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Using real-time PCR to identify pregnancy-associated glycoprotein 2 (PAG-2) in water buffalo (*Bubalus bubalis*) blood in early pregnancy

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ABSTRACT

This study investigates for the first time mRNA pregnancy-associated glycoprotein 2 (PAG-2) expression in blood cells during early pregnancy in water buffalo. The PAGs constitute a large family of glycoproteins expressed in the outer epithelial layer of the placenta in eutherian species. All PAGs are not concomitantly expressed throughout pregnancy; some of them are expressed in the earlier phases, whereas others appear later and are expressed over a shorter period. Twenty-one lactating buffaloes were analyzed—17 females were synchronized with PRID and artificially inseminated (AI), whereas four females were synchronized but not inseminated (control group). Blood was collected at Days 0, 18, 28, 40, and 75 from AI (AI = Day 0). Expression of PAG-2 mRNA in blood samples was measured with real-time polymerase chain reaction. Pregnancy diagnosis was performed on Day 28 (D28) and Day 40 (D40) after AI by ultrasonography (US) and by PAG-1 RIA method. The females diagnosed pregnant at D28 and confirmed pregnant at D40 were defined as D28(+)D40(+) group; the females diagnosed pregnant at D28 but not confirmed pregnant at D40 were defined as D28(+)D40(−) group; and the females that were diagnosed as nonpregnant on either days were defined as D28(−)D40(−) group. PAG-2 mRNA at Day 0 was not observed in any groups. The D28(+)D40(+) group showed the highest expression, starting on Day 18 and increasing progressively up to Day 75. PAG-2 mRNA was also expressed on Day 18 in both D28(+)D40(−) and D28(−)D40(−) groups, but their levels were lower than those of D28(+)D40(+) group and almost constant over time. PAG-2 mRNA was never detected in the control group. The significant difference in the expression of PAG-2 mRNA between the D28(+)D40(+) group and the D28(−)D40(−) group, starting from Day 18, suggests that these animals might have conceived, but have experienced early embryonic loss; therefore, the PAG-2 mRNA was still present in blood circulation although at lower levels, as found in the D28(+)D40(−) group. In conclusion, this study shows that PAG-2 mRNA can be detected in peripheral maternal blood cells earlier than circulating PAG-1 molecules and could be useful for studies on early pregnancy and embryonic mortality.

1. Introduction

Pregnancy diagnosis is an important component of a complete reproduction management program. Early

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detection of serviced females that did not conceive enables us to implement measures that would improve the conception rates of herds [1]. Although it is generally accepted that the earlier pregnancy is diagnosed, the higher the profit margin, it is important to note that the incidence of pregnancy loss can also have a significant negative effect on reproductive performance [2,3]. Therefore, if we are aware of the incidence and frequency of embryonic losses, we can implement management strategies to avoid the negative effects of this phenomenon [4]. In buffalo species, reproductive efficiency is affected by a delayed attainment of puberty, seasonality, and poor estrous expression behavior [5,6]. Therefore, it is essential to distinguish between pregnant and nonpregnant cows, particularly when breeding techniques such as "out of breeding season mating" or artificial insemination are applied. However, it is difficult to carry out studies on early embryonic mortality in buffaloes (as for other ruminant species) because no test is currently available providing valuable information on the survival of an embryo in the uterus during the first 25 days of gestation.

Various methods of pregnancy diagnosis and monitoring of embryonic and/or fetal mortalities have been developed during the last few decades (reviewed by Fricke [4] and Sousa et al.[7]) such as transrectal ultrasonography (US) [8,9], the measurement of P4 concentrations in blood and milk [10], and the determination of pregnancy-associated or pregnancy-specific proteins (PAGs/PSPB) [11–14]. More recently, the expression of interferon-t (*IFN-τ*)–stimulated genes (*ISGs*) has also been investigated [15], although, several studies have shown that the mRNA level of *ISGs* on Day 18 was not an accurate indicator of pregnancy in dairy cows or heifers [16–18].

PAG molecules belong to a large group of placenta-expressed glycoproteins and some of them are secreted into maternal blood [12,19]. Phylogenetic analyses have shown that PAGs can be enclosed into at least two groups known as "ancient" (PAG-II group: PAG-2, -8, PAG-10 to PAG-13) and "modern" (PAG-I group: PAG-1, PAG-3 to -7, PAG-9, PAG-14 to -18, PAG-20 to -22) [20,21]. In general, "modern" PAGs are considered to be limited to binucleate cells, whereas "ancient" PAGs are expressed in both mono- and binucleate trophoblastic cells [21]. All PAGs are not concomitantly expressed throughout pregnancy; some of them are expressed in the earlier phases, whereas others appear later and are expressed over a shorter period [21]. By using the real-time polymerase chain reaction (PCR) technique, it was demonstrated that *PAG-2* mRNA can be detected in placenta in an earlier stage of gestation, which coincides with the beginning of implantation, than *PAG-1* mRNA. Indeed, *PAG-2* mRNA was reported as early as 17 to 19 days of pregnancy in bovine placenta [22], 18 days in caprine [23], and 13 days in ovine [21]. The *PAG-1* mRNA expression has been found at 45 days of gestation and not earlier [21,23].

Eleven distinct PAG molecules from group I were purified and characterized from water buffalo placenta (wbPAG) [24,25], allowing the development of different RIA systems. PAG-1 concentrations can be detected in the blood of water buffalo pregnant females at approximately Day 30 of gestation by performing RIA [25]. Despite reports of 19 different complementary DNAs (cDNAs) of *PAG* genes identified in this species (UniProtKB/TrEMBL), there are no reports concerning the expression of *PAG-1* or *PAG-2* mRNA in buffalo species.

Considering the earlier expression of *PAG-2* mRNA in domestic ruminants, with this study we investigated the *PAG-2* expression in maternal blood cells during the early pregnancy period in buffalo to find a useful marker for studies on early pregnancy and embryonic mortality.

2. Materials and methods

2.1. Animals and experimental design

This study was carried out at the Animal Production Research Centre (CREA–PCM) experimental farm located near Rome, Italy (42°3′ N, 12°37′ E), from February to April, which represents the transition from the breeding season to the nonbreeding season for this species in the northern hemisphere. The animals involved in this experiment were treated in compliance with the animal testing regulations established under Italian law. The experimental design was carried out according to good veterinary practices under farm conditions. The CREA-PCM is authorized to use farm animals for experimental design (as stated in DM 26/96–4 of Italian Welfare Ministry).

Twenty-one lactating Italian Mediterranean buffalo cows, aged 3 to 7 years, were used for determining PAG concentrations and RNA isolation. The animals were kept in an open paddock, fed *ad libitum* on total mixed ration based on maize silage, alfalfa hay, soyabean meal, maize meal, and barley meal (containing 0.90 UFL/kg of dry matter [DM] and 15% crude protein on DM). The buffaloes were milked twice daily.

Before estrus synchronization and artificial insemination program, buffalo cows were subjected to regular clinical examination to exclude animals with diseases such as endometritis, mastitis, and metabolic disorders. The buffalo cows were treated with a progesterone-releasing intravaginal device (PRID; Sanofi, France), containing 1.55 g of natural progesterone kept in place for 10 days. On PRID withdrawal, an intramuscular injection of 500 IU of PMSG (Ciclogonina, Fort Dodge, Italy) and 0.15 mg of cloprostenol ($PGF_{2\alpha}$ analogue; Dalmazin, FATRO, Italy) was administered to each animal. The evening before artificial insemination (AI), the buffaloes were treated intramuscularly with 150-μg gonadorelin (GnRH, Enagon; Intervet, Italy). Seventeen females were artificially inseminated with frozen-thawed semen 72 hours after PRID removal. Four other females, which were used as controls (control group), received the same synchronization treatment but insemination was carried out with straws containing only the extender.

Blood was collected from the jugular vein and put into 10-mL EDTA-coated tubes at Days 0, 18, 28, 40, and 75 from AI (AI = Day 0). To carry out *PAG-2* mRNA quantification, 200-μL of blood were added with 200-μL RNA later stabilizing solutions (Life Technologies, Carlsbad, CA, USA) and stored at −20 °C. For PAG-1 determination, the plasma was separated by centrifugation at ×2500*g* for 10 minutes and stored at −20 °C until assayed. Sampling at Day 75 was only carried out for the animals that were diagnosed as pregnant by US at Day 40.