In vitro oocyte transport through the oviduct of buffalo and crossbred beef cows

Transporte de oócitos in vitro através do oviduto de búfalas e vacas de corte cruzadas

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Abstract

The present study was conducted to verify if the elevation of plasma concentrations of estradiol during superovulatory treatments affects the oocyte transport in buffalo females, as well as if the inferior quality of buffalo oocytes and/or some functional difference on the oviduct of these animals is responsible for the low embryo recovery rate in superovulated buffaloes when compared to cows subjected to the same treatment. Oviducts of 10 buffaloes and 15 of cows, treated to induce a single ovulation were used. The oviducts were placed on Petri dishes and received the following treatments: 5 buffalo oocytes with no E2 (G-BufBuf and G-BovBuf), 5 bovine oocytes with no E2 (G-BufBov and G-BovBov), 5 buffalo oocytes with E2 (G-BufE2Buf and G-BovE2Buf) and 5 bovine oocytes with E2 (G-BufE2Bov and G-BovE2Bov; factorial 2x2x2). Oocytes were incubated for 24h. Subsequently, oviducts were washed and oocytes were recovered and counted. Since no interactions were found between E2 treatment, oviducts and oocytes species, main effects were analyzed separately. Recovery rate and number of oocytes was higher on cattle compared to buffaloes (35.0+8.6% and 1.4+0.3 vs. 10.0±4.6% and 0.5±0.2, respectively; $p<0.05$); no effect of E2 treatment was observed on recovery rate and number of oocytes (29.8±9.0% and 1.3±0.4 vs. 16.9±6.1% and 0.7±0.2, respectively; $p>0.05$); the number of buffaloes and bovine oocytes recovered were similar (1.4±0.4 and 0.6±0.2, respectively; $p>0.05$). Oocytes recovery rate showed a trend ($P=0.07$) to be higher when buffalo oocytes were implanted when compared to bovine oocytes (35.2±9.2% vs. 12.9±5.4%). Present results suggest that oocyte transport by the oviduct of buffaloes and bovine was not dependent on oocytes species or E2 supplementation to the culture medium.

Keywords: MOET. Oocytes. Estradiol. *In vitro*. Buffaloes.

Resumo

O presente estudo foi realizado para verificar se a elevação das concentrações plasmáticas de estradiol durante os tratamentos superovulatórios afeta o transporte dos oócitos em fêmeas bubalinas, bem como se a qualidade inferior dos oócitos de búfalos e/ou alguma diferença funcional no oviduto destes animais é responsável pela baixa taxa de recuperação de embriões em búfalas superovuladas quando comparadas a vacas submetidas ao mesmo tratamento. Foram utilizados 10 ovidutos de búfalas e 15 de vacas, tratadas para a indução de ovulação única. Os ovidutos foram colocados em placas de Petri e receberam os seguintes tratamentos: sem E2 e inseridos com 5 oócitos de búfalas (G-BufBuf e G-BovBuf); sem E2 e com 5 oócitos de vacas (G-BufBov e G-BovBov); com E2 e com 5 oócitos de búfalas (G-BufE2Buf e G-BovE2Buf); e com E2 e com 5 oócitos de vacas (G-BufE2Bov e G-BovE2Bov; fatorial 2x2x2). Posteriormente, foram incubados por 24h e, após esse período, foram lavados para a recuperação e contagem dos oócitos. Como não foi verificado efeito de interação, foram analisados os efeitos principais. O número e a taxa de recuperação de oócitos foi maior em ovidutos de vacas que de búfalas (1,4±0,3/35,0±8,6% vs. 0,5±0,2/10,0±4,6%; $P<0,05$). Foi verificado que o tratamento com ou sem E2 não interferiu no número e na taxa de recuperação de oócitos (1,3±0,4/29,8±9,0% vs. 0,7±0,2/16,9±6,1%; $P>0,05$). Não foi verificada diferença no número de oócitos de búfalas ou de vacas recuperados (1,4±0,4 e 0,6±0,2; $P>0,05$). Observou-se também que houve tendência ($P=0,07$) de maior taxa de recuperação de oócitos de búfalas que de vacas (35,2±9,2% vs. 12,9±5,4%). Os dados são indicativos de que o transporte de oócitos pelo oviduto de búfalas e de vacas independe da espécie do oócito e não é influenciado pelo E2.

Palavras-chave: MOET. Oócitos. Estradiol. *In vitro*. Búfalos.

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Introduction

The birth of buffaloes using Multiple Ovulation and Embryo Transfer (MOET) has been reported in Brazil and other countries[1,2]. However, in large-scale, the use of such technique is limited due to the low embryo recovery rate[1,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21].

Buffalo females respond to superovulatory treatments, however, less embryos are recovered comparing to bovine females. Baruselli[22] verified that the buffalo species respond to the superovulatory treatments with ovulation rates of 62.8%, similarly to that observed by Stock, Ellington and Fortune[23] in the bovine species. However, only 34.8% of ovulations of buffaloes subjected to superestimulation of the follicular growth resulted in the recovery of embryonic structures[24]. Conversely, Adams[25] reported recovery rates between 63 and 80% in superovulated bovine females. These differences between the embryo recovery rates in these species may be associated to failure in the capture process and/or oocytes transport by the oviduct[24,26].

Bellve and McDonald[27] verified in superovulated small ruminant that oviduct retro peristalsis occurred due to high concentration of estradiol (E_2), which could explain the low embryo recovery rate found in buffaloes submitted to the same treatment. Additionally, Kolle et al.[28] found that oviductal epithelium is able to select live oocytes (mature and immature) in mares; however, as soon as the oocyte is degenerated, it floats in the oviductal lumen. This implies that the oviduct is able to select live oocytes. Moreover, the fragile connection between oocytes and granulosa cells may negatively influence transport of these gametes. According to Hunter[29], Lam et al.[30] and Talbot, Shur and Myles[31], the pick up process by the infundibulum, as well as the transport of oocytes across the oviduct depend on the quality of the female gamete.

The elevation on the estradiol/progesterone proportion may damage the interaction between the oocytes and the endossalpinge ciliated cells during ovulations. The absence of this interaction promotes failure in the oocytes capture, because, according to Hunter[29], during the ovulation, the oviduct fluid flows directly to the abdominal cavity. Therefore, the understanding of physiological processes that involve E_2 profile, as well as the quality and transport of oocytes, is extremely important in order to improve MOET efficiency in buffaloes.

The aim of the present study was to test the hypothesis that *in vitro* oocyte transport through the oviduct of buffalo and crossbred beef cows is influenced by oviduct species and/or by gamete species and/or by the presence of E_2 in culture medium. Towards this end, buffalo and bovine oviducts collected during the post-ovulatory period (i.e. period of higher development and activity of oviduct cilia[29]) and incubated in culture medium supplemented or not with E_2, received bovine or buffalo oocytes inserted through the infundibulum.

Material and Method

This project is in accordance with Ethical Principles in Animal Research adopted by Bioethic Commision of the Faculty of Veterinary Medicine and Animal Science of University of São Paulo and was approved "*ad referendum*" (protocol number 175/2002). The first part of the experiment was performed at the Cachoeirinha livestock (Poços de Caldas - MG, Brazil) in July 2005 using six years old crossbred bovine females (n=15; *Bos indicus x Bos taurus*), showing body score condition > 4.0 (scale of 1–5, where 1=very thin and 5=very fat). The second part of the experiment was conducted in January 2006, at the Barra do Capinzal farm (Registro - SP, Brazil), using six years old Murrah buffalo cows (n=10), also showing body score condition > 4.0. These animals were maintained on a *Brachiaria decumbens* pasture with free access to water and mineralized salt.

The experiment was performed in a 2x2x2 factorial arrangement; the factors were oviduct species (buffalo vs. cattle), E_2 treatment (with vs. without) and oocyte species (buffalo vs. cattle).

Estrous cycles synchronization was performed in all animals using an intravaginal progesterone device (P_4; DIB®; MSD Animal Health, Brazil) and 2mg of Estradiol Benzoate (IM; Benzoato de Estradiol®; Tecnopec, Brazil) on Day 0 (AM). Devices were removed after 8 days (Day 8, AM), when animals received a single injection of 0.15mg of prostaglandin (IM; d-cloprostenol; Prolise®; Tecnopec, Brazil) and 400IU of eCG (IM; Novormon®; MSD Animal Health, Brazil). Twenty four hours later (Day 9, AM), animals received 25µg of GnRH (IM; Lecirelina; Gestran-Plus®; Tecnopec, Brazil). Females were slaughtered 48 hours after ovulation induction (Day 11, AM; Figure 1).

Bovine females were slaughtered at the Frigonossa Abattoir (Poços de Caldas - MG), and buffalo females were slaughtered at the Frivale Abattoir (Cajati - SP). Females were eviscerated and the reproductive tract removed. The ipsilateral and contralateral oviducts to the ovary containing the ovulatory follicle were recovered from the ovaries containing one or more CLs and placed into an insulated container in D-PBS solution (glucose 1000 mg, sodium pyruvato 36 mg, penicilin 1.000.000 IU, streptomycin 50 mg and amphotericin B 250 µg), at 38.0ºC. The container was transported to the laboratory in less than 5 hours, which allowed the temperature to remain between 37.0 and 38.0ºC.

In order to optimize and reduce the number of slaughtered animals, both oviducts, ipsilateral and contralateral to the ovulated ovary, were used. According to Havlicek et al.[32], the oviduct contralateral to the ovulated ovary may fulfill the necessary requirements to the embryo culture in the bovine species. Therefore, functional modifications of the ipsilateral oviduct may occur also in the contralateral oviduct since the activities of both oviducts are partially coordinated by hormonal mechanisms affected by systemic concentrations of E_2 and P_4[33].

Bovine oocytes were collected from 20 ovaries obtained from abattoirs; buffalo oocytes were collected by *in vivo* follicular aspiration from 15 buffaloes. In the laboratory (Unidade de Biologia Celular, Centro de Sanidade Animal, Instituto Biológico, São Paulo - SP), both buffalo and cattle cumulus oocyte complexes (COCs) were washed using TCM199 culture medium with HEPES added to 10% bovine fetal serum, 10µl of piruvate, 0.2mM and 25µl of gentamicine. COCs were then matured in 4.5ml of TCM199 medium with bicarbonate added to 10% bovine fetal serum, 10µl of piruvate, 0.2mM and 25µl of gentamicine, 5µl of estradiol, 5µl of FSH and 50µl of LH for 24 hours in a stove/oven with 38.5ºC; 5% of CO_2 and 92% of humidity. Only matured oocytes (i.e. oocytes showing expanded cumulus cells) were used, totalizing 80 cattle and 70 buffalo oocytes. The considered criteria for the CCOs evaluation were the number of layers and the compactation level of the *Cumulus* cells, as

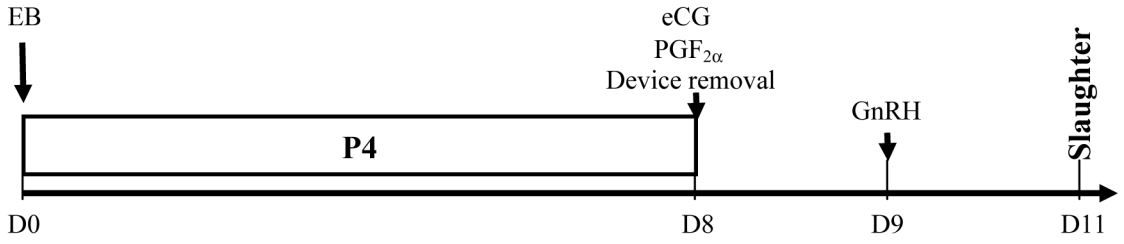

Figure 1 - Schematic diagram of treatments to synchronize ovulation in buffaloes. EB = 2.0 mg Estradiol Benzoate; $PGF_{2\alpha}$ = 0.15 mg d-cloprostenol; eCG = 400 IU equine Chorionic Gonadotropin; GnRH = 25 µg Buserelin acetate

well the cytoplasm aspect as much as the color, homogeneity and integrity[34].

In the laboratory, oviducts were washed in Hanks balanced solution (HBSS) supplemented with 25mM of HEPES. Afterwards, each oviduct was placed in a Petri dish (150 x 25mm) and, in the anterior portion of the infundibulum, 5 matured buffalo oocytes (G-BufBuf, G-BufE2Buf, G-BovBuf and G-BovE2Buf) and 5 matured cattle oocytes (G-BufBov, G-BufE2Bov, G-BovBov and G-BovE2Bov), together with 50µl of maturation medium, were inserted using a 1ml syringe connected to a Tom Cat catheter. All oviducts were immersed in 100ml of maturation medium, supplemented (G-BufE_2Buf, G-BufE_2Bov, G-BovE_2Buf and G-BovE_2Bov) or not (G-BufBuf, G-BufBov, G-BovBuf and G-BovBov) with 10ng/mL of 17β-estradiol (Sigma, Brazil; Table 1).

Each Petri dish, containing the oviducts with the oocytes, was incubated for 24 hours, at 38.5°C in a humidified 5% CO_2 incubator. After incubation, oviducts were washed with 40ml of washing medium (D-PBS) supplemented with 1% bovine fetal serum inserted in the infundibulum with an intramammary cannula. The effluent of each oviduct was collected separately, placed into 100 x 20mm Petri dishes, and evaluated through stereomicroscope (SMZ-2T) at 20-30X magnification in order to quantify the number of oocytes.

All data were evaluated using SAS System for Windows[35]. The effects of oviduct species (buffalo vs. cattle), oocytes species (buffalo vs. cattle) and E_2 treatment (E_2 vs. control), as well as the second and third order interactions, were determined by PROC GLM. Differences between treatments were analyzed using parametric tests (GLM procedure for each factor separately or LSD when combining factors) and non-parametric tests (Wilcoxon), according to the residue normality (Gaussian distribution) and variance homogeneity. Number of recovered oocytes and recovery rate did not have randomly distributed residuals and no transformation was effective; therefore, data were analyzed using non-parametric procedures. A probability value of $p<0.05$ was considered significant. Results were reported as untransformed means ± S.E.M.

Results

Recovery rate and number of oocytes were higher in Group G-BovE_2Buf ($p<0.05$; Table 2) when compared to Groups G-BufBuf, G-BufBov, G-BufE_2Bov. On the other hand, no differences were found between the first and Groups G-BufE_2Buf, G-BovBuf and G-BovE_2Bov ($p>0.05$; Table 2).

Considering that no interactions were found between oviducts species, oocytes species and E_2 treatment for any variables, the main effects of each factor was tested. The oocytes number and recovery rates were higher on bovine oviducts when compared to the buffalo ($p<0.05$; Table 2). However, buffalo oocytes tended to be recovered ($p=0.07$) in higher rates than cattle oocytes. No effect of E_2 supplementation

Table 1 – Experimental groups according to species, treatment and number of oocytes

Groups	Specie (number of oviducts)	Treatments	Number of oocytes matured (animal species)
G-BufBuf	buffaloes (n=3)	without E_2	5 (buffaloes)
G-BufBov	buffaloes (n=4)	without E_2	5 (cattle)
G-BufE2Buf	buffaloes (n=3)	with E_2	5 (buffaloes)
G-BufE2Bov	buffaloes (n=4)	with E_2	5 (cattle)
G-BovBuf	cattle (n=4)	without E_2	5 (buffaloes)
G-BovBov	cattle (n=4)	without E_2	5 (cattle)
G-BovE2Buf	cattle (n=4)	with E_2	5 (buffaloes)
G-BovE2Bov	cattle (n=4)	with E_2	5 (cattle)

Table 2 - Number and recovery rate of buffalo and cattle of oocytes introduced (*in vitro*) through the oviduct of buffaloes and cows and cultured in the presence or abscence of E_2 in the culture medium

Dependent variables	Mean ± SEM								Effects (P > F)					
	G-BufBuf (n=3)	G-BufBov (n=4)	G-BufE2Buf (n=3)	G-BuffE2Bov (n=4)	G-BovBuf (n=4)	G-BovBov (n=4)	G-BovE2Buf (n=4)	G-BovE2Bov (n=4)	Specie	Treatment	Oocyte	Sp.*Treat.	Sp.*Ooc.	Treat.*Ooc.	Sp.*Treat.*Ooc.
Number of oocytes recovered	0.3±0.3[b]	0.2±0.2[b]	1.3±0.9[a,b]	0.2±0.2[b]	1.5±0.5[a,b]	0.5±0.3[b]	2.2±0.8[a]	1.5±0.64[a,b]	0.03	0.09	0.07	0.63	0.71	0.63	0.43
Oocyte recovery rate (%)	6.7±6.7[b]	5.0±5.0[b]	26.7±17.6[a,b]	5.0±5.0[b]	43.3±15.7[a,b]	10.0±5.8[b]	55.0±22.2[a]	31.7±18.3[a,b]	0.02	0.19	0.06	0.74	0.41	0.80	0.46

	MAIN EFFECTS				
	Oviduct of Buffalo	Oviduct of Cattle	Without Estradiol	With Estradiol	Oocyte of Buffalo	Oocyte of Cattle
Number of oocytes recovered	0.5±0.2[b]	1.4±0.3[a]	0.7±0.2	1.3±0.4	1.4±0.4	0.6±0.2
Oocyte recovery rates (%)	10.0±4.6[b]	35.0±8.6[a]	16.9±6.1	29.8±9.0	35.2±9.2[x]	12.9±5.4[y]

[a,b] Means within a line with different superscripts differ (p<0.05)
[x,y] Means within a line with different superscripts indicate a trend (p=0.07)

to the culture medium was observed for both number and rate of oocyte recovery (p>0.05; Table 2).

Discussion

Results of the present experiment demonstrated that the number and rate of oocyte recovery were higher in the oviducts of bovine females when compared to the buffalo; regardless E_2 treatment and oocytes species (Table 2). It is likely that anatomic and/or functional difference between cattle and buffalo oviducts exist, which could have compromised oocyte recovery after *in vitro* flushing of buffalo oviducts. Carvalho[36], in a histology examination, verified that the isthmus of buffaloes showed a thicker muscular layer when compared to bovine cows, which could cause the narrowing of the uterine ostium. This, in turn, could cause the disintegration of some oocytes during the oviduct flushing, resulting in decreased number and rate of oocytes recovery.

Despite the higher oocyte recovery rate observed in the cattle oviducts when compared to the buffalo's (35.0±8.6% and 10.0±4.6%, respectively), results were lower than expected. Because conception rates of bovine and buffalo females submitted to protocols for synchronization of ovulation for fixed time artificial insemination (FTAI) are close to 50%[37,38,39,40,41,42], we expected a recovery rate similar to this value. This could be explained by the culture interval (24 hours), which could have caused modifications on the oviducts, leading to the low recovery rates of both buffalo and cattle oocytes.

E_2 supplementation to the culture medium had no effect on the rate and number of recovered oocytes, independently on the oocytes and oviducts species. Therefore, our hypothesis that E_2 would influence oocytes transit in buffalo cows could not be confirmed *in vitro*.

Results of the present experiment do not agree with previous experiments showing differences on the

quality of oocytes depending on the species. According to Gasparrini[43], due to a more fragile connection between the granulosa cells found in buffaloes COCs, buffalo oocytes are considered to exhibit an inferior quality when compared to cattle oocytes. Therefore, we expected a higher recovery rate of cattle oocytes, instead of the trend found for a higher recovery rate for buffalo oocytes in the present experiment. We believe that oocyte transit throughout the oviduct of both cattle and buffalo cows do not depend on oocyte quality.

Probably, oocyte quality would play an important role on oocyte transport after follicular collapse and further pick up by the infundibulum *in vivo*. Baruselli et al.[44] observed increased embryonic structures recovery rates in superovulated buffaloes previously treated with rBST, which could be related to an improved oocyte quality. On the other hand, Carvalho et al.[45] found low embryonic structures recovery rate two and five days after the first AI in buffaloes submitted to a MOET protocol. The authors suggested that oocyte pick up by the infundibulum but not the oocyte transport through the oviduct would be the key factor to the lower recovery rate in buffaloes. Possibly, within the oviduct, the number of cumulus cells, as well as the degree of adhesion between those cells and the oocyte, renders no influence on oocyte transport. To support this possibility, according to Lam et al.[30], the cumulus cells are eliminated during oocyte transit from the infundibulum to the fecundation point.

Conclusion

Results of the present experiment indicate that, opposite to our hypothesis, *in vitro* oocyte transport through the oviduct of buffalo and crossbred beef cows is not influenced by gamete species or by the presence of 17β-estradiol in the culture medium.

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References

1. BARUSELLI, P. S. Basic requirements for artificial insemination and embryo transfer in buffaloes. **Buffalo Journal**, v. 2, p. 53-60, 1994.

2. DROST, M.; WRIGHT-JUNIOR, J. M.; CRIPE, W. S.; RICHTER, A. R. Embryo transfer in water buffalo (*Bubalus bubalis*). **Theriogenology**, v. 20, p. 549-585, 1983.

3. AMBROSE, J. D; SINGLA, S. K; SAILKHANI, S.; PRAKASH, B. S.; MADAN, M. L. Superovulation response in buffaloes (*Bubalus bubalis*) to different treatment regimes of folltropin. **Theriogenology**, v. 35, p. 181, 1991.

4. BARUSELLI, P. S. **Dinâmica folicular durante o ciclo estral e resposta superovulatória em fêmeas bubalinas (*Bubalus bubalis*)**. 1997. 96 f. Tese (Doutorado em Medicina Veterinária) - Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 1997.

5. BARUSELLI, P. S.; MUCCIOLO, R. G.; ARRUDA, R. P.; MADUREIRA, E. H.; AMARAL, R.; ASSUMPÇÃO, M. E. O. A. Embryo recovery rate in superovulated buffalo. **Theriogenology**, v. 51, p. 401, 1999.

6. BARUSELLI, P. S.; MADUREIRA, E. H.; VISINTIN, J. A.; OHASHI, O. M.; SOUZA, J. S.; PARMEGGIANI, A.; CAMPANILE, G.; ZICARELLI, L. Follicular dynamics and embryo recovery rate in superovulated buffalo. **Arquivos da Faculdade de Veterinária da UFRGS**, v. 27, p. 200, 1999.

7. BARUSELLI, P. S.; MADUREIRA, E. H.; BRAGA, D. P. A. F.; PORTO FILHO, R. M.; CARVALHO, N. A. T.; FRANZOLIN, R. GnRH injection effect on ovulation rates in superovulated buffalo. **Revista Brasileira de Reprodução Animal**, v. 25, p. 549-552, 2001.

8. BARUSELLI, P. S.; MARQUES, M. O.; ARRUDA, R. P.; CARVALHO, N. A. T.; OLIVEIRA, C. A. Plasma estradiol and progesterone concentrations in superovulated buffalo in presence of CIDR-B devices. **Theriogenology**, v. 57, p. 761, 2002.

9. CARVALHO, N. A. T.; BARUSELLI, P. S.; ZICARELLI, L.; MADUREIRA, E. H.; VISINTIN, J. A.; D'OCCHIO, M. J. Control of ovulation with GnRH agonist subsequent to superovulation of follicular growth in buffalo: fertilization and embryo recovery. **Theriogenology**, v. 58, p. 1641-1650, 2002.

10. MADAN, M. L.; DAS, S. K.; PALTA, P. Application of reproductive technology to buffaloes. **Animal Reproduction Science**, v. 42, p. 299-306, 1996.

11. MISRA, A. K.; JOSHI, B. V.; AGRAWALA, P. L.; KASIRAJ, R.; SIVAIAH, S.; RANGAREDDI, N. S.; SIDDIQUI, M. U. Multiple ovulation and embryo transfer in indian buffalo (*Bubalus bubalis*). **Theriogenology**, v. 33, p. 1131-1141, 1990.

12. MISRA, A. K.; KASIRAJ, R.; MUTHA-RAO, M.; RANGA-REDDY, N.S.; JOSHI, B.V. Embryo transfer in buffalo in Índia: progress in the last five years. In: WORLD BUFFALO

CONGRESS, 4., 1994, São Paulo, Brazil. **Proceedings…** São Paulo: WBC, 1994. v. 3, p. 501-503.

13. PRAKASH, B. S.; JAILKHANI, S.; SINGLA, S. K.; MADAN, M. L. Application of a sensitive, heterologous enzymeimmunoassay for progesteronae determination in unextracted buffalo plasma samples collected in na embryo transfer experiment. **Animal Reproduction Science**, v. 27, p. 67-74, 1992.

14. TANEJA, M.; TOTEY, S. M.; ALI, A. Seasonal variation in follicular dynamics of superovulated Indian water buffalo. **Theriogenology**, v. 43, p. 451-464, 1995.

15. TANEJA, M.; SINGH, G.; TOTEY, S. M.; ALI, A. Follicular dynamics in water buffalo superovulated in presence or absence of a dominant follicle. **Theriogenology**, v. 44, p. 581-597, 1995.

16. ZICARELLI, L.; DI-PALO, R.; CAMPANILE, G.; BONI, R.; LANGELLA, M. RBST+FSH-P in superovulatory treatment of Italian mediterranean buffaloes. In: WORLD BUFFALO CONGRESS, 4., 1994, São Paulo, Brazil. **Proceedings…** São Paulo: WBC, 1994. v. 3, p. 459-461.

17. ZICARELLI, L.; BONI, R.; PACELLI, C.; ESPOSITO, L.; SPADETTA, M. Superovulation in italian mediterranean buffaloes using FSH-P with diferent treatments. In: WORLD BUFFALO CONGRESS, 4., 1994, São Paulo, Brazil. **Proceedings…** São Paulo: WBC, 1994. v. 3, p. 462-464.

18. ZICARELLI, L.; BARUSELLI, P. S.; CAMPANILE, G.; DI-PALO, R.; GASPARRINI, B.; NEGLIA, G.; D'OCCHIO, M. J. Embryo recovery in buffalo with timed ovulation and insemination subsequent to follicle superstimulation. In: INTERNATIONAL CONGRESS ON ANIMAL REPRODUCTION, 14., 2000, Stockholm Sweden. **Proceedings…** Stockholm: ICAR, 2000. v. 2, p. 16-19.

19. BARUSELLI, P. S.; CARVALHO, N. A. T. Biotecnologias da reprodução em bubalinos (*Bubalus bubalis*). **Revista Brasileira de Reprodução Animal**, v. 29, n. 1, p. 4-17, 2005.

20. CARVALHO, N. A. T.; NAGASAKU, E. M.; VANNUCI, F. S.; TOLEDO, L. M.; CARVALHO, J. B. P.; CARVALHO, M. I. A. B.; GIMENES, L. U.; AYRES, H.; MARTINS, C. M.; BARUSELLI, P. S. Use of different doses of rBST associated to a protocol for multiple ovulation and embryo transfer in buffalo (*Bubalus bubalis*). **Italian Journal of Animal Science**, v. 6, p. 652-654, 2007.

21. CARVALHO, N. A. T.; BOMBONATO, P. P.; D'ANGELO, M.; BARUSELLI, P. S. Caracterização anatomofuncional do sistema genital de fêmeas bubalinas (*Bubalus bubalis*) e suas implicações na múltipla ovulação e transferência de embriões. **Revista Brasileira de Reprodução Animal**, v. 35, p. 95-103, 2011.

22. BARUSELLI, P. S. Folliculogenesis in buffalo. **Bubalus Bubalis**, v. 4, p. 79-92, 1997.

23. STOCK, A. E.; ELLINGTON, J. E.; FORTUNE, J. E. A dominant follicle does not affect follicular recruitment by superovulatory doses of FSH in cattle but can inhibit ovulation. **Theriogenology**, v. 45, p. 1091-1102, 1996.

24. BARUSELLI, P. S.; MADUREIRA, E. H.; VISINTIN, J. A.; PORTO FILHO, R. M.; CARVALHO, N. A. T.; CAMPANILE, G.; ZICARELLI, L. Failure oocyte entry into oviduct in superovulated buffalo. **Theriogenology**, v. 53, p. 491, 2000.

25. ADAMS, G. P. Control of ovarian follicular wave in cattle: implications for syncronization and superstimulation. **Theriogenology**, v. 41, p. 19-24, 1994.

26. CARVALHO, N. A. T.; VANNUCI, F. S.; D´ANGELO, M.; GALLUPO, A. G.; MELO, G. M.; SOUZA, R. J. ; NICHI, M.; GIMENES, L. U.; SÁ FILHO, M. F.; MARTIZ, C. C.; CASTRICINI, E. S. C.; BARUSELLI, P. S. Oocytes transport across the oviduct of Murrah and Nelore cows. **Italian Journal of Animal Science**, v. 6, p. 649-651, 2007.

27. BELLVE, A. R.; MCDONALD, M. F. Directional flow of fallopian tube secretion in the ewe at onset of the breeding season. **Journal of Reproduction and Fertility**, v. 22, p. 147-149, 1970.

28. KOLLE, S.; DUBIELZIG, S.; REESE, S.; WEHREND, A.; KONIG, P.; KUMMER, W. Ciliary Transport, Gamete Interaction, and Effects of the Early Embryo in the Oviduct: Ex Vivo Analyses Using a New Digital Videomicroscopic System in the Cow. **Biology of Reproduction**, v. 81, p. 267–274, 2009.

29. HUNTER, R. H. F **The fallopian tubes: their role in fertility and infertility**. Berlim, Germany: Springer-Verlag, 1988. p.185.

30. LAM, X.; GIESEKE, C.; KNOLL, M.; TALBOT, P. Assay and importance of adhesive interaction between hamster (*Mesocricetus auratus*) oocyte-cumulus complexes and the oviductal epithelium. **Biology of Reproduction**, v. 62, p. 579-588, 2000.

31. TALBOT, P.; SHUR, B.D.; MYLES, D. G. Cell adhesion and fertilization: steps in oocyte transport, sperm-zona pellucida interactions, and sperm-egg fusion. **Biology of Reproduction**, v. 68, p. 1-9, 2003.

32. HAVLICEK, V.; WETSCHER, F.; HUBER, T.; BREM, G.; MUELLER, M.; BESENFELDER, U. In vitro culture of IVM/IVF embryos in bovine oviducts by transvaginal endoscopy. **Journal of Veterinary Medicine of Austria**, v. 52, p. 94-98, 2005.

33. HAFEZ, E. S. E.; HAFEZ, B. **Reprodução animal**. São Paulo, Brazil: Manole, 2004. 513 p.

34. LEIBFRIED, L.; FIRST, N. L. Characterization of bovine follicular oocytes and their ability to mature *in vitro*. **Journal of Animal Science**, v. 48, p. 76-86, 1979.

35. STATISTICAL ANALYSIS SYSTEM. **SAS-STAT user's guide**. Cary: SAS, v. 2, 1990. p. 891-996.

36. CARVALHO, N. A. T. **Avaliação anatomofuncional do sistema genital de fêmeas bubalinas (*Bubalus bubalis*), e suas implicações na múltipla ovulação e transferência de embriões.** 2006. 145 f. Tese (Doutorado Reprodução Animal) - Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2006.

37. BARUSELLI, P. S.; MADUREIRA, E. H.; VISINTIN, J. A.; BARNABE, V. H.; BARNABE, R. C.; AMARAL, R. Inseminação artificial em tempo fixo com sincronização da ovulação em bubalinos. **Revista Brasileira de Reprodução Animal**, v. 23, p. 360-362, 1999.

38. BARUSELLI; P. S.; MADUREIRA, E. H.; BARNABE, V. H.; BARNABE, R. C.; BERBER, R. C. A.; AMARAL, R. Timed insemination using synchronisation of ovulation in buffalo. In: INTERNATIONAL CONGRESS ON ANIMAL REPRODUCTION, 14., Stockholm Sweden. **Proceedings…** Stockholm: ICAR, 2000. p. 14-18.

39. BARUSELLI, P. S.; AMARAL, R.; BARUFI, F. B.; VALENTIM, R.; MARQUES, M. O. Lecirelin and Buserilin (Gonadotrophin releasing hormone agonists) are equally effective for fixed time insemination in buffalo. **Brazilian Journal of Veterinary Research and Animal Science**, v. 38, p. 142-145, 2001.

40. BARUSELLI, P. S.; CARVALHO, N. A. T.; REICHERT, R. H.; HENRIQUEZ, C. E. P.; NICHI, M. Pre-synchronization with GnRH 7 days before ovsynch protocol for timed insemination in buffalo. In: BUFFALO SYMPOSIUM OF AMERICAS, 1., 2002, Belém, Pará. **Proceedings…** Belém, Pará: BSA, 2002. p. 414-417. 2002.

41. BARUSELLI, P. S.; MARQUES, M. O.; NASSER, L. F. T.; REIS, E. L.; BÓ, G. A. Effect of eCG on pregnancy rates of lactating zebu beef cows treated with CIDR-B devices for timed artificial insemination. **Theriogenology**, v. 59, p. 214, 2003.

42. BARUSELLI, P. S.; MADUREIRA, E. H.; MARQUES, M. O.; RODRIGUES, C. A.; NASSER, L. F. T.; SILVA, R. C. P.; REIS, E. L.; SÁ-FILHO, M. F. Efeito do tratamento com eCG na taxa de

concepção de vacas nelore com diferentes escores de condição corporal inseminadas em tempo fixo (análise retrospectiva). **Acta Scientiae Veterinariae**, v. 32, p. 228, 2004.

43. GASPARRINI, B. In vitro embryo production in buffalo species: state of the art. **Theriogenology**, v. 57, p. 237-256, 2002.

44. BARUSELLI, P. S.; CARVALHO, N. A. T.; CAVALCANTE, A. K. S.; NICHI, M.; ZICARELLI, L. Use of rBST associated to a protocol for multiple ovulation and embryo transfer in buffalo (*Bubalus bubalis*). In: CONGRESSO NAZIONALE SULL'ALLEVAMENTO DEL BUFFALO, 2., 2003, Roma, Italy. **Atti…** 2003. v. 1, p. 269-273.

45. CARVALHO, N. A. T.; ZICARELLI, L.; CAVALCANTE, A. K. S.; NICHI, M.; BARUSELLI, P. S. Embryo recovery rate in buffalo female according to the day of flushing. In: WORLD BUFFALO CONGRESS, 7., 2004, Manila, Philippines. **Proceedings…** Makati:WBC, 2004. v. 2, p. 756-757.