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Establishing an in vitro production program for buffalo embryos (Bubalus bubalis) in Colombia

Establecimiento de un programa de producción in vitro de embriones bufalinos (Bubalus bubalis) en Colombia

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ABSTRACT

Objective. Evaluate the results of the standardization of the in vitro production program of buffalo embryos, using oocytes obtained by ultrasound guided oocyte puncture during the 2012 breeding season in Colombia. Materials and methods. Fifty seven buffalo females were selected for ultrasound guided transvaginal aspiration of follicles, oocytes were identified within follicular fluid, classified and transported to the laboratory and matured in vitro for 18 to 20 hours. Frozen semen of seven Mediterranean bulls were used, motile sperm was obtained using the Percoll technique and oocytes were inseminated with 2 million sperm/ml. Presumptive zygotes were cultured for 6 days, grade 1 embryos obtained were frozen using ethylene glycol. Embryos were transferred to females on day 5 during natural cycle. Results. 97 aspirations were performed on the 57 animals, in 8.2% of the aspirations no oocytes were found. 8 oocytes/aspiration were obtained. Of the 783 oocytes, 92% were classified as viable (721/783) and were fertilized. The cleavage and blastocyst rate were 23% and 19% respectively. 37 embryos were transferred and 11 pregnancies were obtained, confirmed by rectal palpation 60 days after transfer, with a pregnancy rate of 29.7%. Conclusions. The results presented here are comparable with others in literature and shows the feasibility of producing in vitro embryos and pregnancies after the standardization of current protocols, with normal and sexed semen and transfer during natural cycle in buffalo.

Key words: Buffaloes, embryo, fertilization in vitro, reproduction (Source: MeSH).

RESUMEN

Objetivo. Evaluar los resultados de la estandarización de la técnica de producción in vitro de embriones de búfalo, a partir de oocitos obtenidos por punción follicular durante la estación reproductiva del 2012 en una hacienda en Cordoba, Colombia. Materiales y métodos. Cincuenta y siete búfalas fueron seleccionadas para aspiración transvaginal de folículos guiada por ultrasonido, los oocitos fueron identificados y madurados in vitro. Se utilizó semen congelado de 7 búfalos de la raza Mediterráneo para la fertilización in vitro. La fracción móvil fue separada en un gradiente de Percoll, los oocitos fueron inseminados con 2 millones de espermatozoides/ml, los presumptivos cigotos fueron cultivados por 6 días y los embriones grado 1 obtenidos fueron congelados utilizando etilenglicol. Posteriormente,
INTRODUCTION

Buffaloes are an important species due to their great adaptability in extreme environmental conditions and for their production of milk and meat. Muñoz et al (1) reported for Colombia an average milk production at 270 days of 1096±275 kg and the production of fat and protein at 270 days was 7.4 y 5.0%, respectively for lactation. For meat production, animals with increases of up to 1200 g/day are possible, which shows precocity, obtaining a final product low in fat and with a high protein content. Additionally, it has been accepted that buffaloes are more efficient than tractors in pulling conditions up to one ton and its benefits in crops have been shown, especially in palm oil (1).

Buffaloes belong to the Bovidae family, bovines, the Bubalus genus, and the species includes bubalis and carabaensis (2,3). Raising buffalo in Colombia has experienced an exponential increase in the last 15 years, and based on vaccination records, Fedegán, the Colombian Cattle Federation, reported that 160.449 heads exist in the country, females making up 70% of the population (3). Present in Colombia is the Bulbalina race, from the species Bubalus bubalis, comprised of Murrah, Mediterranean and a crossbreed of the first animals that arrived in the country, called buffalypso. From the vaccination model some technologies have been attempted to apply, especially in the area of reproduction, without satisfactory results. In Colombia, improvement programs for the species are being developed, and although reproductive biotechnologies are known and have been applied for more than 10 years, they have not spread widely to breeders (3).

The results of transferring embryos on buffalo ranches have had discouraging results, especially programs involving multiple ovulation and embryo transferring (MOET). Drost (4) reviewed the Hindu experience, including 10 years of superovulation and embryo transferring, finding

los embrones fueron transferidos el día 5 post estro en búfalas en ciclo natural. **Resultados.** En las 57 búfalas, se realizaron 97 aspiraciones foliculares, no se obtuvieron oocitos en el 8.2% de los procedimientos. Se obtuvieron en promedio 8 oocitos por búfala en cada sesión de aspiración. Se recolectaron 783 oocitos de los cuales fueron clasificados como viables el 92% (721/783), se obtuvo una taza de clivaje del 23% y de blastocistos del 19%. De 37 embriones transferidos se obtuvieron 11 preñeces, confirmadas por palpación rectal a los 60 días postransferencia, obteniéndose una taza de preñez del 29.7% **Conclusiones.** Los resultados presentados en este trabajo son comparables con los de la literatura, en la cual se muestra cómo es posible obtener embriones de semen convencional y sexado, además de producir gestaciones con protocolos estándar de fertilización in vitro y transferencia en ciclos con celo natural adaptados para la especie.

**Palabras clave:** Búfalos, embrión, fertilización in vitro, reproducción (Fuente: MeSH)

INTRODUCCIÓN

Los búfalos son una especie importante por su gran adaptabilidad a condiciones medioambientales extremas y por producción de leche y carne. Muñoz et al (1) reportó para Colombia una media de producción de leche a los 270 días de 1096±275 kg y la producción de grasa y proteína a los 270 días fue de 7.4 y 5.0%, respectivamente por lactancia. Para la producción de carne, se tienen animales con ganancias de hasta 1200 g/día, lo que demuestra su precocidad, obteniendo un producto final bajo en grasa y con alto contenido de proteína. Adicionalmente, se ha aceptado que los búfalos son más eficientes que el tractor en condiciones de tiro de hasta una tonelada y se ha demostrado sus bondades en los cultivos, especialmente en los de palma de aceite (1).

El búfalo pertenece a la familia Bovidae, bovino, del que existe el género Bubalus al que pertenecen las especies, bubalis y carabaensis (2,3). La cria del búfalo en Colombia ha experimentado un incremento exponencial en los últimos 15 años, la Federación Colombiana de Ganaderos -Fedegán- basada en los registros de vacunación informó que en el país existen 160.449 cabezas, siendo hembras el 70% de la población (3). En cuanto a la variedad de razas Bubalinas existentes en Colombia, de la especie Bubalus bubalis, se encuentran la Murrah, Mediterráneo y cruzas de los primeros animales que llegaron al país y que se denominan buffalypso. Del modelo vacuno se han tratado de aplicar algunas tecnologías, especialmente en el área de la reproducción, sin resultados satisfactorios. En Colombia, se están construyendo programas de mejoramiento para la especie y aunque las biotecnologías reproductivas se conocen y se aplican desde hace más de 10 años, no están ampliamente difundidas entre los criadores (3).

Los resultados de la aplicación de la transferencia de embriones en los hatos bufalinos han tenido resultados desalentadores, especialmente aquellos programas de múltiple ovulación y transferencia de embriones (MOET). Drost (4), revisó la experiencia
2.3 embryos per animal with a pregnancy rate of 15% and an embryo reabsorption rate of 35%.

Given the low rate of obtaining embryos by MOET, the in vitro production of embryos (PIV) became an alternative to obtaining embryos in these programs. The first buffalo embryo born in vitro was reported in India in 1990. The PIV can considerably increase the amount of embryos obtained, whether it be by slaughterhouse ovaries or live buffaloes. The average oocytes per ovary in the case of slaughterhouse is from 0.43 to 0.70 in India vs 2.4 to 3.3 in Italy (5), but if they are obtained by means of follicular aspiration guided by ultrasound (OPU), this increased up to 2.25 oocytes/ovary (6). When compared with bovines, in spite of being philogenetically similar and having similar general reproductive conditions, buffaloes have some very important differences, among them the presence of less primordial follicles and the formation of gonads (7), in consequence less follicles at birth and less amount of follicles selected in the ovulatory cycle (8).

For many years the selection system in buffalo production has had a strong maternal influence, since the offspring of the best female buffalos are the ones chosen as reproducers, and for that reason transferring embryos is chosen as the technique to be applied. Colombian breeders should increase productivity and efficiency in order to take care of market demands, especially taking advantage of the fact that animal reproduction is very efficient when is evaluated in terms of gestation, birth rate and animal development (9,10).

With this information and the uncertainty of the results, at the end of 2008 (11) the technique of transferring embryos produced in vitro in Colombia began, with variable results. From this first experience the first animals were born in 2011 (12) which prompted an investigation group to establish an embryo production program in Colombia. The objective of this investigative study is to show the results obtained in in vitro production of buffalo embryos, report pregnancies during the 2012-2013 reproductive season, and discuss information obtained in global literature in order to apply this technology and make it a reality for the Colombian buffalo industry.

**MATERIALS AND METHODS**

**Study site.** This study took place from August 2012 and January 2013, on the Praga farm, in the Municipality Pueblo Nuevo, Córdoba, in the agroecological area of a Tropical Rain Forest (8°28’69’’ North 75°16’54’’ West), altitude 60 meters above sea level, annual rainfall 1600 mm.
**Animales.** 57 hembras, 8 buvillas y 49 crías con un mínimo de 90 días posparto fueron utilizadas, pesando de 530 kg y condición corporal superior a 3.5 (escala de 1 a 5: 1 = emaciada y 5 = obesa).

**Follicular suction.** Antes de aspirar los oocitos, la zona perineal fue lavada y desinfectada, y se indujo anestesia epidural mediante la inyección de 4 mL de xilocaina. Las búfalas fueron aspiradas mediante el uso de una aguja calibre 20 guiada por ultrasonido, acondicionada a una sonda transvaginal convexa mediante ecógrafo (Mindray modelo DP 2200). El sistema de aspiración fue lavado constantemente con medio TCM 199 con 25 mM de heparina, suplementado con 100 USP/mL de heparina, 10% de suero fetal bovino y 1% de solución de penicilina y estreptomicina (20000 UI y 20000 µg/mL). Los oocitos aspirados mediante la unión a una bomba de vacío con 80 mmHg fueron colectados en tubos de ensayo de 50 mL con 0.4 mL de heparina (Liquemine, Roche®) e identificados y clasificados en la misma finca. Una vez identificados, los oocitos fueron clasificados como viables y transportados al laboratorio en medio de maduración en una incubadora a 37.5°C en medio gasificado, TCM 199 con 25 mM de heparina, suplementado con 0.3mM cisteína, 50 µM cisteamina, 0.5 µg/ml FSH, 5 µg/mL LH y 1 µg/mL 17 β estradiol (14). Todas las estructuras enviadas fueron sometidas a maduración, no se incluyeron algunas estructuras redondeadas, que aparentemente no tienen citoplasma.

**Laboratory procedure.** A menos que se mencione explícitamente, todos los reactivos para la cultura fueron de la firma Sigma-Aldrich (Sigma Chemical Co, St Louis, Mo, USA).

Una vez en el laboratorio, los oocitos fueron transferidos a cajas de Petri, para continuar su maduración en grupos de máximo 20, hasta el momento de la fertilización a las 20 horas post aspiración.

**MATERIALES Y MÉTODOS**

**Sitio de estudio.** El presente estudio se realizó entre agosto del 2012 y enero del 2013, en la hacienda Praga, localizada en el Municipio de Pueblo Nuevo, Córdoba, condición agroecológica de Bosque Húmedo Tropical (8°28'69” Norte 75°16’54” Oeste), altitud 60 m.s.n.m, régimen anual de lluvias 1600 mm.

**Animales.** Fueron utilizadas 57 hembras, 8 buvillas y 49 crías con mínimo 90 días posparto, peso de 530 kg y condición corporal superior a 3.5 (escala de 1 a 5: 1 = emaciada y 5 = obesa).

**Aspiración folicular.** Antes de aspirar los oocitos, la zona perineal fue lavada y desinfectada, y se indujo anestesia epidural mediante la inyección de 4 mL de xilocaina. Las búfalas fueron aspiradas mediante el uso de una aguja calibre 20 guiada por ultrasonido, acondicionada a una sonda transvaginal convexa mediante ecógrafo (Mindray modelo DP 2200). El sistema de aspiración fue lavado constantemente con medio TCM 199 con 25 mM de heparina, suplementado con 100 USP/mL de heparina, 10% de suero fetal bovino y 1% de solución de penicilina y estreptomicina (20000 UI y 20000 µg/mL). Los oocitos aspirados mediante la unión a una bomba de vacío con 80 mmHg fueron colectados en tubos de ensayo de 50 mL con 0.4 mL de heparina (Liquemine, Roche®) e identificados y clasificados en la misma finca. Una vez identificados, los oocitos fueron clasificados como viables y transportados al laboratorio en medio de maduración en una incubadora a 37.5°C en medio gasificado, TCM 199 con 25 mM de heparina, suplementado con 0.3mM cisteína, 50 µM cisteamina, 0.5 µg/ml FSH, 5 µg/mL LH y 1 µg/mL 17 β estradiol (14). Todas las estructuras enviadas fueron sometidas a maduración, no se incluyeron algunas estructuras redondeadas, que aparentemente no tienen citoplasma.

**Procedimiento de laboratorio.** A menos que se mencione explícitamente, todos los reactivos para la preparación de los medios de cultivo fueron de la firma Sigma-Aldrich (Sigma Chemical Co, St Louis, Mo, USA).

Una vez en el laboratorio, los oocitos fueron transferidos a cajas de Petri, para continuar su maduración en grupos de máximo 20, hasta el momento de la fertilización a las 20 horas post aspiración.

Para la fertilización se utilizó semen congelado convencional y sexado, proveniente de la central italiana COFA (Centro Fecundacione...
cultured in the fertilization environment between 20 and 24 hours at 38.5°C, in a 5% atmosphere of CO₂, 90% humidity.

Later the oocytes were denuded and the zygotes were taken to half culture, synthetic oviductal fluid (SOF) to which essential and nonessential amino acids and bovine seric albumin were added. The first environmental change was 4 days after the aspiration; due to the characteristics of the buffalo oocytes it is not possible to identify the quantity of blastomers in each structure and due to this, only the non-inseminated structures are removed. The second environmental change is done at day 6, when the first blastocysts can be observed. The formation of blastocysts begins on day 6 obtaining those that are the best quality and go to day 8, when they were frozen by means of the ethylene glycol slow freezing method (12) for direct transfer.

Some embryos were transferred to receptors in a natural cycle, day 6 post-estrum in the ipsilateral horn to the corpus luteum of this cycle’s ovulation.

Statistical analysis. Data was shown with statistically descriptive values, in comparative cases the proportion comparison test was sued and a non-parametric correlation analysis was done between the variables, p>0.05 was considered statistically significant; GraphPad Prism 5 (GraphPad software, San Diego, CA) program was used.

RESULTS

Between July 2012 and January 2013, 97 follicular aspirations were performed on 57 animals, 49 multi-birth buffalos and 8 young cows. The age of the buffalos was on average 96 months with a range from 14.5 to 206 months. The general results of the program are presented in table 1.

Of the 57 buffalos, 36 were only aspirated once. 97 aspirations were done in 8 procedures.

| Table 1. Comparison between young cows and multiple birth cows within the parameters described for production of in vitro embryos in water buffalo. |
|-----------------|-----------------|-----------------|---------------|---------------|
| Animals        | Young cows      | Multiple birth cows | Total | Valor of p |           |
| Animals        | 8               | 49               | 57   | -           |           |
| Aspiration     | 15              | 82               | 97   | No difference (ND) |           |
| Total          | 95              | 688              | 783  | ND          |           |
| Oocytes / aspiration | 6.3             | 11.1             | 8.07 | <0.05       |           |
| Viables        | 89              | 632              | 721  | ND          |           |
| Oocytes / aspiration | 5.7             | 10.2             | 7.4  | ND          |           |
| Blastocyst at day 6-7 | 12              | 123              | 135  | <0.05       |           |
| % of blastocysts at day 6-7/ viable oocytes | 13.30%           | 18.30%           | 19%  | ND          |           |

Algunos embriones fueron transferidos a receptoras en ciclo natural, el día 6 post estro en el cuerno ipsilateral al cuerpo lúteo de la ovulación de ese ciclo.

Análisis estadístico. Los datos se mostraron con valores de estadística descriptiva, en los casos de comparación se usó la prueba de comparación de proporciones y se realizó análisis de correlación no paramétrica entre las variables, se consideró significancia estadística un p>0.05, se utilizó el programa GraphPad Prism 5 (GraphPad software, San Diego, CA).

RESULTADOS

Entre julio del 2012 y enero del 2013, se realizaron 97 aspiraciones folliculares a 57 animales, 49 búfalas pluríparas y 8 buvillas. La edad de las búfalas fue en promedio 96 meses con un rango de 14.5 hasta 206 meses. Los resultados generales del programa se presentan en la tabla 1.
no oocytes were obtained, the maximum of oocytes per aspiration was 35. 783 oocytes were recovered, of these 721 (92%) were classified as viable and were used to produce embryos. 8.07 total oocytes/buffalo and 7.4 oocytes/viable per aspiration. No significant differences were found in the production of oocytes/buffalo during the season, with a range from 8 to 11.

126 oocytes were fertilized with sexed semen and there were no significant differences in cleavage or in the production of embryos when compared with non-sexed semen, 20% and 17% respectively.

Of the oocytes fertilized on day 4 171 (23%) cleaved and on days 7 and 8 of the culture, 135 (19%) reached the blastocyst stage; all the embryos were frozen with ethylene glycol for direct transfer.

1.35 embryos were obtained by aspiration with a variation from 0 to 7. Of the 135 embryos obtained, 88 (65%) became blastocysts on day 6, and 47 (35%) on day 7. The production of embryos in the first aspiration in September 2012 was 10% and the last reported one in January 2013 was 20%, a significant difference (p<0.05).

In 11 female buffalos (11.3%) in which at least one oocyte was recovered no embryos were obtained, these represented 67 oocytes, 29% of these suspected zygotes did not cleave, 56% reached the state of 2 cells and 15% went from 4 to 8 cells on day 4 but stopped developing.

Additionally, buffalos that had given birth produced more oocytes and embryos than the young cows, 11.1 vs. 6.3 oocytes/buffalo and 18.3 % vs 13.3% buffalo blastocysts respectively (p<0.05). Concerning embryo development, the young cow embryos reached the blastocyst state on day 7, greater proportion than the buffalos that had given birth (56% vs 14%).

Finally 37 embryos were transferred from which 11 pregnancies were started (29%), confirmed by rectal palpation 60 days after transfer.

**DISCUSSION**

This study presents for the first time in world literature the use of PIV of buffalo embryos and the transfer of frozen embryos during natural heat for production of high quality buffalos.

De las 57 bufalas, 36 fueron aspiradas solamente una vez. Se realizaron 97 aspiraciones, en 8 procedimientos no se obtuvieron oocitos, el máximo de oocitos por aspiración fue de 35. Fueron recuperados 783 oocitos, de estos 721 (92%) fueron clasificados como viables y se utilizaron para producción de embriones. Se obtuvieron 8.07 oocitos totales/búfala y 7.4 oocitos/viables por aspiración. No se encontraron diferencias significativas en la producción de oocitos/búfala durante toda la estación, con un rango de 8 a 11.

Se fertilizaron 126 oocitos con semen sexado y no se obtuvieron diferencias significativas en el clivaje ni en la producción de embriones cuando se comparó con semen no sexado, 20% y 17% respectivamente.

De los oocitos fertilizados al día 4 clivaron 171 (23%) y en los días 7 y 8 de cultivo alcanzaron el estado de blastocisto 135 (19%); todos los embriones fueron congelados en medio con etilenglicol para transferencia directa.

Se obtuvieron 1.35 embriones por aspiración, con una variación de 0 a 7. De los 135 embriones obtenidos, 88 (65%) se convirtieron en blastocistos en el día 6 y 47 (35%) en el día 7. La producción de embriones en la primera aspiración en septiembre del 2012 fue del 10% y en la última informada en enero 2013 fue del 20%; siendo esta diferencia significativa (p<0.05).

En 11 búfalas (11.3%) en las que al menos se recuperó un oocito no se obtuvieron embriones, estos representaron 67 oocitos, 29% de estos presuntos zigotos no clivaron, el 56% alcanzaron el estado de 2 células y el 15% llegó de 4 a 8 células al día 4 pero detuvieron su desarrollo.

Adicionalmente, las búfalas paridas produjeron más oocitos y embriones que las buvillas, 11.1 vs 6.3 oocitos/búfala y 18.3 % vs 13.3% blastocistos búfala respectivamente (p<0.05). En cuanto al desarrollo embrionario los embriones de buvilla llegan al estado de blastocisto el día 7, mayor proporción que las búfalas paridas (56% vs 14%).

Finalmente fueron transferidos 37 embriones de los que se obtuvieron 11 preñeces (29%) confirmadas por palpación rectal el día 60 post transferencia.

**DISCUSIÓN**

En este trabajo se presenta por primera vez en la literatura mundial el uso del PIV de embriones bufalinos y la transferencia de embriones congelados durante un celo natural para la producción de búfalos de calidad superior.
It is seen to be a safe and reproducible procedure, complications were only seen in 1.7% of the cases; an edema could be explained as the anaphylactic reaction to the epidural anesthesia used, which was resolved spontaneously. Post aspiration complications were not present, such as adherences.

It should be kept in mind that to obtain oocytes of young animals the size of the vaginal canal was a limiting factor, since the transducer could not be used on 7 young cows (data not shown), and it is probable that in very young animals the transducer would damage the vagina canal and these animals cannot be aspirated. It was also seen that 8 buffalos could not be aspirated for anatomic reasons due to a narrow vagina or adherences of the internal organs.

The rate for obtaining oocytes in this study was 8.07 oocytes/buffalo by aspiration, which is superior to that informed in Italy 2.3 (13), Brazil 4.1-6.7 (16,17) and 1.2 in India (18). A possible explication could be the fact that the aspirations were done during the reproductive season, while some of these authors sampled their study during the whole year. The viability of this study was superior (92%) to that reported in literature (44%,45%), (16,18).

Neglia et al (19), suggest that the length of the needle and the suction system have a great influence in the quality of the oocytes found, indicating that the cells of the cumulus, poorly adhered, are detached during the aspiration process. For this study a short needle was used, that goes directly connected to the conduction system, which could explain the high viability of the oocytes obtained, even more when one of the principal parameters of classification of the oocytes are the grainy layers surrounding it.

Also, it is important to highlight that when evaluating the oocytes there are very few that can be classified as type A, since a large quantity of the oocytes do not have the appearance of a complex with adequate granular and cytoplasm; in some cases they appear as rounded structures that apparently do not have cytoplasm that would be like an atretic oocyte, which in this study weren’t taken into account for the analysis. This limitation meant that almost all the oocytes obtained were used for fertilization. In conditions in the field, it is necessary to use grade A, B, C oocytes to produce embryos, and it is possible to seek ways to correctly classify oocytes of this species.

Se demuestra que es un procedimiento seguro y reproducible, sólo se presentaron complicaciones en 1.7% de los casos, un edema que podría ser explicado como una reacción anafiláctica a la anestesia epidural utilizada, el cual se resolvió espontáneamente. Tampoco se presentaron complicaciones post aspiración como adherencias.

Se debe tener en cuenta que para obtener ocotitos de animales jóvenes el tamaño del canal vaginal fue un limitante, ya que en 7 buñulas no se pudo pasar el transductor (datos no mostrados), es probable que en animales muy jóvenes, el paso del transductor lacere el canal vaginal y esos animales no se puedan aspirar. Se constató que 8 búfalas no pudieron ser aspiradas por razones anatómicas, debido a la estrechez de la vagina o adherencias de los órganos internos.

Neglia et al (19), sugirieron que la longitud de la aguja y del sistema de succion tiene una gran influencia en la calidad de los oocitos encontrados, indicando que las células del cúmulus, pobremente adheridas se desprendan durante el proceso de aspiración. En este trabajo para la aspiración fue utilizada una aguja corta, que va directamente conectada al sistema de conducción, esta razón podría explicar la alta viabilidad de los oocitos obtenidos, más aún cuando uno de los principales parámetros de clasificación de los oocitos son las capas de granulosa que lo rodean.

También, es importante resaltar que al evaluar los oocitos son muy pocos los que se pueden clasificar como tipo A, debido a que una gran cantidad de oocitos no tienen la apariencia de un complejo con la granulosa y citoplasma adecuados; en algunos casos aparecen unas estructuras redondeadas que aparentemente no tienen citoplasma que semejarían un ocoto atrésico que en este trabajo no fueron tenidos en cuenta para el análisis. Esta limitación hace que prácticamente todos los oocitos obtenidos sean utilizados para la fertilización. En las actuales condiciones de trabajo de campo, se hace necesario el uso de los oocitos grado A, B, C para producir embriones, cabe la posibilidad de buscar formas para la clasificación correcta de los oocitos en esta especie.
It has been reported that a greater quantity of embryos are produced when oocytes collected from live animals are used for embryo production, rather than using ovaries from a slaughterhouse (30.6±4.3% versus 18.5±1.8%). However, in literature consulted, no differences were observed in the maturation or cleavage of oocytes (18,20) when comparing these two sources of oocytes.

Pregnancy rates obtained are superior to those reported by our group in 2011 (25% vs 29%) although this difference is not significant and is in line with other reports in literature (21, 22).

Results obtained in this study using sexed semen differ from those reported by Lu et al (21), in which significant differences were observed in fertilization when sexed and conventional semen are used.

It is fundamental to understand that the production of blastocysts in vitro within a production system for in vitro embryos is not only influenced by biological factors, such as the development of follicles and the quality of oocytes, but also by factors related to laboratory procedures, the mother's nutrition and handling of the animals.

The use of frozen embryos for direct transfer facilitates establishing and managing these programs since in the properties where artificial insemination program are established, the only requirement that is needed is to change from inseminating to transferring the embryo, with time and luteal evaluation adjustments that an experienced technician can do without significantly increasing costs. From this experience, it can be said that with buffalo, biotechnological procedures that are performed with natural reproduction are successful. The pregnancy rate in this study was 29% compared with 20% (12) obtained when the receptors for transferring embryos were synchronized.

When the results of the program are analyzed from the point of view of the breeder based on the data reported here: 1.35 embryos are obtained by aspiration and a pregnancy rate of 29%, resulting in the probability of pregnancy for each aspirated buffalo of 0.39%, which means that three sessions of follicular aspiration should be done to guarantee at least one pregnancy for each animal that undergoes aspiration, calculating that to ensure at least one pregnancy 4 to 6 transferrable embryos should be produced per cycle (23). This means that for now, to see some improvement, one pregnancy 4 to 6 transferrable embryos should be produced per cycle (23). This means that for now, to see some improvement, one pregnancy 4 to 6 transferrable embryos should be produced per cycle (23). This means that for now, to see some improvement, one pregnancy 4 to 6 transferrable embryos should be produced per cycle (23). This means that for now, to see some improvement, one pregnancy 4 to 6 transferrable embryos should be produced per cycle (23). This means that for now, to see some improvement, one pregnancy 4 to 6 transferrable embryos should be produced per cycle (23). This means that for now, to see some improvement, one pregnancy 4 to 6 transferrable embryos should be produced per cycle (23). This means that for now, to see some improvement, one pregnancy 4 to 6 transferrable embryos should be produced per cycle (23). This means that for now, to see some improvement, one pregnancy 4 to 6 transferrable embryos should be produced per cycle (23). This means that for now, to see some improvement, one pregnancy 4 to 6 transferrable embryos should be produced per cycle (23). This means that for now, to see some improvement, one pregnancy 4 to 6 transferrable embryos should be produced per cycle (23). This means that for now, to see some improvement, one pregnancy 4 to 6 transferrable embryos should be produced per cycle (23). This means that for now, to see some improvement, one pregnancy 4 to 6 transferrable embryos should be produced per cycle (23). This means that for now, to see some improvement, one pregnancy 4 to 6 transferrable embryos should be produced per cycle (23). This means that for now, to see some improvement, one pregnancy 4 to 6 transferrable embryos should be produced per cycle (23). This means that for now, to see some improvement, one pregnancy 4 to 6 transferrable embryos should be produced per cycle (23).
aspiration should be done with a minimum of five donors. Investigators should strive to improve embryo production conditions and analyze donors in order to increase the possibilities of generating embryos for the improvement program.

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