Ultrasonography and Reproduction in Buffalo

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Abstract: Ultrasonography is a simple, reliable and non-invasive imaging technique without secondary effects. Application of real time ultrasonography in veterinary practice has developed to become the most efficient diagnostic tool for managing reproduction. The objectives of current work are to offer an overview of the uses and utility of ultrasonography for the buffalo evaluation of physiological and pathological conditions and for the application of assisted reproductive technologies. Assessment of pregnancy status and fetal viability early postbreeding to identify cows that fail to conceive improves reproductive efficiency by decreasing the interval between artificial insemination services and increasing artificial insemination service rate. Ovarian and uterine pathologies, not accurately detected *via* rectal palpation, can easily be visualized by ultrasound and appropriate therapies can be implemented. Determination of fetal sex in utero is useful when coupled with a management decision that justifies the expense of fetal sexing. Development of integrated reproductive management systems that combine ultrasound with new and existing reproductive technologies will further enhance the practical applications of ultrasonography. Development of extension education programs to train practitioners to use ultrasound for routine reproductive examinations is a critical step toward rapid implementation of this technology into the dairy industry.

Keywords: Follicles, ovary, corpus luteum, pregnancy, foetal viability, foetal age, foetal gender, reproductive management.

INTRODUCTION

The application of real-time ultrasonography for studying reproductive functions in animal sciences is a recent development and nowadays ultrasonography is no longer an elite technique for few selected breeders but a widely recognized and used key tool in reproductive management and research.

Ultrasonography is a simple, reliable, non-invasive imaging technique without side effects and since its development in the early 1950s, medical ultrasound has gained a major share of the imaging market next to X-ray (Figure **1**). Thus, applications of ultrasonography in veterinary practice has developed, from a limited use for pregnancy diagnosis and detection of some pathological conditions like hydrometra or metritis, to the most efficient diagnostic tool for checking the reproductive health of both males and females and for planning and performing the reproductive management of the herd.

Moreover, in the last years, the use and the yields of assisted reproduction techniques (ART) have found a substantial benefit in the implementation of high-resolution transrectal ultrasonographic techniques. First, in research, for the study and determination of the limiting factors and for the adjustment of the protocols in artificial insemination and embryo production and transfer. Second, in practice, for the election and management of both donors and recipients. An additional advantage is that in all the aspects related to management and improvement of reproduction, ultrasonography permits a close relationship between research and practice, as findings in research can be directly translated to the field. On the other hand, the most restrictive factors of ultrasonography are that its effciency is always dependent on the expertise of the operator.

Obviously, the use of ultrasonography in ruminant reproduction has generated a lot of information by now. The objective of this work is to offer an overview of the use and usefulness of real-time ultrasonography in the assessment of reproduction in buffalo cows. Thus, we provide an overview of the utility of ultrasonography for the evaluation of physiological and pathological conditions and in the application of ART. Some useful pictures will be reported regarding the use of ultrasonography for minitoring buffalo reproduction. A skilled veterinarian using ultrasound can now provide real-time information not available with any other method, which adds value for the dairy producer and the veterinarian, resulting in a more successful reproduction program [2].

Today, in buffalo females, ultrasound is used for the following examinations:

- Ovarian status determination
- Onset of puberty determination

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Figure 1: From left to right: Changes in image quality from 1985, 1990 to 1995 respectively. There were improvements in spatial and contrast resolution, background noise reduction, dynamic range, and near and far field visualization [1].

- Follicular monitoring for diagnosis or pharmacological treatments
- Ovulatory follicles and ovulation time determination
- Ovulation time or anovulatory condition determination
- Corpus luteum monitoring
- Stage of the oestrus cycle determination
- Luteal persistence and anovulatory conditions differentiation
- Establishment of optimal time for artificial insemination
- Oocytes recovery through ultrasound Ovum Pick-up
- Recipients testing for MOET programmes
- Early diagnosis of pregnancy
- Embryo growth characterization
- Foetal viability and age determination
- Foetal number and gender determination
- Post-partum uterine involution determination
- Embryonic death rate (by lack of heartbeat) determination

OVARIAN FOLLICLES

Before the ultrasound evaluation of ovarian follicles was limited to palpation, laparoscopy or visual examination of excised ovaries. With the advent of ultrasound, however, non-invasive, repeated monitoring of follicular (Figure **2**) and luteal development became possible.

The improvement of the reproductive performance of buffalo cow needed a better understanding of the mechanisms controlling ovarian follicular growth and development.

The era of study of follicular dynamics in cattle by ultrasound began with the classic publication by Pierson and Ginther [3] concluding that ultrasonography “offers much research potential for critical testing of biological hypotheses.”

This original work was followed in rapid succession by more detailed ultrasonographic studies of the bovine ovary. These studies were conducted at Cornell University [4-6], at University College, Dublin, Ireland [7, 8] and at the University of Wisconsin [9, 10]. The studies also included endocrine (progesterone, estradiol, and LH) data that were correlated with ovarian follicular morphology.

According to this early work, application of ultrasonic imaging has revealed that ovarian follicular turnover during an unstimulated oestrous cycle in buffalo is similar to that observed in cattle and is characterized by waves of follicular recruitment, growth and regression [11]. The number of follicular waves during an oestrous cycle can vary from 1 to 3 in buffaloes, with 2 waves being the most common (63%); (Figure **3**) [12-14]. There has been no report of 4 follicular waves within an oestrous cycle in buffaloes, although 4 waves do occur in cattle [15]. These follicular waves consist of a group of antral follicles begin to grow to 4 mm and from there there is a selection of a dominant follicle, which continues to grow, while others become subordinate follicles and initiate a process of atresia. The emergence of the first follicular wave, either in cycles of 2 or 3 waves, occurs immediately after ovulation, while the second wave occurs on days 10-11 for 2-waves cycles; in a cycle with three waves, the waves emerge, on average, at days 1, 9 and 16. The maximum size of each dominant

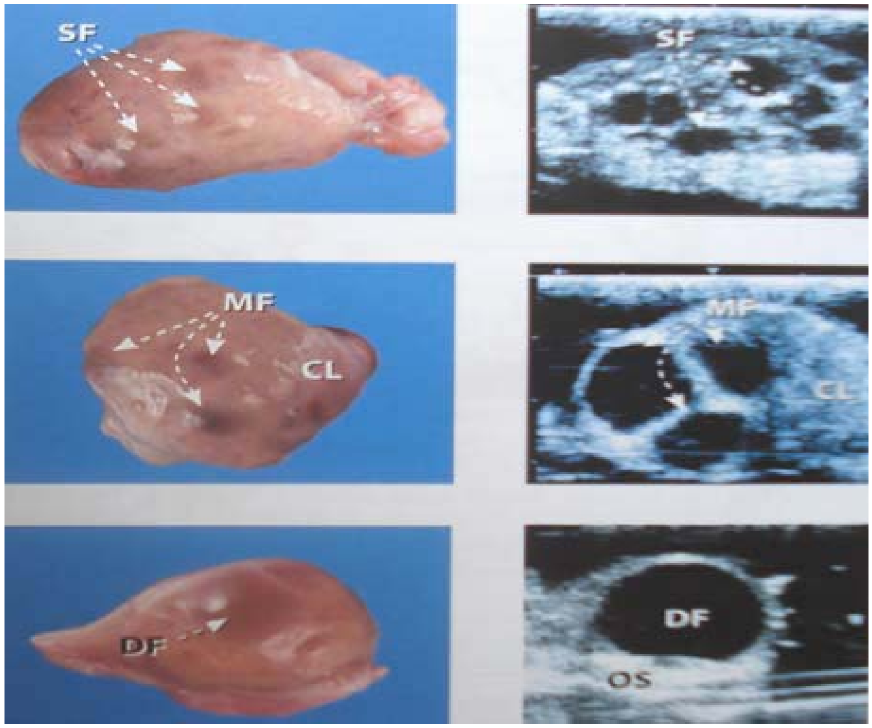


Figure 2: Ultrasonographic images of buffalo ovaries showing small follicles (SF; upper image) medium follicles and corpora lutea (MF, CL; middle image) and dominant follicle (CL; down image).

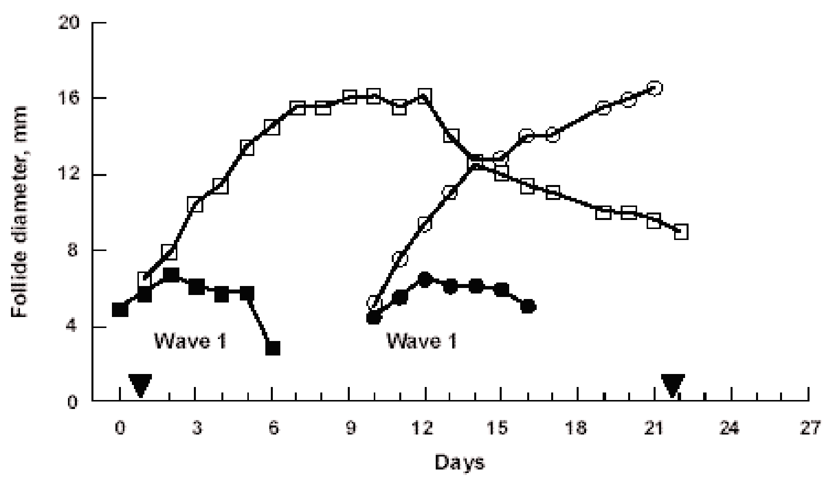


Figure 3: Buffalo oestrus cycle characterized by two follicular waves [2].

follicle is around 15 mm [16]. Two- and three-wave cycles were significantly different with regard to the average length of intervals to oestrus and ovulation and the average length of luteal phase.

Wave-like patterns of follicular growth were also observed, by ultrasonography, in pre-pubertal Mediterranean Italian buffaloes at 5–9 months of age [17], in primiparous and pluriparous Mediterranean Italian buffaloes postpartum [18] and during pregnancy [1]. The application of ultrasonic imaging has revealed that the number of follicles recruited into a follicular wave is lower in buffaloes than in cattle [2, 19]. The relatively small number of follicles, typical of follicular waves in buffaloes, could be related to the smaller pool of primordial follicles reported for buffalo heifers (12,636) compared with cattle (50,000) [20]. Buffalo heifers also tend to have a greater rate of follicle atresia (67%; [20]) relative to cattle (50%; [21]). The number of antral follicles in swamp buffaloes was reported to be only 20% of antral follicles in cattle [22]. The number of non-atretic follicles (>1.7 mm) was between 1 and 5 (average 3) for buffaloes and 17 and 32 (average 22) for cattle [22].

OVULATION AND CORPUS LUTEUM

Determination of ovulation by ultrasound examination has been reported [23, 24]. In this last work, the ovaries of 55 buffalo cows were monitored *via* transrectal ultrasonography twice daily, during oestrus to ovulation to examine the size of ovulatory follicle and timing of ovulation. Ovulation was defined when the previously ultrasonic identified follicle (>9 mm) disappeared on a subsequent ultrasound scan and subsequently confirmed by the development of corpus luteum at the same spot. The usefulness of ultrasonography performed at 2-hourly intervals for detecting the onset of ovulation has also been demonstrated [25]. As in buffalo the corpus luteum (CL) is deeply embedded in the ovary, its ultrasonic detection may be more sensitive than detection by palpation (Figures **4** and **5**), this being dependent on the experience of the individual performing rectal palpation [26]. The ultrasonic characteristics of corpora lutea (CL) have been described [25, 27]. Generally, a CL is identified ultrasonically from the third day after ovulation. A developing CL appears on the ultrasound image as a poorly defined, irregular, greyish-black structure with echogenic spots all within the ovary; a mid-cycle CL is a well defined granular, greyish echogenic structure with a demarcation line visible between it and the ovarian stroma; in a regressing CL the demarcation line is faint, owing to the slight difference in echogenicity between the tissues [28]. On thirty adult buffaloes synchronized by PRID+PMSG+PGF2α and artificially inseminated, [27] examined ultrasonografically the ovaries to study the preovulatory follicle (PF) and the successive corpus luteum (CL). On the basis of echogenic appearance, the corpora lutea (CLs) were classified as belonging to grade 1, 2 or 3. Significant difference was found in the diameter of PF and in that of CL in relation to the CL echogenic appearance. Well defined granular CL, distinguishable from the ovarian stroma and dark grey in colour (grade 3) resulted to have a bigger diameter compared with CLs belonging to a grade 1 or 2, and resulted to derive from a larger PF. Positive correlation was found between plasma P4 concentration and CL diameter. The PF diameter was larger in buffaloes become pregnant after AI respect to the non-pregnant

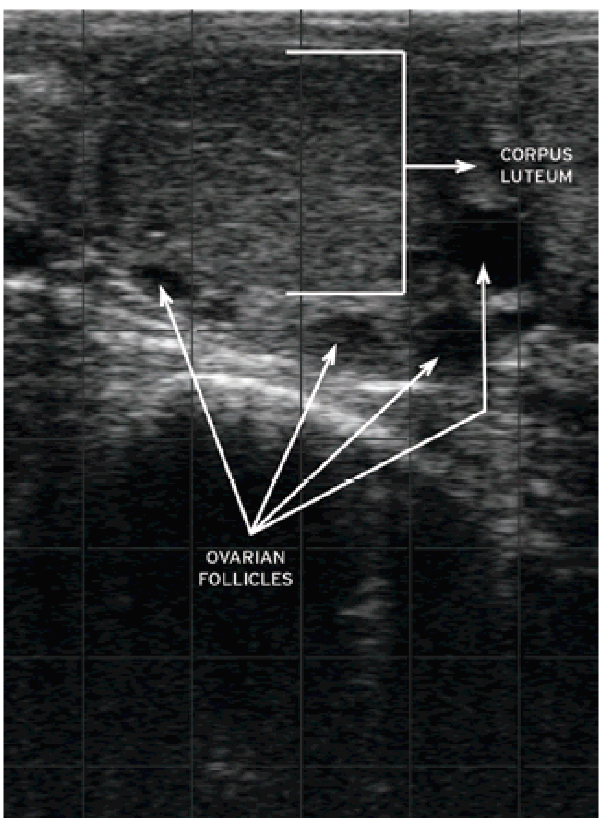


Figure 4: Ultrasound image of ovary with surrounding follicles [29].

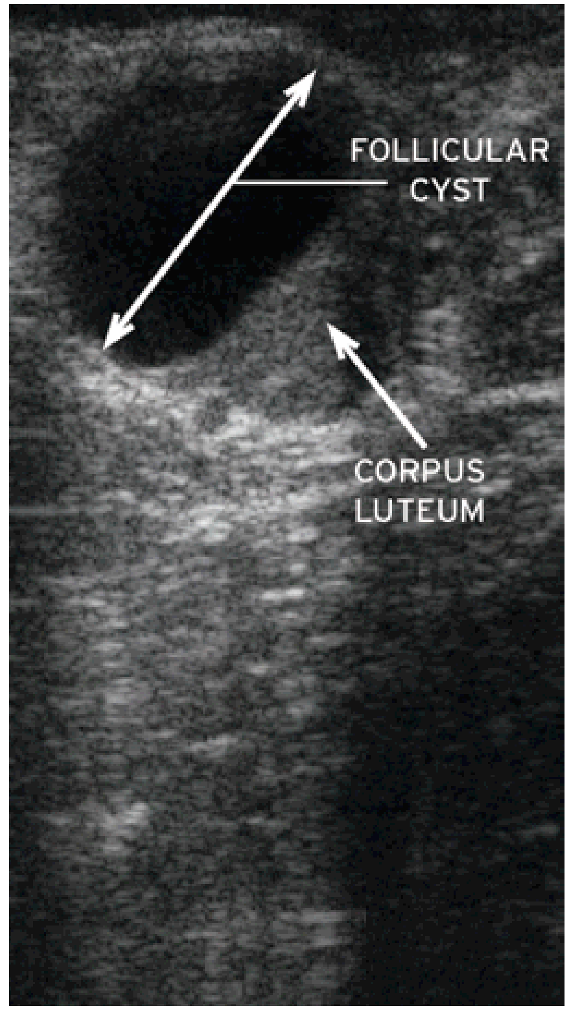
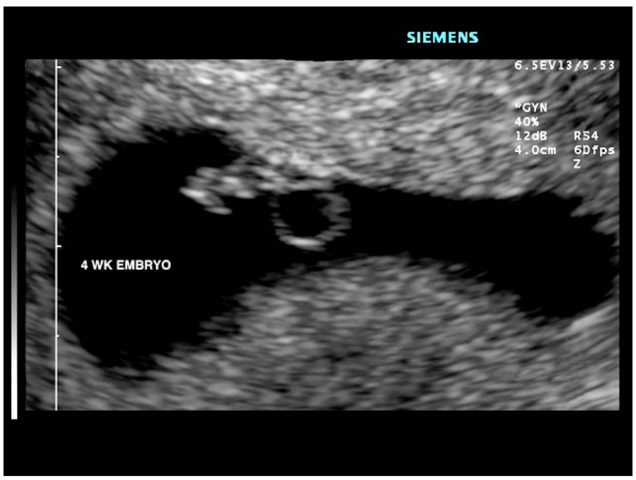


Figure 5: Ultrasound image of ovary with a follicular cyst and corpus luteum [29].

ones. The CL diameter, also, was significantly larger in the pregnant buffalo compared to the non-pregnant ones since the first observation at day 7.

EARLY PREGNANCY, FOETAL VIABILITY AND FETAL AGE

Early pregnancy diagnosis can improve reproductive performance by decreasing the interval between successive artificial insemination services and coupling a non-pregnancy diagnosis with an aggressive strategy to rapidly rebreed the animal. Pregnancy diagnosis in buffalo can be achieved by ultrasonography quickly, easily and accurately and the foetus appears as an echogenic structure inside a non-echogenic structure [40]. Under most on-farm conditions, pregnancy can be diagnosed as early as 26 days post artificial insemination (AI) [2] (Figures **6** and **7**).



Figue 6: Ultrasound image of a 26-day pregnancy.

This is approximately seven days earlier than pregnancy diagnosis by manual palpation. However, diagnosis of pregnancy at an early stage should be considered with caution due to the possibility of early embryonic loss.

To compensate for embryonic mortality, buffaloes diagnosed pregnant early post breeding must undergo one or more subsequent pregnancy examinations to identify and rebreed animals that experience embryonic mortality. Thus, farmers who have implemented early pregnancy diagnoses must consider the timing and frequency of subsequent pregnancy examinations to confirm foetal viability and to maintain the reproductive performance of the herd. Published rates of embryonic/foetal death in the first two months of pregnancy are variable and depend on the individual study. However, rates range from 10-20% [30, 31] in

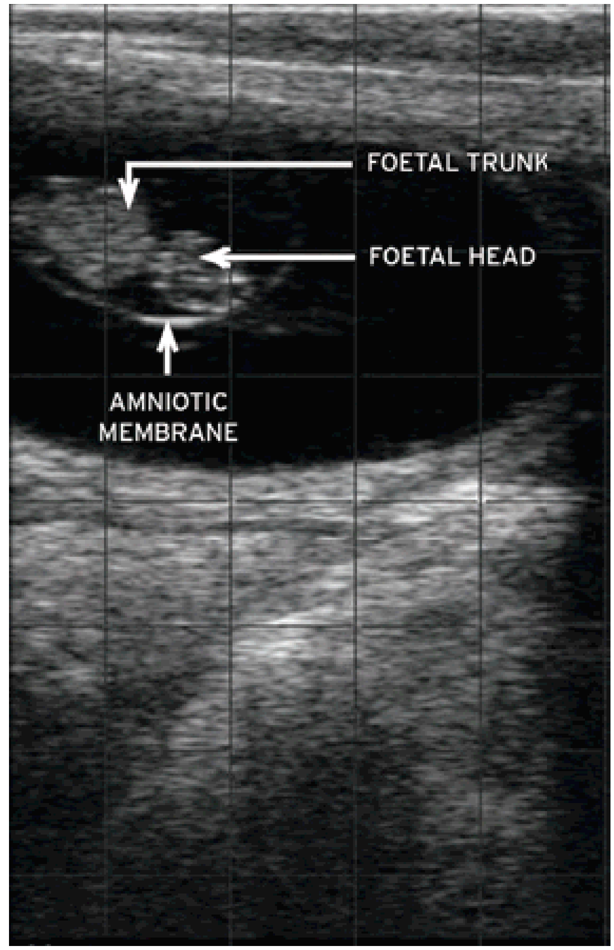


Figure 7: Ultrasound image of a 38-day pregnancy.

adult buffalo cows. This represents a significant factor affecting overall reproductive performance of the herd. Several indicators of foetal death can be identified through the use of ultrasound, including: lack of foetal heartbeat/foetal movement that is readily apparent with ultrasonography; presence of flocculent material in the amniotic (Figure **8**) or chorioallantoic fluid and separation of the chorioallantois from the uterine wall [32].

Early detection of embryonic/foetal death will decrease the time to re-breeding of the non-pregnant cows and therefore improving profitability.

Estimation of foetal age, monitoring of foetal growth across time and diagnosis of pregnancy disorders can be performed by ultrasonographic foetometry. Various ultrasound methods for estimating animal foetal growth have also been described in the literature [33, 34]. These techniques are based on serial measurements of specific somatic parameters in the foetus (Figure **9**), such as *crown rump lenght* (CRL), *biparietal diameter of the cranium* (BPD), *abdominal diameter* (AD), *muzzle-*

Figure 8: Image of flocculent material in the pregnancy fluids and of the degradation of the dead fetus. On palpation there would still be cardinal signs of pregnancy, including a membrane slip.

occipital length (MOL) and the *lengths of femur, tibia and metatarsal bone*. [2], in order to begin the development of a buffalo fetal growth chart, used six synchronized dry Italian Mediterranean buffaloes and pregnancy was diagnosed on the 27th day after artificial insemination by means of a ultrasonographic scan with a 7.5 MHz transrectal transducer. The animals were examined from the 27th through the 70th day of gestation. These examinations were carried out at intervals of ten days till to 48th day of gestation and at intervals of approximately three days till to 70th day of gestation. The animals were examined and each study lasted 10-15 minutes. The examinations included the presence of an heartbeat and for each foetus the following parameters were recorded: crown rump length (CRL, from the 27th through the 57th day), abdominal diameter (AD, from the 37th through the 70th day), biparietal diameter of the cranium (BPD, from the 48th through the 70th day), muzzle-occipital length (MOL, from the 52th through the 70th day) and femur length (FL, from the 55th through the 70th day) (Figure **9**).

A total of 200 examinations were made (about 33 per animal) by the same operator. The relationship between gestational age and the recorded parameters has been described by regression equations showed in Table **1**.

The age prediction by ultrasonographic foetal measurements in buffalo could provide a relatively easy means for observing the events of pregnancy for research purpose and a practical and economic tool for breeders. Furthermore, multiple measurements of foetal parts can be used to construct reference curves for normal growth, which can then be used to estimate

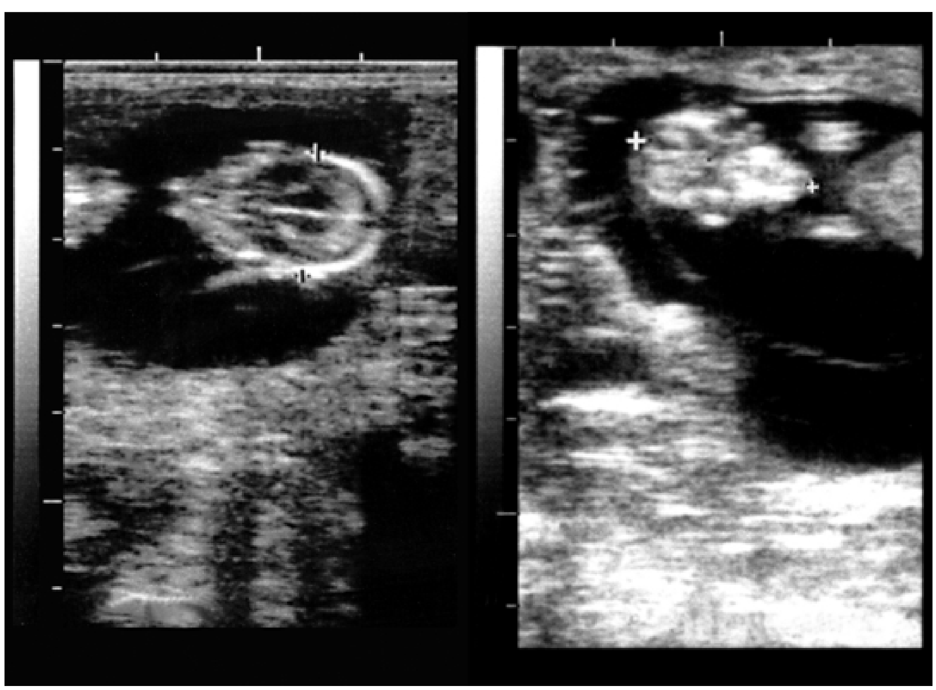


Figure 9: Measurement of BPD (left image) and MOL (right image).

Table 1. Relationship Between Gestation Age and Crown Rump Length, Abdominal Diameter, Biparietal Diameter, Muzzle-Occipital Length and Femur Length [35]

Foetal structure	regression equation	r =	P<
Crown Rump length (mm)	$y = 22.71153-1.16234x+0.02753x^2$	0.95	0.0001
Abdominal diameter (mm)	$y = -14.3452+0.5631x$	0.94	0.0001
Biparietal diameter (mm)	$y = -11.2431+0.4455x$	0.91	0.0001
Muzzle-Occipital length (mm)	$y = -21.4779+0.8063x$	0.89	0.0001
Femur length (mm)	$y = -10.3454+ 0.2631x$	0.76	0.0001

gestation length in animals with an unknown service date or, conversely, to investigate the normality of foetal growth and development in pregnancies of known gestational age.

FOETAL GENDER

Early pregnancy diagnosis and fetal gender determination by ultrasound is one of the modern trends in reproduction science and has become a

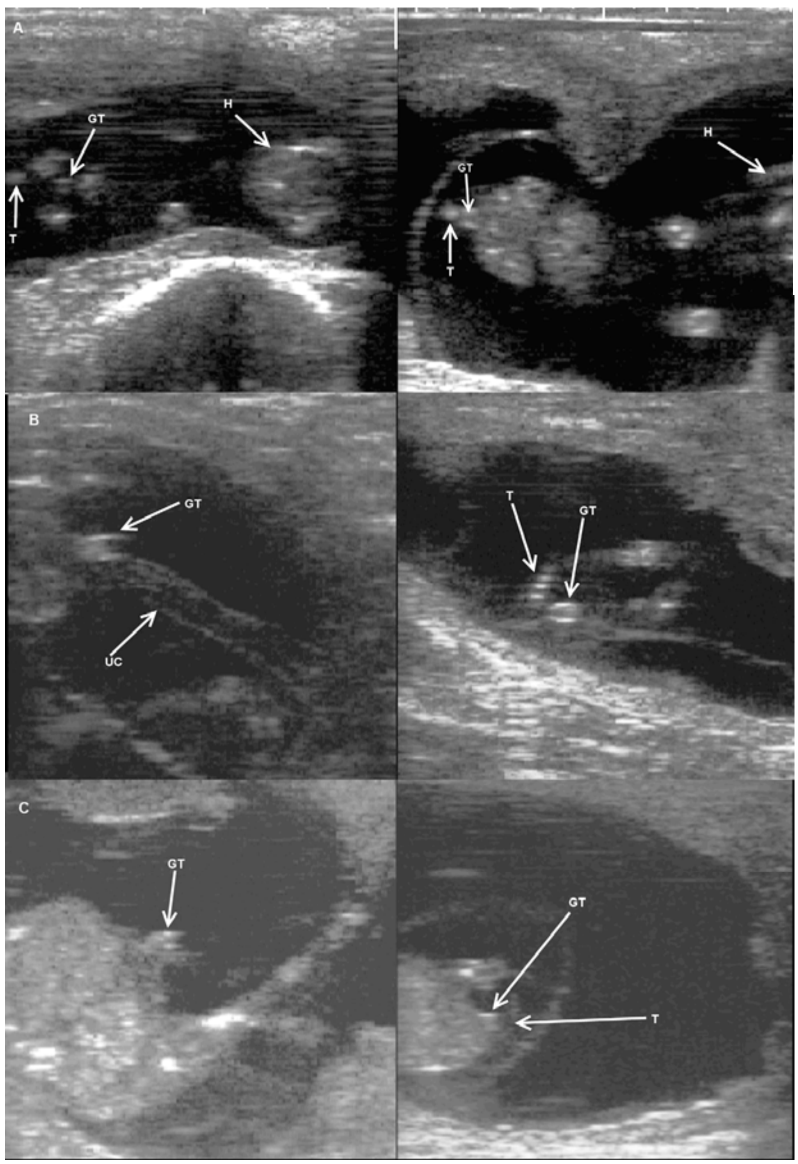


Figure 10: Ultrasound image carried out on longitudinal (**a**), cross-sectional (**b**) and sagittal (**c**) scans of male (left) and female (right) fetal genitalia. GT, genital tubercle; T, tail; H, head, UC, umbilical cord [39].

reliable and efficient tool in veterinary practice. The increasing importance of buffaloes, worldwide, for milk production and for meat comsuption makes this technique appealing to the practitioner for a better service to the buffalo breeders. The gender of foetuses can be detected by visualisation of the morphology and location of the genital tubercle (precursor to the penis and clitoris) (Figure **10**) [36] or the scrotum and mammary glands [37].

Ultrasound imaging of 28 buffalo foetuses on day 50 to 65 (period considered critical for foetal gender determination) has been performed every day [38]. The position of the genital tubercle was considered to be diagnostically relevant for both males (n=16) and females (n=12) by day 57, with confirmation of the sex to occur by day 59. The hyperechogenic image of the buffalo genital tubercle did not show any appreciable differences from the bovine genital tubercle. A good flat ventral view of the foetus at day 57 was essential for gender determination and at this stage a good view was always reached within two minutes of ultrasound scanning for each animal. Echographic confirmation of gender was performed from day 65 to 67 and 100 percent efficiency was verified. The ultrasound transducer must be manipulated within the rectum to provide a frontal, cross-sectional or sagittal image of the ventrum of the foetus. The umbilicus and tail serve as excellent landmarks when determining the location of the genital tubercle or the presence or absence of the scrotum (Figure **10**).

TRANSVAGINAL ULTRASOUND-GUIDED FOLLICULAR PUNCTURE

Increased interest in the "*in vitro*" embryo-production (IVEP) technologies, for faster propagation of superior germoplasm, has led to the development of the transvaginal ultrasound-guided follicular puncture (Ovum pick-up or OPU): this latter technique, combined with the IVEP technology, has great potential for improving the genetic development of this species through the maternal lineage. Within this framework, transvaginal oocyte recovery by puncture and aspiration of antral follicles has become a routine procedure in most laboratories where IVEP is part of the services offered to breeders.

The technique of OPU in buffalo, as in cattle, consists in the transvaginal recovery of the oocytes by

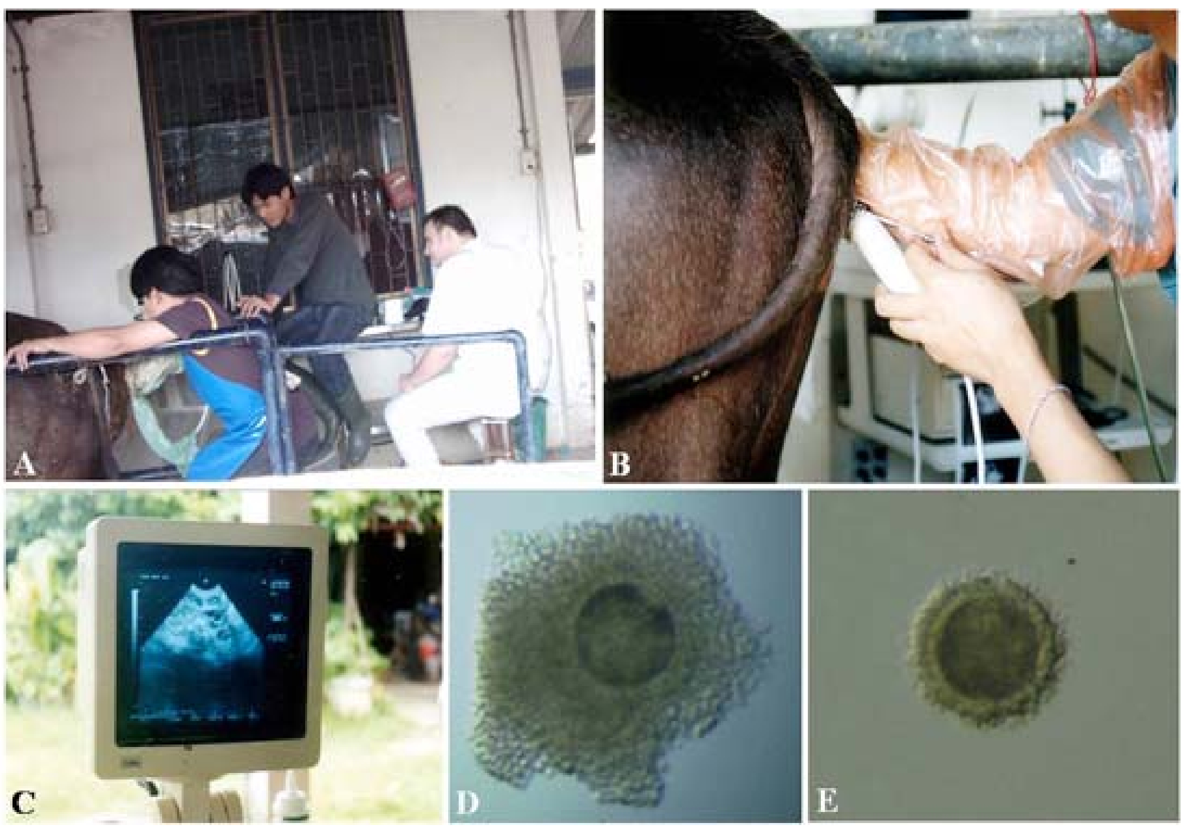


Figure 11: Ovum pick-up technique for oocyte collection from live female buffalo. (**A**) The cow is held in the enforcement case that facilitates rectal palpation. (**B**) An ultrasound probe with a long needle connected to a vacuum pump is passed through the vagina while the other hand holds the ovary by rectal palpation. (**C**) Real time ultrasound image. (**D**) Aspirated cumulus oocyte complex (**E**) Oocyte with incomplete cumulus cell layers [40].

Figure 12: Image of flocculence representing purulent material in the lumen of the uterus.
Although in heat this animal would clearly not be fertile.
On palpation this uterus would feel identical to that of a normal uterus.

follicular aspiration under ultrasound guide followed by *in vitro* fertilisation and culture of the fertilized oocytes (Figure **11**).

The OPU technique is a non invasive and repeatable procedure for recovering immature oocytes from individual known donors. The possibility of collecting large numbers of meiotically competent oocytes, suitable for (IVEP), renders the OPU*IVEP technique competitive to Superovulation (SO) for embryo production. Furthermore, the OPU technique can be performed in non cyclic females, in pregnant cows, in subjects with patent oviducts or genital tract infections, in animals not responsive to hormonal stimulation. It can also be employed as a means of obtaining embryos from clinically infertile but valuable animals.

OPU has been successfully applied in the buffalo species since 1994 [41] and subsequent studies dealing with this technique have been reported by several authors [42, 43] showing a low yield of good quality oocytes per ovary, compared to cattle (on average 2.4 *vs* 10.0, respectively) [44, 45].

UTERINE PATHOLOGY

Routine scanning of the uterus during scheduled reproductive exams will permit speedy diagnosis of several abnormalities such as**:** unviable fetus that can be determined by the absence of a heartbeat, abnormal uterine fluid or a degenerating fetus; pyometra that can be quickly and easily determined by the cloudiness or echogenicity of the uterine fluid and a distended uterus; abscesses, tumors, adhesions, mucometra. Ultrasound can assess the quality of intra-uterine fluid – purulence vs. mucous (Figure **12**). It can also detect very small amounts of fluid that would not be palpable in subclinical metritis cases. In clinical cases it can determine if the discharge is due to metriris or vaginitis.

Rare fetal anomalies such as schistosomus reflexus, multiple heads, fetal ascites and extreme arthrogryposis are devastating if allowed to go to term. Ultrasound, particularly at the stage for fetal sexing, can often identify these anomalies much earlier.

OTHER APPLICATIONS

Apart from oocytes aspiration, the transvaginal ultrasound-guided puncture may be also used in others applications: aspiration of fetal fluids for sex determination, biochemical and hormonal analysis; sampling of uterine contents for diagnostic purposes; sampling of follicular fluid and fluid from central cavity of CL; injection of substances into the ovaries, follicles and uterus.

CONCLUSION

The impact of real-time ultrasound on the study of animal reproduction has been dramatic and the development of portable ultrasound machines has given clinicians an added tool for diagnostic reproductive management. Ultrasound is commonly used to monitor uterine anatomy, involution, and pathology. In addition, it has been used to detect pregnancy, to study embryonic mortality, to monitor fetal development, and to determine fetal sex. The applications of ultrasound used by scientists include the ability to monitor follicular characteristics, ovarian function, and aid in follicular aspirations and oocyte retrieval. To-day, as technology has improved, technicians have an opportunity to use the internet or video conferencing for ultrasound image analyses. With every new technological development, scientists, veterinarians, and producers discover new possibilities for the use of reproductive ultrasound to enhance the scientific merit of research or improve reproductive efficiency in animal operations.

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