvate, supplemented with 50μg/mL heparin and 10μg/mL penicillamine, hypotaurine and epinephrine (PHE). For the fertilization, 0.5mL straws of frozen semen from only one *Bos taurus taurus* bull were thawed in water bath at 37℃. The progressive motility and the spermatic vigor were evaluated. After thawing, the semen was deposited on the surface of the 45% (v/v) Percoll solution (Parrish *et al*. 1995) placed above the 90% (v/v) one and centrifuged (600x g, 10min). The supernatant was discarded and the spermatozoa were suspended in 4mL TALP-Sperm. The semen was centrifuged again (200x g, 10min), 10μL of the sediment (200μL) being removed for determination of the sperm cells concentration by counting in Neubauer chamber (dilution 1:20). The COC were inseminated with a $1x10^6$ spermatozoa/mL concentration. The co-culture (spermatozoa and COC) was carried out in an incubator for 18 to 22 hours, at 39℃ in an atmosphere of 5% CO_2 in air and saturated humidity.

In vitro culture (IVC) of embryos

The presumptive zygotes were transferred to drops of 100μL modified TCM-199 medium with 5.95mg/mL HEPES (25mM), 0.025mg/mL sodium pyruvate and 10% (v/v) ECS and submitted to the partial or total mechanical denudation, through repeated aspirations with automatic pipette (Shioya *et al*. 1988), for approximately 90 seconds. After that the zygotes were adequately washed in TCM-HEPES medium and transferred to drops of 200μL synthetic oviductal fluid (SOFaaci) medium added of amino acids, citrate and inositol (Holm *et al*. 1999) containing 5% (v/v) ECS and 0.025mg/mL sodium pyruvate, under mineral oil in 100x15mm petri dish. The probable zygotes were cultured SOFaaci medium with or without 100μM ascorbic acid. The culture until D2 was carried out in an incubator at 5% CO_2 in air and according the treatments on IVM, as well as on IVC. The culture after cleavage evaluation on D2 was performed with two gaseous atmospheres: in an incubator at 5% CO_2 in air (20% O_2 in air) or in an incubator in the bag system at 5% CO_2, 5% O_2 and 90% N_2 (5% O_2), at 39℃ and saturated humidity.

Evaluation of the embryonic development

The stages of *in vitro* embryonic development evaluated were the following: cleavage on the second day (D2); early blastocysts, blastocysts, expanded blastocysts, hatching blastocysts and hatched blastocysts on day 7 (D7); and, expanded blastocysts, hatching blastocysts and hatched blastocysts on day 9 (D9) of culture, considering the fertilization day as day zero (D0), according to the International Embryo Transfer Society classification (IETS; Robertson & Nelson 1998). After the evaluation of the cleavage rate on D2, the structure that was not cleaved was put into separate drops (50μL) corresponding to their respective groups. For cell counting, hatching and hatched blastocysts obtained on D9 were fixed in 2% (w/v) paraformaldehyde and loaded in 0.5mL straws. Subsequently, the embryos were stained with Hoechst (33342) in the final concentration of 10μg/mL PBS for nuclei visualization and counting in epifluorescence microscope equipped with excitation filter (365mm) and emission filter (410nm).

Statistical analysis

To analyze the percentage of cleaved zygotes on D2, the effects of ascorbic acid addition on maturation, ascorbic acid addition on culture and the interaction between the two factors were considered. In the analysis of the embryonic development on D7 and D9 the effects of presence of ascorbic acid on maturation, presence of ascorbic acid on culture, the gaseous atmosphere and the interaction among these factors were evaluated. In this study 10 replications were performed and the effect of the routine day of the *in vitro* production maintained in the model analysis. In the analysis of the cell number only the hatched blastocysts were included due to the low number ($n<5$) of hatching blastocysts in each group. The data of embryonic development on D7 and D9 were analyzed after arc sine square root transformation and the data of blastocysts cell counting after logarithmic transformation of base 10. The analyses were performed by GLM procedure of SAS statistical package version 9.1.3 (2003) and the averages compared by Tukey test, being considered significant the value of $P<0.05$.

Results

There was no effect of the interaction (P>0.05) between the ascorbic acid addition to maturation medium and the addition to culture medium on the cleavage rate. The cleavage rate on D2 was similar (P>0.05) between groups cultured in the presence and absence of ascorbic acid, both on maturation and culture (Table 1). There was no effect of the interaction (P>0.05) among the factors studied (ascorbic acid addition to maturation medium, ascorbic acid addition to culture medium and atmosphere of culture) on the embryonic development on D7 and D9. There was no significant effect (P>0.05) of any of the three factors assessed on the blastocysts percentage on D7 and D9 (Table 2). The hatched blastocysts cell number was higher (P<0.05) with the ascorbic acid on culture medium than in the absence of the antioxidant (Table 3).

Table 1 Cleavage rate (% mean ± S.E.M) on D2 of bovine *cumulus*-oocyte complexes matured (IVM) and cultured (IVC) *in vitro* in the absence or presence of 100µM ascorbic acid (AA)

AA on IVM	AA on IVC		Mean number
	100µM	0	
100µM	81 ± 9.5 (343)	85 ± 6.8 (359)	83 ± 8.4[a]
0	80 ± 11 (331)	81 ± 9.6 (339)	80 ± 10[a]
Mean number	81 ± 10[a]	83 ± 8.5[a]	

Numbers in parentheses indicate the number of cleaved embryos.
There was no effect of the interaction (P>0.05).
[a] in the line or column indicates that there was no difference between the means (P>0.05).

Table 2 Embryonic development on D7 and D9 (% mean ± S.E.M) of bovine embryos *in vitro* produced in the absence or presence of 100µM ascorbic acid (AA) on maturation (IVM) and on culture (IVC) under different atmospheric conditions

AA on IVM	Atmosphere	AA on IVC		Mean	Mean
		100µM	0	AA on IVM	Atmosphere
		Development on D7			
100µM	5% CO_2	20 ± 8.3 (42)	31 ± 12 (64)	25 ± 11[a]	5% CO_2
	Bag system	24 ± 11 (48)	26 ± 10 (56)		24 ± 12[a]
0	5% CO_2	21 ± 13 (46)	23 ± 13 (48)	25 ± 14[a]	Bag system
	Bag system	29 ± 16 (59)	27 ± 14 (57)		26 ± 13[a]
Mean AA on IVC		24 ± 12[a]	27 ± 12[a]		
		Development on D9			
100µM	5% CO_2	19 ± 11 (40)	26 ± 14 (54)	22 ± 11[a]	5% CO_2
	Bag system	19 ± 8.9 (42)	25 ± 10 (55)		22 ± 12[a]
0	5% CO_2	21 ± 11 (46)	21 ± 14 (44)	23 ± 12[a]	Bag system
	Bag system	22 ± 12 (46)	28 ± 9.8 (59)		24 ± 10[a]
Mean AA on IVC		20 ± 10[a]	25 ± 12[a]		

Numbers in parentheses indicate the number of blastocyst.
5% CO_2 in air and saturated humidity (20% O_2).
Bag system= 5% CO_2, 5% O_2, 90% N_2 and saturated humidity (5% O_2).
There was no effect of the interaction (P>0.05).
[a] in the line or column indicates that there was no difference between the means (P>0.05).

Table 3 Mean cell number ± S.E.M of hatched blastocysts visualized by Hoechst fluorescent staining, obtained from bovine *cumulus*-oocyte complexes *in vitro* matured (IVM) and cultured (IVC), with or without 100µM ascorbic acid (AA) addition, in different atmospheric conditions

AA on IVM	Atmosphere	AA on IVC		Mean AA on IVM	Mean Atmosphere
		100µM	0		
100µM	5% CO_2	169 ± 38 (12)	155 ± 38 (21)	166 ± 48[a]	5% CO_2
	Bag system	180 ± 54 (15)	166 ± 55 (24)		159 ± 50[a]
0	5% CO_2	168 ± 71 (18)	149 ± 45 (21)	161 ± 53[a]	Bag system
	Bag system	181 ± 51 (20)	146 ± 39 (20)		168 ± 51[a]
Mean AA on IVC		175 ± 55[a]	155 ± 45[b]		

Numbers in parentheses indicate the number of blastocyst.
5% CO_2 in air and saturated humidity (20% O_2).
Bag system= 5% CO_2, 5% O_2, 90% N_2 and saturated humidity (5% O_2).
There was no effect of the interaction (P>0.05).
[a,b] in the line or column indicates difference between the means (P<0.05).

Discussion

One of the differences between *in vitro* and uterine environments is the oxygen tension. The oxygen concentration in air (20%) generally employed in embryo culture systems is considerably greater than the oxygen concentration in the oviduct and uterus of most mammals (Fischer & Bavister 1993). However, many culture systems for embryos *in vitro* produced use SOFaaci medium with 5% O_2 (Lonergan *et al*. 1999; Van Soom *et al*. 2002; Ali *et al*. 2003). This system eliminates the need for co-culture, but increases the manipulation to remove *Cumulus oophorus* cells after *in vitro* fertilization (IVF), exposing the zygotes to stress, which could also affect their development potential (Holm *et al*. 1999).

Ascorbic acid (vitamin C) is an important water-soluble antioxidant that reduces sulfhydryl radicals, scavenges free radicals and protects against endogenous oxidative DNA damage (Fraga *et al*. 1991). However, the ascorbic acid can also act as a pro-oxidant, maintaining iron and other metals in a reduced form and, thus, promoting lipid peroxidation (Yamamoto & Niki 1988; Guérin *et al*. 2001). Therefore, it has been added to *in vitro* maturation and culture media with much debate about its embryotoxic effects (Hossein *et al*. 2007).

In this experiment, the cleavage rate was similar when 100μM of ascorbic acid was added to *in vitro* maturation and/or culture media. Previous studies have also reported similarity in the cleavage rate of bovine oocytes (Blondin *et al*. 1997) and porcine (Tatemoto *et al*. 2001) *in vitro* matured in the presence or absence of 0.05mg/mL of ascorbic acid, or of 250μM of a stable ascorbic acid derivative, the 2-O-α-glucoside, respectively, after culture in 20% of oxygen in air. Also in porcines, Hossein *et al*. (2007) did not observe difference in the cleavage rate with 200μM of L-ascorbic acid in the culture medium in an atmosphere of 5% of oxygen.

It seems that the protective effect of antioxidants is more necessary in high concentrations of oxygen, when the culture is carried out with 5% CO_2 in air. Presumably it is more difficult, although more physiologically relevant, to show protective effect of antioxidant with 5% CO_2, 5% O_2 and 90% N_2 than with 5% CO_2 in air (Olson & Seidel 2000). However, in this study, the embryonic development was similar when the maturation or culture media were added of 100μM of ascorbic acid compared to groups without it, independently of the atmospheric condition of culture. Equally, in previous studies, bovine oocytes *in vitro* matured with 0.05mg/mL (Blondin *et al*. 1997) or 5mmol/L (Dalvit *et al*. 2005) of ascorbic acid, cultured under 20 or 5% of oxygen, respectively, showed meiotic maturation, *in vitro* fertilization and blastocyst rates similar to the groups without ascorbic acid. Mattos *et al*. (2007) also observed that the supplementation with 50μM of ascorbic acid to culture medium of bovine embryos under high concentration of oxygen (20% in air), resulted in blastocyst rates (35%) comparable to the group without antioxidant (30%).

Because the ascorbic acid is easily oxidized in aqueous solutions (Tolbert *et al*. 1975), it seems that when added to the medium it degrades rapidly, and may not show protective effect against the ROS. The addition of large quantities of ascorbic acid, in an attempt to offset its rapid degradation might, however, result in toxic effects to the embryos. In culture conducted under 20% of oxygen in air, embryotoxic effect of 200 and 400μM concentrations of ascorbic acid for mice embryos (Wang *et al*. 2002) and 500 and 750μM for porcine embryos has been reported (Tatemoto *et al*. 2001). The lack of positive effect with the ascorbic acid addition observed in this study may indicate that the 100μM concentration was insufficient to scavenge the ROS production at the level necessary to improve the embryonic development. Considering the possible degradation of ascorbic acid, an one determined moment of the culture, it does not seem to protect the embryos and, perhaps, its inclusion for

more times in the culture medium would be necessary during the period of embryo development. In fact, Hossein *et al*. (2007) showed that the supplementation of 200μM of L-ascorbic acid to culture medium of porcine embryo (0, 48, 96 or 120 h) under an atmosphere of 5% of oxygen, did not increase the blastocyst development. Nonetheless, when the concentration was divided in two equal halves (100 and 100μM) and added in two times of culture (0 and 48, 0 and 96, or 48 and 96 h), there was greater development until blastocyst stage, compared to the embryos cultured in the absence of antioxidant. These data show the importance of concentration and duration of embryotrophic effects of ascorbic acid, indicating that its addition at various moments of the culture, will be necessary to scavenge the ROS, being more appropriate to improve the embryonic development, that its inclusion in only one moment of culture.

In several studies, the deleterious effect of high oxygen concentration has been observed, occurring an increase in the embryonic development when the *in vitro* culture is carried out at 5% O_2 (Luvoni *et al*. 1996; Takahashi *et al*. 2000; Yuan *et al*. 2003; Kitagawa *et al*. 2004). It is suggested that low tension (5%) of oxygen can reduce the ROS formation and, consequently, reduce the oxidative stress leading to further development of the embryo. In the present study, however, deleterious effect of culture under high oxygen concentration on embryonic development wasn't observed. In other studies (Khurana & Niemann 2000; Booth *et al*. 2005; Corrêa *et al*. 2008), also did not notice differences in the blastocyst rate when the culture was performed under an atmosphere of 5 or 20% O_2. Corrêa *et al*. (2008) studied the production and expression of genes related to oxidative stress, in different atmospheres, and did not notice any difference in the cleavage rate of bovine embryos cultured under high (20% in air; 77%), or low (5%; 80%) oxygen tension.

The number of embryonic cells has been extensively used as an indicator of embryo quality (Khurana & Niemann 2000; Pereira *et al*. 2005). The hatched blastocyst cell number was not affected by culture atmosphere, which confirms the observations of Corrêa *et al*. (2008). However, Hossein *et al*. (2007) demonstrated beneficial effect in the total cell number, number of cells in the inner cell mass (ICM) and the trophectoderm (TE) of porcine embryos, when two doses of 100μM ascorbic acid were included at two different times of the culture instead of an only dose of 200μM. In this study, the 100μM ascorbic acid concentration was not sufficient to increase the embryonic development, but its presence in the culture resulted in the

production of a greater cell number in the blastocysts, and thus, improved the embryo quality.

In conclusion, the 100µM ascorbic acid addition on maturation and/or culture media did not increase the blastocyst production, but it increased the hatched blastocyst cell number, independently of embryos that were cultured in an atmosphere with 5% or 20% of oxygen.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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4 DISCUSSÃO GERAL

Embora tenha ocorrido grande avanço nos procedimentos de maturação, fecundação e cultivo *in vitro* de oócitos bovinos, a porcentagem de embriões produzidos *in vitro* com potencial de desenvolvimento continua menor que os produzidos *in vivo*. Assim, um sistema de cultivo adequado que forneça maior taxa de desenvolvimento *in vitro* de embriões com melhor qualidade é de grande interesse para a aplicação tanto na pesquisa como comercial desta biotecnologia.

O estresse oxidativo durante o cultivo tem sido enfatizado como um dos principais fatores responsáveis pela menor taxa de produção e baixa qualidade dos embriões produzidos *in vitro*. Fatores ambientais tais como exposição à luz e maior concentração de oxigênio durante o cultivo podem induzir trocas no metabolismo, conduzindo ao desequilíbrio na produção e remoção das espécies reativas de oxigênio (ROS) (KITAGAWA et al., 2004; DALVIT et al., 2005). Estas modificações podem causar estresse oxidativo e alterar importantes funções celulares incluindo o controle da expressão de diversos genes (MOUATASSIM et al., 1999), conseqüentemente pode ocorrer comprometimento da qualidade dos embriões e dos resultados obtidos com a técnica. Para proteger oócitos e embriões do estresse oxidativo durante o cultivo *in vitro*, vários antioxidantes podem ser adicionados ao meio (ALI et al., 2003). No entanto, manter o equilíbrio antioxidante-pró-oxidante nos embriões através de tal suplementação é um processo complexo (GUÉRIN et al., 2001).

Trocas rápidas na fisiologia e metabolismo do embrião se alternam durante o desenvolvimento embrionário inicial (GARDNER; LANE, 2002). Os zigotos têm baixa atividade metabólica e exibem baixos níveis de consumo de oxigênio, enquanto após a formação da blastocele o metabolismo do embrião aumenta significativamente (LEESE, 1995; HARVEY et al., 2002). Assim, a produção de espécies reativas de oxigênio pelos embriões iniciais e sua susceptibilidade a estas varia com o estágio de desenvolvimento. Portanto, a exigência da adição de antioxidantes ao meio de cultivo pode não ser a mesma nos diferentes estágios do desenvolvimento. Ao avaliar a produção de blastocistos (Capítulo 1) após maturação

in vitro com 100, 250 ou 500μM de ácido ascórbico, ou na ausência de antioxidante, observa-se que houve diferença somente no desenvolvimento embrionário no dia 9 de cultivo. No meio com concentração de 100μM a taxa de blastocistos (39%) foi superior ao grupo-controle (29%). Entretanto, quando essas concentrações foram adicionadas ao meio de cultivo *in vitro*, houve tendência ($P<0.07$) de maior produção de blastocistos já no dia 7. A produção de blastocistos com a concentração de 100μM (32%) foi superior a de 500μM (20%) de ácido ascórbico. Esta superioridade também se manteve no dia 9 de cultivo (28% x 14%, respectivamente). É provável ainda, que os efeitos benéficos da adição do ácido ascórbico tenham sido melhor demonstrados quando o antioxidante foi adicionado durante o período de cultivo *in vitro*, devido ao maior tempo (9 dias) em que os embriões foram expostos ao antioxidante, em relação ao período de maturação (22-24h). Estes resultados indicam a função protetora do ácido ascórbico no embrião produzido *in vitro*, que pode refletir uma função similar *in vivo*.

Um mecanismo antioxidante potencial no embrião inicial pode residir no seu próprio metabolismo. A produção de ATP no embrião inicial provém geralmente da glicólise, com menos de 30% sendo produzido via fosforilação oxidativa (TRIMARCHI et al., 2000). Isto sugere que o metabolismo “mais anaeróbico” do embrião inicial pode auxiliar a minimizar a produção de espécies reativas de oxigênio pela mitocôndria, e, portanto, reduzir o risco de estresse oxidativo. Este fato provavelmente explica a similaridade na taxa de clivagem entre os grupos maturados ou cultivados *in vitro* na presença de ácido ascórbico (Capítulo 1).

Poucos estudos têm pesquisado a função do ácido ascórbico na produção *in vitro* de embriões, especialmente em bovinos. Dessa forma, o ácido ascórbico tem sido usado a várias concentrações (TARÍN et al., 1994). As concentrações 100, 250 e 500μM de ácido ascórbico utilizadas neste estudo (Capítulo 1), evidenciam o efeito dose-dependente no desenvolvimento embrionário, principalmente quando o antioxidante foi adicionado ao meio de cultivo, fase em que as estruturas permanecem por maior período de tempo no mesmo ambiente. Na presente pesquisa verificou-se que à medida que a concentração de ácido ascórbico foi aumentada, menor foi a taxa de produção de blastocistos. Este fato pode ter ocorrido pelo acúmulo de ácido ascórbico que se tornou prejudicial; ou ainda, porque a quantidade de ROS neutralizada foi superior à quantidade necessária para as funções celulares fisiológicas. Entretanto, tem-se observado que a concentrações

fisiológicas as ROS participam no processo celular fisiológico (HANCOCK et al., 2001).

Ao avaliarem o efeito embriotóxico do ácido ascórbico em meio de cultivo de camundongos, Wang e colaboradores (2002) também observaram que o desenvolvimento embrionário foi afetado de maneira dose-dependente. Concentrações entre 50 e 100μM resultaram em taxas de blastocistos similares ao grupo-controle (sem antioxidante), enquanto altas concentrações (200 e 400μM) foram embriotóxicas. Em camundongos, Lane et al. (2002) demonstraram que a inclusão de 100μM de ascorbato na criopreservação de embriões em estágios de clivagem e de blastocistos, é benéfico para o subseqüente desenvolvimento embrionário, enquanto a concentração de 500μM, que é significativamente elevada comparada com os níveis fisiológicos é prejudicial ao desenvolvimento embrionário. Isto sugere que a terapia antioxidante pode ter efeitos negativos e indesejáveis se uma dosagem limite segura do antioxidante é excedida.

O ácido ascórbico apresenta atividade pró-oxidante a baixas concentrações, por manter íons e outros metais em estado reduzido e, portanto promovendo a peroxidação lipídica. Entretanto, estudos *in vitro* sugerem que altas concentrações estejam relacionadas à regeneração direta do alfa-tocoferol pela redução dos radicais tocoferil em um ciclo redox (PACKER et al.,1979; MACHLIN; GABRIEL, 1980; HALLIWELL; GUTTERIDGE, 1990). A disponibilidade relativa de metais e oxigênios livres reduz a meia-vida da vitamina C no corpo de semanas a poucas horas no cultivo, conduzindo a produção de oxidantes no meio de cultivo de células (KIPP; SCHWARTZ, 1990). Mesmo com oxigênio reduzido a 5%, é possível que a vitamina C tenha atuado em determinados momentos como pró-oxidante, e não como antioxidante, em nosso sistema de cultivo.

Ao testarmos o efeito simultâneo da adição de 100μM de ácido ascórbico aos meios de maturação e/ou cultivo *in vitro*, sob alta (20% em ar) ou baixa (5%) concentração de oxigênio durante o cultivo (Capítulo 2) observamos que não houve interação entre esses fatores. A taxa de clivagem e o desenvolvimento embrionário no D7 e D9 não diferiram entre os grupos com e sem antioxidante, independente da concentração de oxigênio durante o período de cultivo. Uma das diferenças entre ambientes *in vivo* e *in vitro* é a concentração de oxigênio. Sabe-se que sua concentração na tuba uterina e útero na maioria dos mamíferos é inferior a empregada nos sistemas de cultivo de embriões *in vitro* (FISCHER; BAVISTER,

1993), e que altas concentrações são prejudiciais ao desenvolvimento do embrião, provavelmente devido ao acúmulo de espécies reativas de oxigênio (LUVONI et al., 1996). Entretanto, neste experimento, tal fato não foi observado, possivelmente devido a presença do ácido ascórbico.

Neste estudo, as taxas de blastocistos no D7 e no D9 (Capítulo 2) foram similares entre as diferentes concentrações de oxigênio. Resultados similares foram relatados por Khurana; Niemann (2000), Booth et al., (2005) e Corrêa et al., (2008), que não observaram diferença na taxa de blastocistos quando o cultivo foi realizado com 5 ou 20% de oxigênio. Entretanto, a maioria dos estudos relata o efeito deletério de altas concentrações de oxigênio, mostrando aumento no desenvolvimento do embrião em várias espécies quando o cultivo é realizado a 5% de oxigênio (LUVONI et al., 1996; TAKAHASHI et al., 2000; GUÉRIN et al., 2001; YUAN et al., 2003; KITAGAWA et al., 2004; PETERSEN et al., 2005). Os autores sugerem que a concentração de 5% de oxigênio pode diminuir a formação de ROS e conseqüentemente diminuir o estresse oxidativo e conduzir ao maior desenvolvimento do embrião. Para evitar o estresse oxidativo durante o cultivo de oócitos e embriões é importante limitar a geração de ROS e facilitar sua neutralização.

A maioria dos sistemas de cultivo para embriões produzidos *in vitro* utilizam o meio SOFaaci com 5% O_2 (VAN SOOM et al., 2002; ALI et al., 2003) eliminando o requerimento de co-cultivo, porém exige manipulação para remoção das células do *Cumulus oophorus* após a fecundação *in vitro* (FIV). Este procedimento expõe os zigotos ao estresse que também pode afetar seu potencial desenvolvimento (HOLM et al., 1999). Entretanto, maior desenvolvimento de blastocistos é observado quando este meio é utilizado em concentrações superiores a 5% de oxigênio, sem remoção das células do *Cumulus oophorus* após a FIV (PEREIRA et al., 2005). As células do *Cumulus oophorus*, mantidas com o zigoto após a fecundação podem funcionar como neutralizadoras através da remoção de substâncias tóxicas do meio de cultivo (FUJITANI et al., 1997). Logo, o estresse oxidativo causado pela maior concentração de oxigênio pode ser minimizado na presença destas células. Isto explica a similaridade no desenvolvimento embrionário neste estudo (Capítulo 2) entre as diferentes concentrações de oxigênio utilizadas durante o período de cultivo, independente da produção *in vitro* na presença do antioxidante.

Uma alternativa para aumentar a taxa de produção de blastocistos pode ser o

uso de células em co-cultivo (MÉNÉZO et al., 1990), provavelmente por permitirem a manutenção do equilíbrio entre antioxidantes e pró-oxidantes no meio de cultivo. O efeito benéfico das células em co-cultivo pode ocorrer devido a redução da concentração de O_2 no meio, ou pela detoxicação das espécies reativas de oxigênio pelo sistema antioxidante (e.g. superóxido dismutase, glutationa peroxidase) presente nestas células. Em adição, sob condições aumentadas de estresse oxidativo, a expressão de algumas destas enzimas antioxidantes pode ser induzida. Dessa forma, os resultados contraditórios observados previamente na literatura podem ser explicados, pois um componente pode ter efeitos benéficos no desenvolvimento do embrião em um meio definido, mas não ser efetivo (ou mesmo exercer ação deletéria) em um meio diferente (GUÉRIN et al., 2001).

Em suínos, TAO et al. (2004) verificaram que 50, 250 ou 750μM de L-ácido ascórbico podem substituir parcialmente as células do *Cumulus oophorus* em oócitos desnudos por promoverem a maturação meiótica, especialmente da metáfase I à metáfase II. Entretanto, em complexos *cumulus*-oócitos (CCO) a maturação meiótica não foi alterada, aparentemente devido a existência das células do *Cumulus oophorus* circunvizinhas. Em bovinos, estas células contribuem para a síntese da glutationa (GSH) no oócito durante a maturação *in vitro* (DE MATOS et al., 1997), importante na proteção celular contra o estresse oxidativo (MEISTER, 1983). Assim, quando CCO bovinos são fecundados *in vitro* na presença de peróxido de hidrogênio (H_2O_2), as células do *Cumulus oophorus* protegem contra o estresse oxidativo por catabolizar espécies reativas de oxigênio em componentes menos tóxicos (FATEHI et al., 2005).

O número de células embrionárias tem sido extensivamente usado como um indicador da qualidade do embrião. Neste estudo, o número total de células de blastocistos produzidos *in vitro* não diferiu entre as diferentes concentrações de ácido ascórbico avaliadas e o grupo-controle (Capítulo 1). Entretanto, com a inclusão de 100μM ácido ascórbico no cultivo (Capítulo 2), o número de células dos blastocistos eclodidos foi maior ($P<0,05$) do que na ausência do antioxidante. Ao considerarmos as diferentes condições atmosféricas, neste estudo (Capítulo 2) e no de Corrêa et al., (2008), o número total de células dos embriões produzidos *in vitro* nos dois sistemas (5 ou 20% O_2) foi similar, sugerindo que a qualidade dos embriões produzidos não foi afetada pelas condições de cultivo. Em adição, Hossein et al. (2007) também não observaram diferença no número total de células com 200μM de

ácido ascórbico no meio de cultivo sob 5% de oxigênio quando comparado ao grupo de oócitos controle (sem antioxidante). Entretanto, o número de células aumentou quando este antioxidante foi dividido e adicionado em duas metades iguais (100 e 100μM).

Fica claro que os sistemas de cultivo constituem condições limitadas para o desenvolvimento do embrião e que o estresse oxidativo é inevitável. O entendimento dos mecanismos antioxidantes presentes no embrião e sua vizinhança irá auxiliar a identificação de meios de cultivo mais adequados e que proporcionem melhor qualidade aos embriões produzidos *in vitro*.

Para otimizar a produção de embriões *in vitro*, o estresse oxidativo deve ser controlado também durante o cultivo *in vitro*. Oócitos e embriões devem ser protegidos reduzindo-se a concentração de oxigênio no ambiente gasoso e/ou adicionando substâncias ao meio de cultivo.

5 CONCLUSÕES

1. A concentração de 100µM de ácido ascórbico durante a maturação ou cultivo *in vitro* aumenta a produção de blastocistos no D9.

2. A taxa de blastocistos produzidos no D9 diminui com a adição de 500µM de ácido ascórbico durante o período de cultivo.

3. As concentrações 100, 250 e 500µM de ácido ascórbico na MIV ou CIV produziram embriões com qualidade morfológica semelhante ao dos embriões produzidos em meio sem antioxidante.

4. Não existe interação entre a presença de ácido ascórbico na maturação, a presença de ácido ascórbico no cultivo e a atmosfera gasosa.

5. A produção de embriões não se modifica em meio de maturação e/ou cultivo com 100µM de ácido ascórbico, independentemente da atmosfera de cultivo.

6. O número de células dos blastocistos eclodidos aumenta quando 100µM de ácido ascórbico é adicionado durante o CIV.

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