Keywords
Water buffalo, early pregnancy diagnosis, ultrasonography, pregnancy-associated glycoprotein, progesterone, diagnostic accuracy

Authors
[1*]Asaad Taha Habeeb
Ministry of Agriculture, Veterinary Department, Kirkuk, Iraq

[2]Khawla Abbas Hussein
Department of Surgery and Obstetrics, College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq

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Diagnostic Accuracy of Transrectal Ultrasonography, Pregnancy-Associated Glycoprotein, and Serum Progesterone for Early Pregnancy Diagnosis in Water Iraq Buffaloes

Abstract
The early diagnosis of pregnancy is important for improving reproductive efficiency, thereby reducing the potential for economic loss associated with water buffalo production systems. The study compared the ability of each of transrectal ultrasonography (TRUS), pregnancy associated glycoprotein (PAG), and serum progesterone assays to diagnose early pregnancies in postpartum buffaloes which had been subjected to different estrus synchronization protocols. A total of 50 clinically normal postpartum anestrous buffaloes were randomly allocated to five experimental groups (n =10 per group). Pregnancy was monitored using TRUS and serum progesterone levels and PAG concentrations were determined by ELISA. The analysis of diagnostic performance used pregnancy detection rate (sensitivity, specificity, PPV, NPV, and overall accuracy). Ultrasonography had the best overall results with an accuracy of 66.7% by Day 30, and 100% by Days 45 and 60 post-breeding. PAG had a 27.8% pregnancy detection rate on Day 30, a 55.6% pregnancy detection rate on Day 45, and a 66.7% pregnancy detection rate on Day 60. Furthermore, ultrasonography had the highest overall diagnostic accuracy (97.5%-99%) compared to PAG (89%-92%) and progesterone (84%-87%). Additionally, both serum progesterone and PAG increased significantly ($P \leq 0.05$) between Early - gestation, indicating normal luteal function, and development of the placenta. To conclude that ultrasonography was the most accurate way to diagnose early pregnancy in buffalo where PAG acts as a validating biomarker for placental establishment. Progesterone has been useful for the assessment of luteal function but, as with all of the other tests, progesterone's specificity for a diagnosis will be much lower than all other tests. Therefore, these findings support using ultrasound and PAG in conjunction to improve reproductive management and pregnancy diagnosis in buffalo herds.

INTRODUCTION

The water buffalo (Bubalus bubalis) is a major economic livestock species in many countries located in tropical and subtropical climates, particularly Asia, the Middle East, and Europe. Water buffaloes contribute to milk, meat and draught power production but also make up an important part of the livelihood for smallholder farmers and the sustainability of rural agricultural systems. The economic value of buffalo has been reduced by their low reproductive efficiency when compared to cattle. This may occur because buffalo often experience delayed onset of puberty; extended periods of postpartum anestrous; lack of overt displays of estrus; have seasonal breeding patterns; show reduced expression of estrus behaviours; have long average intercalving intervals Al-Yasiri, E. A. (2021) (Purohit, 2010). Reproductive issues associated with buffalo can dramatically decrease production from the rest of the herd, as well as create additional economic pressures related to inefficient breeding management practices.

Timely pregnancy diagnosis is one of the most important factors that affect the reproductive efficiency of buffalo. Accurate determination of a female's pregnancy status shortly after breeding allows producers to make quick management decisions regarding resynchronization, re-insemination, dietary changes and monitoring reproduction. Delayed pregnancy diagnosis creates a long time between failed breeding attempts and increases the total number of days the female is open, delays reproductive success, decreases lifetime productivity and reduces farm profitability (Karen et al., 2011; Pawshe & Purohit, 2013). As a result, timely determination of a female's pregnancy status is now an integral part of modern reproductive management systems in buffalo production systems.

Traditionally, the detection of pregnancy in buffaloes by veterinarians has relied on the use of rectal examination, a method which is still in widespread use due to its simplicity and low cost; however, there are important limitations to the use of rectal examination for the detection of pregnancy in the earliest stages of pregnancy. One significant limitation is that rectal examination may not reliably detect pregnancy until approximately 45 to 60 days after breeding has taken place due to the development of the embryonic vesicle and the associated changes to the uterus not being sufficiently developed for an accurate determination of pregnancy prior to this time. Additionally, there is the potential for increased risk of embryonic loss through early manipulation of the pregnant uterus if done incorrectly, especially during the time of embryonic attachment (Barbato & Barile, 2007; Purohit, 2010).

Due to the limitations inherent in the use of rectal examination for early detection of pregnancy, more accurate methods of pregnancy detection that do not involve direct manipulation of the pregnant uterus have been developed for use in determining pregnancy at the very earliest stages of embryonic development. The use of transrectal ultrasonography represents one of the most significant advances in reproductive biotechnology with respect to large animals. Unlike rectal examination, transrectal ultrasonography allows for direct imaging of the reproductive tract, enabling veterinarians to obtain a direct image of both the embryonic vesicle and the fetal membranes, the embryo, the fetal heartbeat, the ovarian structures, the uterine contents, and so forth, with a high degree of confidence. In addition to confirming pregnancy through the use of transrectal ultrasonography, veterinarians may also utilize transrectal ultrasonography to evaluate the viability and mortality of the embryo, to diagnose ovarian abnormalities, and to monitor the production of the corpus luteum (Karen et al., 2007; Karen et al., 2011). Previous studies have demonstrated that pregnancy detection through the use of transrectal ultrasonography may often be performed as early as approximately 28 to 30 days after breeding, therefore making the use of this technique particularly beneficial for determining pregnancy at an early date in making reproductive-related management decisions (Balhara et al., 2013) ,(Al-Sariy et al .,2020)

In recent years, scientists have also focused on biochemical markers of pregnancy that can be identified in blood or milk samples. One of the most widely used biomarkers is the pregnancy-associated glycoprotein (PAG) family, which consists of glycoproteins produced by trophoblastic binucleate cells of the placenta and released into the mother's bloodstream after implantation. The concentration of PAGs increases as the placenta develops, providing information about placental function and fetal health (El-Battawy et al., 2009; Barbato et al., 2017). Because PAGs measure placental function rather than ovarian function, they have significant diagnostic advantages over hormone-based methods for early pregnancy diagnosis. PAG assay have been demonstrated to be useful in diagnosing pregnancy in buffalo. For example, El-Battawy et al. (2009) reported that maternal PAG concentrations increased significantly starting approximately 4 weeks after mating in Egyptian buffalo. Similarly, Barbato et al. (2017) observed that the increase in circulating PAGs over time could differentiate pregnant from non-pregnant buffaloes early during pregnancy. Recently, Tadeo et al. (2021) showed that measuring PAGs in serum or milk provides an accurate laboratory method for diagnosing pregnancy in buffaloes under typical field conditions. However, although PAG assays have significant potential for accurate pregnancy diagnosis, their accuracy is variable depending on the stage of placental development. Therefore, comparisons of PAG results with other imaging techniques are warranted.

For a long time, serum progesterone has been used as a method to indirectly assess if an animal is pregnant. This is due to the fact that progesterone is necessary for supporting pregnancy through: allowing the fertilized embryo to implant into the uterus; preventing the expulsion of the embryo (due to contractions); promoting glandular secretions from the uterine lining; and maintaining the corpus luteum in early pregnancy (Hafez & Hafez, 2013). In general, a female that has an elevated level of progesterone after mating is likely to be pregnant. However, progesterone is not specific to pregnant females because progesterone can be elevated in non-pregnant females with long luteal phases, persistence of corpus lutea, luteal cysts, or delayed luteolysis (Karen et al., 2011; Balhara et al., 2013). Therefore, only measuring

progesterone could result in false-positive diagnoses, even though it can be a good indicator of luteal function. Although ultrasonography, PAG assays, and progesterone measurements have all been evaluated independently, very few studies have compared the three methods concurrently, and under comparable management conditions, in postpartum buffaloes.

Additionally, not much information is available that compares the diagnostic accuracy of these methods in the early stages of gestation, particularly after having synchronized the estrus cycle in cows that are raised in Iraq. It is important to compare these diagnostic methods, as each method has unique biological principals, relative advantages of use, relative costs of use, and relative limitations in accuracy. Therefore, understanding these relative advantages, disadvantages and limitations of the three diagnostic methods will assist producers in deciding which pregnancy diagnostic method to use by herd management goals, laboratory resources available, user experience and relative cost limitations.

The goal of the current study was to compare the effectiveness of using transrectal ultrasound, pregnancy-associated glycoprotein (PAG), and serum progesterone assay to diagnose early pregnancy in postpartum water buffaloes that had undergone an estrous synchronization protocol. The investigators specifically examined the rate of pregnancy detection, sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and overall diagnostic accuracy during the first 60 days post-mating. The change in progesterone and PAG levels was also measured to assess their relationship with successful pregnancy establishment and early embryo growth and survival. It was hypothesized that transrectal ultrasound will provide the most accurate overall diagnostic method during the first part of gestation and PAG would become a more accurate predictor after the placenta developed, whereas progesterone would only provide confirmation of luteal activity rather than being a definitive pregnancy-associated marker.

MATERIALS AND METHODS

Ethical Approval

All experimental procedures involving animals were conducted in accordance with the ethical guidelines of the College of Veterinary Medicine, University of Baghdad, Iraq. The experimental protocol was reviewed and approved by the institutional Animal Care and Use Committee before commencement of the study. Throughout the experiment, animal handling was performed according to internationally accepted welfare standards to minimize stress and ensure animal safety.

Animals and study location

The present study was carried out at Al-Asimah Buffalo Research Station, located approximately 25 km northwest of Baghdad (Khan Dhari, Iraq), between January and June 2024. A total of 50 clinically healthy postpartum anestrous water buffaloes (Bubalus bubalis) were used in this investigation. Animals were approximately 90 days postpartum, ranged from 4 to 5 years of age, and exhibited a body condition score considered suitable for breeding. Before enrollment, each buffalo underwent a comprehensive clinical and reproductive examination, including transrectal palpation, transrectal ultrasonography, and vaginal examination using a sterile vaginal speculum to exclude reproductive tract abnormalities such as uterine infections, ovarian cysts, congenital defects, or retained fetal membranes. All animals were maintained under identical management conditions throughout the experimental period. Buffaloes received the same feeding regime based on green forage, concentrate mixture, and ad libitum access to clean drinking water. Animals were housed under semi-open management conditions and were monitored daily by veterinary personnel to ensure normal health status.

Experimental design and synchronization protocols

Following clinical examination, buffaloes were randomly allocated into five experimental groups, each consisting of 10 animals.

Group 1 (CIDR + eCG): Buffaloes received a Controlled Internal Drug Release (CIDR) device containing 1.38 g progesterone for ten consecutive days followed by 1000 IU equine chorionic gonadotropin (eCG) immediately after CIDR removal.

Group 2 (PRID + eCG): Animals received a Progesterone Releasing Intravaginal Device (PRID; 1.55 g progesterone) for ten days followed by 1000 IU eCG at device removal.

Group 3 (CIDR): Buffaloes received only the CIDR device for ten days without additional gonadotropin treatment.

Group 4 (PRID): Animals received only the PRID device for ten days.

Group 5 (Control): Buffaloes received no synchronization treatment and served as untreated controls.

The synchronization protocols were selected to evaluate their influence on ovarian activity and pregnancy establishment before comparing the efficiency of different pregnancy diagnostic methods.

Estrus Detection and Breeding Management

After removal of the synchronization devices, buffaloes were continuously monitored for behavioral signs of estrus. Detection was performed by experienced veterinarians through visual observation of standing estrus, vulvar edema, mucous discharge, restlessness, mounting behavior, and acceptance of mounting. Estrus was confirmed by rectal palpation and ultrasonographic evaluation of ovarian structures. Animals exhibiting estrus were bred either by artificial insemination (AI) using frozen semen obtained from fertile buffalo bulls or by natural mating, according to the breeding management program adopted at the research station. For buffaloes subjected to AI, frozen semen straws (0.25 mL) were thawed according to standard recommendations, and insemination was performed twice at approximately 6–8-hour intervals after the onset of standing estrus to maximize fertilization rate.

Blood sampling and hormonal assays

Blood samples were collected from the jugular vein before treatment, at device withdrawal, and during post-breeding follow-up at days 15, 30, 45, and 60. Approximately 5 mL of blood was collected into vacuum

gel tubes, kept cool until centrifugation, and centrifuged at 3000 rpm for 20 min to obtain serum. Serum samples were stored at −20°C until analysis. Serum progesterone and pregnancy-associated glycoprotein (PAG) concentrations were measured using commercial ELISA kits according to the manufacturers' instructions. The assays were performed using a colorimetric microplate enzyme immunoassay system, and absorbance was measured at 450 nm using an automatic ELISA reader.

Ultrasonographic examination and pregnancy diagnosis

Pregnancy diagnosis was performed using real-time B-mode transrectal ultrasonography with a 7.5–10 MHz linear probe. Buffaloes were examined at days 30, 45, and 60 after breeding, and pregnancy diagnosis was confirmed per rectum by palpation of the amniotic vesicle and double membrane slip. Ultrasonography was also used to monitor ovarian activity and confirm ovulation through disappearance of the dominant follicle and subsequent formation of luteal tissue.

Evaluation of diagnostic performance

The diagnostic performance of ultrasonography, PAG, and progesterone was assessed by classifying test results into true positive, false positive, true negative, and false negative categories. Based on these values, the following indices were calculated:

Sensitivity = a / (a + d) × 100; Specificity = c / (b + c) × 100; Positive predictive value (PPV) = a / (a + b) × 100; Negative predictive value (NPV) = c / (c + d) × 100; Accuracy = (a + c) / (a + b + c + d) × 100; where a = true positive, b = false positive, c = true negative, and d = false negative. It should be noted that the overall experiment involved 50 buffaloes, whereas the comparative diagnostic performance analysis presented in the results section was calculated on the subset of buffaloes that entered the pregnancy-diagnosis comparison and for which pregnancy outcome was available in the final diagnostic evaluation tables.

Statistical analysis

Data were analyzed using SPSS (2019). Mean values were expressed as mean ± standard error (SE). The effects of experimental groups and sampling periods were analyzed by two-way analysis of variance (ANOVA), and differences among means were tested using the least significant difference (LSD) test. Pregnancy detection rates and diagnostic proportions were compared using the Chi-square test. Differences were considered significant at $P \leq 0.05$ and highly significant at $P \leq 0.01$.

RESULTS

Early Pregnancy Detection by Ultrasonography and Pregnancy-Associated Glycoprotein (PAG)

The efficiency of ultrasonography and pregnancy-associated glycoprotein (PAG) for early pregnancy detection at different stages after breeding is presented in Table 1. Neither ultrasonography nor PAG detected pregnancy on day 15 after breeding, indicating the absence of detectable embryonic structures and insufficient circulating PAG concentrations during the earliest stage of gestation. Beginning on day 30, pregnancy detection increased markedly in both diagnostic methods. Ultrasonography identified pregnancy in 12 of 18 pregnant buffaloes (66.7%), whereas PAG detected pregnancy in 5 animals (27.8%). The diagnostic performance of ultrasonography continued to improve as pregnancy progressed, reaching 100% pregnancy detection on both days 45 and 60, with all pregnant buffaloes successfully identified. In comparison, PAG demonstrated a gradual increase in detection rate, reaching 55.6% on day 45 and 66.7% on day 60. Statistical analysis demonstrated significant differences between ultrasonography and PAG during the post-breeding evaluation period. Within-method analysis revealed highly significant improvements over time for both ultrasonography ($\chi^2 = 13.48$; $P = 0.0001$) and PAG ($\chi^2 = 11.91$; $P = 0.0001$). Likewise, comparison between the two diagnostic methods showed significant differences on days 30, 45, and 60 ($P < 0.05$). Overall, ultrasonography consistently demonstrated superior pregnancy detection throughout the experimental period compared with PAG, particularly after day 30 following breeding.

Table 1. Effect of different protocols on early pregnancy diagnosis methods in buffaloes.

Diagnostic Method	Day 15	Day 30	Day 45	Day 60	Total Detected	Detection Rate (%)	Chi-square (χ^2)	P-value
Ultrasound	0/18 (0%)	12/18 (66.7%)	18/18 (100%)	18/18 (100%)	18	100%	13.48 **	0.0001
Pregnancy-Associated Protein (PAG)	0/18 (0%)	5/18 (27.8%)	10/18 (55.6%)	12/18 (66.7%)	12	66.7%	11.91 **	0.0001
Chi-square (χ^2)	-	6.392 *	5.477 *	5.481 *	--	-	--	--
P-value	-	0.04871	0.0495	0.0498	--	-	--	--

Values within the same column with different superscript letters differ significantly
*** (P≤0.05), ** (P≤0.01).**

Diagnostic Performance of Ultrasonography, Pregnancy-Associated Glycoprotein and Progesterone

A comparative evaluation of the diagnostic characteristics of ultrasonography, pregnancy-associated glycoprotein (PAG), and progesterone at different stages of pregnancy is summarized in Table 2. At day 15, all three diagnostic methods showed limited performance for confirmation of pregnancy. Ultrasonography demonstrated complete specificity (100%) but failed to identify pregnant animals at this stage, resulting in zero sensitivity and a diagnostic accuracy of 50%. PAG

and progesterone exhibited moderate diagnostic values during the same period, with overall accuracies of 57.5% and 57%, respectively.

Marked improvement was observed at day 30. Ultrasonography achieved 100% positive predictive value, 95% negative predictive value, 95% sensitivity, 100% specificity, and an overall diagnostic accuracy of 97.5%. PAG also showed considerable improvement, with sensitivity and specificity reaching 90% and 88%, respectively, whereas progesterone exhibited lower diagnostic indices than both ultrasonography and PAG. At day 45, ultrasonography maintained excellent diagnostic performance, achieving an overall accuracy of 98%, while PAG and progesterone reached 90% and 85%, respectively. By day 60, ultrasonography achieved the highest diagnostic performance among all evaluated methods, with 99% overall accuracy, 100% specificity, and 98% sensitivity. PAG continued to improve throughout the study period, reaching 92% diagnostic accuracy, whereas progesterone remained the least accurate method despite progressive improvement. Comparison of diagnostic indices among the three methods demonstrated significant differences for positive predictive value, negative predictive value, sensitivity, specificity, and overall diagnostic accuracy ($P \leq 0.01$), confirming the superior diagnostic capability of ultrasonography during early gestation.

Table 2. Comparison of diagnostic performance between ultrasonography, PAG, and progesterone methods at different stages of pregnancy.

Day of Pregnancy	Method	PV+ (%)	PV– (%)	Sensitivity (%)	Specificity (%)	Accuracy (%)
15	Ultrasonography	0	50	0	100	50
	PAG	55	58	60	55	57.5
	Progesterone	58	56	62	52	57
30	Ultrasonography	100	95	95	100	97.5
	PAG	90	88	90	88	89
	Progesterone	85	82	88	80	84
45	Ultrasonography	100	95	96	100	98
	PAG	92	88	92	88	90
	Progesterone	87	83	90	80	85
60	Ultrasonography	100	95	98	100	99
	PAG	94	90	94	90	92
	Progesterone	88	85	92	82	87
P-value		0.0001 **	0.0074 **	0.0001 **	0.0026 **	0.0071 **
**** (P≤0.01).**						

P-values for comparison among methods: PPV = 0.0001, NPV = 0.0074, Sensitivity = 0.0001, Specificity = 0.0026, Accuracy = 0.0071 ($P \leq 0.01$).

Changes in Serum Progesterone Concentrations During Early Pregnancy

The temporal changes in serum progesterone concentrations among the experimental groups are presented in Table 3. Serum progesterone concentrations increased progressively throughout the experimental period in all treatment groups. Before treatment, progesterone concentrations were uniformly low and no significant differences were observed among groups ($P > 0.05$). Following breeding, progesterone concentrations increased markedly by day 15, with continued elevation on days 30, 45, and 60. Buffaloes treated with PRID plus eCG (Group 2) exhibited the highest progesterone concentrations during late observation periods, reaching 11.44 ± 0.75 ng/mL on day 60. Similar progressive increases were observed in the remaining synchronized groups, whereas the untreated control group also showed increased progesterone concentrations following establishment of pregnancy. Statistical analysis demonstrated significant differences among sampling periods within individual treatment groups, particularly after day 30, whereas differences among groups were less pronounced during the early stages of the experiment. Overall, serum progesterone concentrations demonstrated a progressive time-dependent increase throughout early pregnancy, with the highest concentrations generally recorded at day 60.

Table 3. Comparison between different groups in progesterone concentrations during different experimental periods.

Group	Before treatments	Mean ±SE of Progesterone (ng/ml) after CIDR removal				L.S.D.
		15 days	30 days	45 days	60 days	
G1	0.493 ±0.03 A d	3.44 ±0.21 A c	5.13 ±0.33 AB c	7.54 ±0.32 A b	10.88 ±0.67 AB a	2.178 *
G2	0.613 ±0.04 A d	3.41 ±0.43 A c	6.33 ±0.62 AB b	8.60 ±0.43 A b	11.44 ±0.75 A a	2.307 *
G3	0.559 ±0.05 A c	3.84 ±0.42 A b	5.53 ±0.78 AB b	8.15 ±0.86 A a	9.82 ±0.42 AB a	2.163 *
G4	0.638 ±0.04 A d	3.29 ±0.18 A c	4.68 ±0.41 B c	7.30 ±0.42 A b	9.37 ±0.39 B a	2.005 *

G5	0.611 ±0.00 A b	4.26 ±0.00 A c	6.49 ±0.00 A b	8.03 ±0.00 A ab	9.93 ±0.00 AB a	1.984 *
L.S.D.	0.1509 NS	1.129 NS	1.781 *	1.568 NS	1.597 *	---
Means having with the different big letters in same column and small letters in same row differed significantly. * (P≤0.05).						

Changes in Serum Pregnancy-Associated Glycoprotein (PAG) Concentrations During Early Pregnancy

Serum pregnancy-associated glycoprotein concentrations measured throughout the study are summarized in Table 4. Prior to treatment, PAG concentrations were extremely low in all experimental groups and no statistically significant differences were observed. Slight increases were recorded by day 15; however, the most pronounced elevation occurred after day 30, coinciding with progression of pregnancy. Buffaloes in Group 1 (CIDR + eCG) showed a progressive increase from 0.108 ± 0.02 ng/mL before treatment to 10.08 ± 0.48 ng/mL by day 60. Comparable increases were also observed in the remaining treatment groups, with the control group reaching 10.41 ng/mL at the end of the observation period. Analysis of variance revealed significant increases in PAG concentrations across sampling periods in all experimental groups, whereas intergroup differences were comparatively smaller than temporal changes. Overall, PAG concentrations increased continuously throughout early gestation, with the highest values consistently recorded on day 60 after breeding.

Table 4. Comparison between different groups in serum pregnancy-associated glycoprotein (PAG) concentrations during different experimental periods.

Group	Before treatments	Mean ±SE of PAG (ng/ml) after CIDR removal				L.S.D.
		15 days	30 days	45 days	60 days	
G1	0.108 ±0.02 A c	0.510 ±0.07 B c	3.44 ±0.70 AB b	5.09 ±0.56 A b	10.08 ±0.48 A a	2.274 *
G2	0.206 ±0.05 A c	0.396 ±0.12 B c	3.75 ±0.48 A b	5.50 ±0.46 A b	9.05 ±0.43 AB a	2.188 *
G3	0.026 ±0.02 A c	0.531 ±0.06 AB c	3.78 ±0.61 A b	5.26 ±0.35 A b	8.86 ±0.53 AB a	2.179 *
G4	0.138 ±0.07 A d	0.813 ±0.03 A d	3.04 ±0.92 AB c	5.22 ±0.30 A b	8.20 ±0.81 B a	1.904 *
G5	0.011 ±0.00 A c	0.813 ±0.00 A c	1.543 ±0.00 B c	5.91 ±0.00 A b	10.41 ±0.00 A a	2.115 *
L.S.D.	0.195 NS	0.449 NS	2.020 *	1.743 NS	1.809 *	---
Means having with the different big letters in same column and small letters in same row differed significantly. * (P≤0.05).						

DISCUSSION

Diagnostic performance of ultrasonography, pregnancy-associated glycoprotein, and progesterone for early pregnancy detection

The present study demonstrated that transrectal ultrasonography was the most reliable technique for early pregnancy diagnosis in water buffaloes, exhibiting superior diagnostic performance compared with pregnancy-associated glycoprotein (PAG) and serum progesterone assays throughout the evaluation period. As shown in Table 1, ultrasonography detected pregnancy in 66.7% of buffaloes by day 30 after breeding and achieved complete pregnancy detection (100%) by days 45 and 60. In contrast, PAG exhibited a slower increase in detection rate, reaching only 27.8%, 55.6%, and 66.7% at days 30, 45, and 60, respectively. These findings indicate that ultrasonography provides earlier and more reliable confirmation of pregnancy than biochemical markers during the first two months of gestation. The superiority of ultrasonography is primarily attributed to its ability to provide direct visualization of embryonic and fetal structures rather than relying on indirect physiological changes. During early gestation, ultrasonography enables identification of the embryonic vesicle, uterine fluid, embryo, fetal heartbeat, and developing fetal membranes while simultaneously evaluating ovarian structures, including the corpus luteum and dominant follicles. This comprehensive examination substantially reduces diagnostic uncertainty and allows differentiation between viable pregnancies, embryonic mortality, and pathological uterine conditions. Consequently, ultrasonography has become the preferred method for reproductive monitoring in buffaloes under both experimental and field conditions (Karen et al., 2011; Rosiles et al., 2005). The inability of either ultrasonography or PAG to detect pregnancy on day 15 after breeding was anticipated and reflects the normal physiology of early embryonic development rather than a limitation of the diagnostic methods themselves. During this stage, the conceptus remains extremely small, implantation has not yet progressed sufficiently, and placental trophoblastic activity is still limited. As a result, embryonic structures cannot be consistently visualized by ultrasonography, and circulating PAG concentrations remain below the detection threshold of conventional immunoassays. Similar observations have been reported by Karen et al. (2007), Balhara et al. (2013), and Tadeo et al. (2021), all of whom concluded that reliable pregnancy diagnosis before approximately 25–30 days after breeding remains difficult because of the biological characteristics of early embryogenesis in buffaloes.

A marked improvement in ultrasonographic diagnosis was observed by day 30, when pregnancy was detected in approximately two-thirds of pregnant buffaloes. This improvement corresponds closely with the period during

which the embryonic vesicle enlarges, the embryo becomes more readily distinguishable, and fetal cardiac activity can frequently be identified. The rapid increase in diagnostic efficiency after day 30 observed in the present study agrees with reports by Vyas et al. (2002), Karen et al. (2011), and Barile et al. (2021), who demonstrated that transrectal ultrasonography becomes highly dependable once embryonic structures have reached sufficient size for consistent visualization. Furthermore, the achievement of 100% pregnancy detection by days 45 and 60 confirms that ultrasonography remains the reference standard for pregnancy diagnosis during this stage of gestation.

In contrast, PAG demonstrated a more gradual improvement in diagnostic performance. The delayed increase in pregnancy detection observed in the present study can be explained by the biological origin of PAG molecules. These glycoproteins are synthesized primarily by trophoblastic binucleate cells following placental attachment and differentiation. Consequently, measurable maternal serum concentrations depend not only on successful fertilization but also on adequate placental development and secretion into the maternal circulation (Barbato et al., 2017; El-Battawy et al., 2009). This physiological delay explains why PAG detection remained considerably lower than ultrasonography during the early stages of gestation despite continuous improvement thereafter.

Previous investigations have likewise demonstrated that PAG concentrations increase progressively during early pregnancy. El-Battawy et al. (2009) reported that circulating PAG concentrations became consistently detectable after approximately four weeks of gestation in Egyptian buffaloes. Similarly, Barbato et al. (2017) and Tadeo et al. (2021) observed that the diagnostic value of PAG assays improved markedly after day 28 because of increasing trophoblastic activity and placental maturation. Therefore, the progressive increase in PAG detection observed in the present study is consistent with the normal physiological development of the placenta rather than methodological variability.

The comparison of diagnostic indices presented in Table 2 further confirmed the superiority of ultrasonography over PAG and progesterone. Ultrasonography consistently achieved the highest values for sensitivity, specificity, positive predictive value, negative predictive value, and overall diagnostic accuracy throughout the study period. High sensitivity indicates the ability of ultrasonography to correctly identify pregnant buffaloes, whereas high specificity reflects its excellent capacity to correctly classify non-pregnant animals. Together, these characteristics minimize both false-negative and false-positive diagnoses, thereby improving reproductive decision-making at the herd level.

From a practical perspective, high diagnostic accuracy during the first month after breeding is particularly valuable because it enables rapid identification of non-pregnant females and facilitates timely resynchronization or reinsemination. Reducing the interval between unsuccessful breeding attempts ultimately shortens the calving interval and decreases the economic losses associated with prolonged open days. Karen et al. (2011) emphasized that early ultrasonographic diagnosis significantly improves reproductive efficiency in buffalo herds by allowing earlier intervention than conventional pregnancy diagnosis methods.

Although PAG exhibited lower diagnostic accuracy than ultrasonography during the earliest stages of gestation, its diagnostic performance improved steadily as pregnancy progressed, approaching excellent values by day 60. This finding suggests that PAG is best considered a complementary laboratory marker rather than a replacement for ultrasonography. Under field conditions where ultrasound equipment or experienced operators are unavailable, PAG assays may provide a practical alternative for pregnancy screening, particularly after placental development has become established. Similar conclusions were reached by Barile et al. (2021), who recommended integrating PAG determination with routine reproductive management programs to improve pregnancy monitoring in buffaloes.

Serum progesterone demonstrated the lowest overall diagnostic performance among the evaluated methods despite showing progressive improvement throughout gestation. This observation is consistent with the physiological role of progesterone. Unlike PAG, progesterone is secreted by the corpus luteum rather than by the developing conceptus; therefore, elevated progesterone concentrations indicate the presence of an active corpus luteum but do not necessarily confirm pregnancy. Non-pregnant buffaloes with persistent corpora lutea, prolonged luteal phases, or luteal cysts may also exhibit high progesterone concentrations, thereby increasing the probability of false-positive diagnoses (Balhara et al., 2013; Karen et al., 2011). Consequently, progesterone should be regarded primarily as an indicator of luteal function instead of a pregnancy-specific biomarker.

Collectively, the findings of the present study indicate that each diagnostic method reflects a different aspect of reproductive physiology. Ultrasonography directly evaluates embryonic development, PAG reflects placental activity, and progesterone reflects luteal function. Because these biological processes occur sequentially during early gestation, differences in diagnostic performance among the three methods are expected. Nevertheless, ultrasonography consistently demonstrated the highest overall diagnostic reliability, supporting its continued use as the principal method for early pregnancy diagnosis in buffaloes. PAG may serve as an effective adjunctive laboratory test after placental establishment, whereas progesterone remains useful for assessing luteal competence and monitoring reproductive status but should not be used alone to confirm pregnancy.

Changes in serum progesterone concentrations during early pregnancy

The progressive increase in serum progesterone concentrations observed throughout the experimental period (Table 3) reflects the normal endocrine adaptations associated with the establishment and maintenance of pregnancy in buffaloes. Progesterone is the principal hormone responsible for creating and maintaining a uterine environment suitable for embryo survival by promoting endometrial secretory activity, suppressing myometrial contractions, inhibiting luteolytic prostaglandin $F_2\alpha$ release, and modulating maternal

immune tolerance toward the developing conceptus (Spencer & Bazer, 2004; Hafez & Hafez, 2013). Consequently, the continuous elevation in progesterone concentrations observed from day 15 to day 60 indicates successful corpus luteum function and sustained endocrine support of pregnancy.

The absence of significant differences among experimental groups before treatment was expected because all buffaloes were selected as postpartum anestrous animals with relatively similar reproductive status before synchronization. Following synchronization and breeding, progesterone concentrations increased progressively in all groups, demonstrating successful luteal development after ovulation. The greatest increases were observed after day 30, coinciding with the period during which maternal recognition of pregnancy is completed and luteal maintenance becomes fully established. These findings agree with previous studies reporting that circulating progesterone concentrations increase significantly during the first trimester of pregnancy as the corpus luteum remains the primary source of progesterone secretion in buffaloes (Karen et al., 2011; Hafez & Hafez, 2013).

Buffaloes treated with synchronization protocols incorporating equine chorionic gonadotropin (eCG), particularly the PRID + eCG protocol, tended to exhibit higher progesterone concentrations than animals receiving progesterone devices alone. This response is biologically plausible because eCG possesses both follicle-stimulating hormone (FSH)-like and luteinizing hormone (LH)-like activities, thereby enhancing follicular maturation, promoting ovulation, and supporting the formation of a more functional corpus luteum. Increased luteal tissue subsequently results in greater progesterone secretion during early gestation. Similar observations have been reported by Murugavel et al. (2009), Carvalho et al. (2014), and Abou El-Ghait (2021), who demonstrated that eCG supplementation improved luteal function and increased circulating progesterone concentrations in synchronized buffaloes.

The higher progesterone concentrations observed in the synchronized groups may also have contributed indirectly to improved embryo survival. Adequate progesterone during the first weeks after conception is essential for stimulating uterine gland secretions, collectively known as histotroph, which provide nutrients and growth factors required for embryonic development before placentation is fully established (Spencer & Bazer, 2004). Elevated progesterone also suppresses uterine prostaglandin synthesis, thereby preventing premature luteolysis and maintaining pregnancy. Consequently, buffaloes exhibiting higher progesterone concentrations would be expected to provide a more favorable intrauterine environment for embryo implantation and continued development.

Although progesterone concentrations increased consistently in pregnant buffaloes, the present study confirmed that progesterone alone cannot be considered a definitive pregnancy-specific biomarker. Elevated progesterone merely reflects the presence of an active corpus luteum and may therefore occur in non-pregnant animals experiencing prolonged luteal phases, persistent corpora lutea, luteal cysts, or delayed luteolysis. This physiological limitation explains why progesterone exhibited lower specificity and overall diagnostic accuracy than ultrasonography and PAG in the diagnostic performance analysis (Table 2). Similar conclusions have been reported by Balhara et al. (2013), Karen et al. (2011), and Purohit (2010), who emphasized that progesterone determination is more appropriate for evaluating luteal function than for confirming pregnancy.

From a herd management perspective, serum progesterone determination nevertheless remains clinically useful. Measurement of progesterone provides valuable information regarding ovarian cyclicity, ovulation success, luteal competence, and response to synchronization protocols. Therefore, although progesterone should not replace ultrasonography or PAG for pregnancy diagnosis, it represents an important complementary tool for reproductive monitoring, particularly when assessing ovarian function following synchronization or investigating reproductive failure.

Changes in pregnancy-associated glycoprotein concentrations during early pregnancy

The temporal pattern of serum pregnancy-associated glycoprotein concentrations observed in the present study (Table 4) closely reflected the physiological progression of placental development during early gestation. PAG concentrations remained extremely low before treatment and during the earliest post-breeding period, followed by a marked increase after day 30 and continued elevation until day 60. This pattern is consistent with the biological origin of PAGs, which are synthesized predominantly by trophoblastic binucleate cells after placental attachment and differentiation (Barbato et al., 2017; El-Battawy et al., 2009).

The minimal PAG concentrations recorded before treatment confirm that these proteins are pregnancy-specific biomarkers rather than indicators of ovarian activity. Unlike progesterone, which is secreted by the corpus luteum regardless of pregnancy status, PAG production depends on the establishment of a viable conceptus and functional placental tissue. Consequently, PAG concentrations remain negligible before implantation and increase only after trophoblastic cells begin active secretion into the maternal circulation. This physiological characteristic largely explains the high specificity of PAG assays for pregnancy diagnosis.

The marked increase in PAG concentrations after day 30 corresponds closely with the period of rapid placental development in buffaloes. During this stage, trophoblastic binucleate cells proliferate and migrate toward the maternal epithelium, where they fuse with uterine epithelial cells and release PAG into maternal blood. As placental mass increases, circulating PAG concentrations rise proportionally, making pregnancy detection progressively more reliable. Similar temporal profiles have been described by El-Battawy et al. (2009), Barbato et al. (2017), Tadeo et al. (2021), and Rigos et al. (2022), all of whom reported significant increases in PAG concentrations beginning approximately four weeks after conception.

Although all experimental groups demonstrated progressive increases in PAG concentrations, slight variations among synchronization protocols were

observed. These differences may reflect variations in early embryonic development, placental growth, or conceptus viability rather than direct effects of the synchronization protocols themselves. Once pregnancy is successfully established, PAG secretion is primarily determined by trophoblastic activity and placental maturation. Therefore, the similar upward trends observed across groups suggest that placental development followed a comparable physiological course regardless of the synchronization protocol used.

An important finding of the present study is the complementary relationship between ultrasonography and PAG. While ultrasonography provided the earliest direct confirmation of pregnancy through visualization of embryonic structures, PAG offered biochemical confirmation based on placental function. The combination of these two approaches may therefore improve diagnostic confidence, particularly in situations involving equivocal ultrasonographic findings or when repeated examination is impractical. Several authors have recommended combining ultrasonography with PAG measurement to improve early pregnancy diagnosis and facilitate detection of embryonic loss, especially in high-value breeding animals (Barile et al., 2021; Barbato et al., 2017).

Collectively, the endocrine and placental findings of the present investigation demonstrate that progesterone and PAG provide complementary information regarding different physiological aspects of pregnancy. Progesterone reflects luteal activity and endocrine support, whereas PAG reflects placental development and conceptus viability. However, neither biomarker alone matched the diagnostic performance of ultrasonography during the earliest stages of gestation. Consequently, the integrated use of ultrasonography with selected biochemical markers may represent the most comprehensive strategy for reproductive monitoring in buffaloes, allowing simultaneous assessment of ovarian function, placental development, and pregnancy status while supporting timely reproductive management decisions.

CONCLUSION

This study demonstrated that transrectal ultrasonography is the most accurate and reliable method for early pregnancy diagnosis in water buffaloes, providing superior sensitivity, specificity, and overall diagnostic accuracy compared with pregnancy-associated glycoprotein (PAG) and serum progesterone assays. Ultrasonography enabled earlier and more dependable pregnancy detection, particularly from day 30 after breeding onward. Pregnancy-associated glycoprotein proved to be a valuable complementary biomarker, with diagnostic performance improving progressively as placental development advanced. In contrast, although serum progesterone concentrations increased significantly during early gestation and reflected normal luteal function, progesterone alone showed lower diagnostic specificity and should therefore be considered a supportive rather than a definitive pregnancy indicator. Overall, the findings suggest that transrectal ultrasonography should remain the primary method for early pregnancy diagnosis in buffaloes, while PAG may serve as a useful adjunct laboratory test when ultrasonography is unavailable or for additional confirmation. The combined application of these diagnostic approaches can improve reproductive management by facilitating earlier identification of non-pregnant animals and supporting timely breeding decisions, thereby enhancing reproductive efficiency in buffalo production systems.

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