Endometrial Cytology and Bacteriological Isolates From Buffaloes With Retained Fetal Membranes and Their Effects on the Reproductive Efficiency

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Abstract: This study aimed to determine if the buffaloes with retained fetal membranes (RFM) and without systemic involvement had an effect on the subsequent reproductive efficiency. One hundred buffaloes with or without placental retention were allocated into 4 groups, including 25 buffaloes at day 15 post-calving had RFM (1st group), 25 buffaloes at 45 days post-calving had RFM (2nd group), 25 buffaloes without RFM at day 15 post-calving as control (3rd group) and 25 buffaloes without RFM at day 45 post-calving as control (4th group). The intrauterine perfusion fluid (10ml) was collected and examined bacteriologically and cytologically to evaluate the intrauterine environment. The reproductive parameters were determined in both buffaloes with or without retained fetal membranes. The detection rate of bacterial spp. was significantly ($P<0.05$) higher in buffaloes with RFM collected at day 15 after parturition than those in other groups. All 25 buffaloes with RFM at 15 days post-partum (100.0 %) showed positive results. From 22 of them (88.0%), more than one bacterial species was isolated. An *Archanobacterium pyogenes* (A. pyogenes) was isolated from 56.0% of buffaloes with RFM after 15 days post-calving. On the other hand, 5 (20.0%) out of 25 buffaloes with RFM at 45 days post-partum showed positive results. Nine out of 25 (36.0%) buffaloes without RFM at 15 days post-partum showed positive results. Moreover, 4 out of 25 (16.0%) control buffaloes at 45 days post-partum showed positive results. The bacterial species most frequently isolated was *Lactobacillus spp*. The number of buffaloes with ≥70% PMNs or ≤ 40% lymphocytes cells was higher (24/25, 96%) in the 1st group (RFM) at 15 days than those in 2nd group (RFM) at 45 days post-calving. The number of buffaloes with ≥70% PMNs or ≤ 40% lymphocytes cells was also significantly ($P<0.01$) higher in control group (17/25, 65%) at 15 days than those in control group (6/25, 24%) at 45 days. There were no significant variations among the groups of the buffaloes with retained placenta and the control groups at 15 and 45 days post-calving in postpartum uterine involution, the number of days from parturition to initial insemination, the number of days to conception and the number of services per conception. The overall conception rate was 15(60%) and 16(64%) in the RFM group, meanwhile, it was 19(76%) and 20(80%) in the control groups. It could be concluded that, in most buffaloes, the retained fetal membranes without systemic involvement had no major effect on the postpartum reproductive performance. [Journal of American Science. 2010;6(11):115-121]. (ISSN: 1545-1003).

Keywords: Buffaloes, Bacteriologically, Cytologically, Insemination, Conception.

1. Introduction

Risk factors for acute metritis were categorized by Sheldon and Dobson (2004) as intrauterine damages (Stillbirth, dystocia, twins, caesarean section, retained placenta, delayed uterine involution), metabolic disorders (milk fever, ketosis, left displaced abomasums) and the balance between pathogencity and immunity (disruption of neutrophil function, type of bacterial flora, progesterone and glucocorticoids administration, early formation of corpus luteum, level of hygiene). The infection and to some extent the inflammation of the uterine wall during and after parturition must be accepted as a physiological process (Lewis, 1997; Hertl et al., 2010). Pathogenic species for metritis isolated from the uterine cavity are *Escherichi coli*, *A. pyogenes* and obligate anaerobic species *Fusobacterium* (F) *necrophorum* and *Prevotella spp*. (Lewis, 1997; Sheldon et al., 2004; Bicalho et al., 2010). Besides the quantity and quality of bacteria in the uterus, the efficiency of uterine defense mechanisms determines the severity of metritis. The uterine defense mechanisms consist of anatomical and physical barriers i.e. the vulvar and cervical closure as well as the cell mediated and humeral immune systems. The initial cellular response to an infection of the uterine wall is an influx of PMNs and macrophages. Immunoglobulins and opsonins are released from the endometrium (Bondurant, 1999; Dhaliwal et al., 2001; Földi et al., 2006). Knowledge and characteristics of the intrauterine environment following placental retention is needed to establish effective measures for an improving the reproductive efficiency in cattle with retained placenta (Joosten et al., 1988; Salama et al., 1993). This study aimed to determine if the buffaloes with retained fetal

membranes (RFM) and without systemic involvement had an effect on the subsequent reproductive efficiency based on the bacteriological and cytological examinations of intrauterine perfusion fluid.

2. Material and Methods

2.1. Animals

This study was conducted "between" November, 2008 to October, 2009 using 800 Egyptian buffaloes housed in barn stalls belonging to private farms related to Balkas, Dakahlia Province. The age of the animals ranged between 3-8 years. The animals stall fed and had unrestricted access to hay and 8-10 kg concentrate feed for each. Fifty buffaloes had not expelled the placenta after 24 hours post calving were assigned to the experimental group (RFM), while the other fifty had expelled the placenta without manual interference were used as a control group (C). No treatment was administered to the buffaloes with placental retention (expect those animals showing systemic involvement associated with fever, were both systemically and locally treated and excluded from the study). One hundred buffaloes with or without placental retention were allocated into 4 groups, including 25 buffaloes at day 15 post-calving had RFM (1^{st} group), 25 buffaloes at 45 days post-calving had RFM (2^{nd} group), 25 buffaloes without RFM at day 15 post-calving as control (3^{rd} group) and 25 buffaloes without RFM at day 45 post-calving as control (4^{th} group).

2.2. Collection of intrauterine perfusion fluid

The intrauterine perfusion fluid was collected once only from four groups by the method performed by Kaneko et al. (1996). A vaginal speculum was inserted into the vagina after cleaning of the vulva with disinfection (Betadine). The tip of a balloon catheter (Terumo Inc. Tokyo, Japan Fr. 22) was inserted into the cervix as deep as possible without touching the vaginal wall. The vaginal speculum was removed, and then the balloon catheter was advanced into the uterus using the recto-vaginal method. The balloon was inflated with air. Sterile physiological saline (100 ml) was infused into the uterus through a balloon catheter and recovered by gentle massaging of the uterus.

2.3. Bacteriological examination of the intrauterine perfusion fluid

The perfusion fluid (10 ml) was centrifuged at 1000 rpm for 10 minutes and after removal of the supernatant, the sediment was resuspended in 1 ml of physiological saline. An allocate of the resuspended sediment (100 µl) was applied to soy agar with 5% sheep blood and incubated for 2-7 days at 37ºC in both aerobic and anaerobic atmospheres. Using the criteria of Kaneko et al. (1996), samples showing growth of more than 50 identical colonies were defined as positive control for bacteria and were considered to indicate bacteriological deterioration of the intrauterine environment. Gram-negative, a typical, pine leaf-like rods, which showed hemolytic reaction on sheep blood containing agar medium were negative to the catalase test, were judged to the A. pyogenes. Samples showing the growth of a more than one A. pyogenes colony were defined as positive for A. pyogenes. All bacterial isolates were identified according to Bergey's manual of a systemic bacteriology (Holt et al., 1994).

2.4. Cytological examination of the intrauterine perfusion fluid

The perfusion fluid (10 ml) was centrifuged as described above and the sediment was smeared on a glass slide, dried in air, fixed for 3 minutes with methyl alcohol and then the sediment was stained with Giemsa. A total of 200 cells was counted at x1000 in each specimen and classified into PMNs, eosinophils, lymphocytes and macrophages like cells. The percentage of PMNs and lymphocytes were calculated and recorded. Specimens with a PMNs ratio exceeding 70% or a lymphocytes ratio below 40% were considered to indicate a poor cytologically intrauterine environment (Kaneko et al., 1996).

2.5. Investigation of the postpartum reproductive performance

The postpartum uterine involution, number of days from parturition to initial insemination, number of days until conception and number of inseminations required to achieve conception, as well as the overall conception rate, were determined in both buffaloes with or without placental retention.

2.6. Statistical analysis

The positive rates for bacteria and for A. pyogenes were compared between groups by using Chi-square test. The PMNs ratio and the lymphocytes ratio in sediment, inflammatory cells and the reproductive performance were compared by a student's T-test according to Snedecor & Cochran (1982).

3. Results

The detection rate of *Streptococcus spp*., *Bacterioids melaninogenias, Fusebacterium necrophorum, Escherichia coli, Pasteurella multocida, Proteus vulgaris, Lactobacillus spp., Staphylococcus aureus, Enterococcus spp.* and *A. pyogenes* was significantly ($P<0.05$) higher in buffaloes with RFM collected at day 15 after parturition than that in other groups (Table 1). All 25

RFM buffaloes at 15 days post-partum (100.0 %) showed positive results. From 22 of them (88.0%), more than one bacterial species was isolated. A mixed culture of *E. coli, Streptococcus spp., Pasteurella multocida, A. pyogenes* and *Fusebacterium necrophorum* were most common. The bacterial species most frequently isolated was *E. coli*, (25 isolates, (100.0%), followed by *Streptococcus spp*, (22 isolates, 88.0 %), *Pasteurella multocida* (15 isolates, 60.0%) and *A. pyogenes*, (14 isolates, 56.0%). Other bacteria as Bacterioids melaninogenias and Fusebacterium necrophorum were found at lower frequencies (10 isolates for each one) as presented in Table 1. On the other hand 5 (20.0%) out of 25RFM buffaloes at 45 days post-partum showed positive results. The bacterial species most frequently isolated was *Staphylococcus* spp., *Pasteurella multocida and Proteus vulgaris.*

Whereas 9 (36.0%) out of 25 control buffaloes at 15 days post-partum, showed positive results. From 4 of them (16.0%), more than one bacterial species was isolated. A mixed culture of *E. coli, Streptococcus spp., Lactobacillus spp*. and *Enterococcus spp.*were most common. The bacterial species most frequently isolated was *E. coli and Lactobacillus spp*. (4 isolates for each, 16.0%), followed by *Streptococcus spp. and Enterococcus spp.* (3 isolates for each, 12.0 %) and Fusebacterium necrophorum (2 isolates, 8.0%). Moreover, 4 out of 25 (16.0%) control buffaloes at 45 days post-partum showed positive results. The bacterial species most frequently isolated was Lactobacillus spp. (Table 1).

Table 1. Proportion of buffaloes with or without placental retained showing positive cultures of aerobic and anaerobic bacteria in the intrauterine perfusion fluid collected at 15 and 45 days after parturition

Group	Bacterial species	No. /Frequency of isolation (%)
Buffaloes with retained fetal membranes		
at 15 days post-partum	*Streptococcus spp.*	22/25 (88.0)
	Bacterioids melaninogenias	10/25 (40.0)
	Fusebacterium necrophorum	10/25 (40.0)
	Escherichia coli	25/25 (100.0)
	Pasteurella multocida	15/25 (60.0)
	A. pyogenes	14/25 (56.0)
Total positive		25/25 (100.0) [a]
at 45 days post-partum	*Streptococcus spp.*	2/25
	Staphylococcus aureus	1/25
	Pasteurella multocida	2/25
	Proteus vulgaris	2/25
Total positive		5/25 (20.0)[c]
Control groups		
at 15 days post-partum	*Escherichia coli*	4/25 (16.0)
	Streptococcus spp.	3/25 (12.0)
	Fusebacterium necrophorum	2/25 (8.0)
	Lactobacillus spp.	3/25 (12.0)
	Enterococcus spp.	3/25 (12.0)
Total positive		9/25 (36.0)[b]
at 45 days post-partum	*Streptococcus spp.*	2/25 (8.0)
	Lactobacillus spp.	4/25 (16.0)
Total positive		4/25 (16.0)[c]

Different superscripts in the same column (Total positive) mean significant difference P<0.05

The main number of PMNs in the 1st group (RFM) at 15days post-calving was higher (P<0.01) than any those in other groups. There was also significant different in the percentage of PMNs (P<0.01) between control groups at 15 and 45 days. The mean number of a lymphocytes was significantly lower (P<0.01) in 1st group (RFMs) at 15 days than those in other groups. Also, there was a significant differences (P<0.01) between control groups at 15 and 45 days. The number of buffaloes with ≥70% PMNs or ≤ 40% lymphocytes cells was higher (24/25, 96%) in the 1st group (RFM) at 15 days than those in 3rd group (RFM) at 45 days post-calving. The number of buffaloes with ≥70% PMNs or ≤40% lymphocytes cells was also significantly (P<0.01) higher in control group (17/25, 65%) at 15 days than

those in control group (6/25, 24%) at 45 days (Table 2).

Table 2. The mean value of PMNs and lymphocytes (mean ± SD) in the intrauterine perfusion fluid of the buffaloes with and without RFM, collected 15 and 45 days after parturition

Groups	No.	PMNs Mean ± SD	Lymphocytes Mean ± SD	No. (%)
Buffaloes with retained fetal membranes				
at 15 days post-partum	25	83.5±17.7^{a}	14.4±16.0^{d}	25/25 (100.0)^{a}
at 45 days post-partum	25	52.4±29.5^{c}	41.7±27.4^{b}	5/25 (20.0)^{c}
Control groups				
at 15 days post-partum	25	63.9±26.0^{b}	31.02±3.3^{c}	9/25 (36.0)^{b}
at 45 days post-partum	25	36.9±26.3^{d}	55.4±27.1^{a}	4/25 (16.0)^{c}

Different superscripts in the same column (Total positive) mean significant difference P<0.05

There were no significant variations among the groups of the buffaloes with retained placenta and the control groups at 15 and 45 days post-calving in postpartum uterine involution (29.30±1.21 and 28.47±1.38 vs. 28.41±1.04 and 27.95±1.60), the number of days from parturition to initial insemination (90.0±28.5 and 83.0±3.22 vs. 84.0±20.6 and 79.2±28.7), the number of days to conception (124.7±56.4 and 131.2±66.0 vs. 114.11±19.46 and 116.0±31.51), and the number of services per conception (1.78±1.03 and 1.97±1.06 vs. 1.62±1.02 and 1.53±1.03), respectively. The overall conception rate was 15(60%) and 16(64%) in the RFM group, meanwhile, it was 19(76%) and 20(80%) in the control groups (Table 3).

Table 3. Postpartum reproductive performance in buffaloes with or without retained fetal membranes

Groups	Buffaloes number	Uterine involution	No. of days to initial insemination	No. of days to conception	No. of services per conception	Overall conception rate
Buffaloes with retained fetal membranes						
RFM15	25	29.30±1.21^{a}	90.0±28.5^{b}	124.7±56.4^{c}	1.78±1.03^{d}	15(60%)
RFM45	25	28.47±1.38^{a}	83.0±3.22^{b}	131.2±66.0^{c}	1.97±1.06^{d}	16(64%)
Control buffaloes						
C15	25	28.41±1.04^{a}	84.0±20.6^{b}	114.11±19.46^{c}	1.62±1.02^{d}	19(76%)
C45	25	27.95±1.60^{a}	79.2±28.7^{b}	116.0±31.51^{c}	1.53±1.03^{d}	20(80%)

The same superscripts within the same column means non-significant difference P<0.05
RFM: Retained Fetal Membrane C: Control

4. Discussion

The period immediately after calving is very important in the reproductive life cycle of buffalo because of the vast influence on reproductive efficiency. A normal uterine involution and the re-establishment of the ovarian function postpartum are crucial to obtain short calving to conception interval that is required to optimize milk and calf production. Retention of foetal membranes with the dominance of *E. coli* in the uterine lumen might favor the colonization of other bacteria including facultative anaerobic and strictly anaerobes in the uterine wall of buffaloes (Paisley et al. 1986; Hussain 1989). High prevalence of bacterial isolation from buffaloes after 15 days of calving in both buffaloes with or without RFM revealed mainly *E. coli*. This suggests that the bacterial contamination which is mainly *E. coli* present in the uterus shortly after parturition. This observation is in agreement with Dohmen and Sheldon (Dohmen et al. 2000; Sheldon et al. 2006) in cattle. This suggests that the bacterial contamination which is mainly *E. coli* present in the uterus after parturition might favor the development of uterine infection by other highly pathogenic organisms. While other facultative anaerobic bacteria and strictly anaerobic bacteria, which lack the ability to invade intact epithelium, are usually considered facultative pathogens (Dohmen et al. 1995; Sheldon et al. 2004).

The development of uterine disease depends on the immune response of the cattle, as well as the species and number (load and challenge) of bacteria (Sheldon et al. 2006). Therefore, damage to epithelium is usually required to establish infection (Cohen et al. 1995; Sheldon and Dobson 2004; Sheldon et al. 2004) either by *E. coli* infection or damaged epithelium resulting from obstetrical manipulation (Paisley et al. 1986; Hussain 1989). A. pyogenes and strictly anaerobic bacteria were never isolated from buffaloes with normal parturition after 15 days of parturition. A. pyogenes was isolated from 56.0% of buffaloes with RFM after 15 days post-calving. A pyogenes induces metritis by synergism with gram-negative bacilli such as F. necrophorum and prevotella spp., where F. necrophorum is known to produce a potent leukocidal endotoxin, these toxins facilitate tissue invasion by A. pyogenes which in turn produces growth stimulating factor for the species of bacteriodes which seen to have unusual potent lipopolysaccharide molecules (Ruder et al., 1981; Kaneko et al., 1997; Lewis, 1997; Sheldon et al., 2004).

Bacterial isolation from RFM buffaloes after 15 days of calving included non-specific bacteria mainly *E. coli*, A. pyogenes, Fusebacterium necrophorum, Bacterioids melaninogenias, Streptococcus spp., and Pasteurella multocida. These results were concordant with those reported by Azawi and Taha (2002); Jadon et al. (2005); Azawi (2006). Retained foetal membranes diminish uterine ability to eliminate contaminated organisms. The exact causes of uterine infections during the postpartum period remain unknown (Lewis 1997 and Azawi, 2008).). The detection rate of bacteria and A. pyogenes were decreased at day 45 after parturition and were no longer significantly different from those of the control animals. From the previous results, it is clear that infection and to some extent the inflammation of the uterine wall during and after parturition must be accepted as a physiological process (Lewis, 1997; Stephen et al., 2008). Lactobacillus spp. was isolated only from the uterus of buffaloes after 15 and 45 days of calving in buffaloes without RFM (control groups). These bacteria were never isolated from buffaloes with RFM. This result suggests that the presence of Lactobacillus sp. in the uterus indicated a healthy uterus (Bondurant 1999).

In the cytological examination, the number of buffaloes with ≥70% PMNs or ≤ 40% lymphocytes cells was higher (24/25, 96%) in the 1st group (RFM) at 15 days than those in 2nd group (RFM) at 45 days post-calving. The number of buffaloes with ≥70% PMNs or ≤ 40% lymphocytes cells was also significantly ($P<0.01$) higher in control group (17/25, 65%) at 15 days than those in control group (6/25, 24%) at 45 days (Table 3). The high percent of PMN in the uterus of RFM buffaloes suggests both direct and indirect effects of bacterial toxins to attract or stimulate PMN infiltration in the uterus. These observations were in agreement with the earlier observations of Zerbe et al. (2001). Others provided an evidence that placenta attracts PMN to uterine lumen (Hoedemaker et al. 1992). Moreover chemo-attractive properties of uterine fluid have been described in vitro and the uterus response quickly to an antigen with release of PMN-chemotactic mediators, which results in a rapid migration of PMNs into the uterine lumen (Pycock, 1994 and Watson et al., 1987). It could be suggested that the combined effect of RFM and bacterial infection and their toxins in the uterus attracts high number of PMN in uterine discharge of RP buffaloes. It appears that retained placenta does adversely affect the postpartum intrauterine environment but the injury is repaired at day 45 after parturition. Mechanical aspects of the uterine defense system are currently believed to be a major contributor in uterine clearance of a bacteria and inflammatory products (Troedsson & Liu, 1991; Troedsson et al., 1993; Stephen, 2008).

Retained placenta has been reported as risk factors to induce metritis and thereby to reduce subsequently fertility (Coleman et al., 1985; Dohoo & Martin, 1984; Erb et al., 1981; Halpern et al., 1985; Heinonen & Heinonen, 1989; Sheldon & Dobson, 2004). In this study, non significant difference in reproductive performance was found between the buffaloes with or without RFM. It has been reported that fertility was not affected in the buffaloes with retained placenta if metritis was not induced after placental retention or if the cattle had recovered from metritis by the time of an insemination (Stevenson & Call, 1988; and Werven et al., 1992 and Claire & Chery, 2007). *In conclusion*, as any system in the body, bacteria are regularly present in the genital tract of normal buffaloes during the different reproductive stages. These bacterial flora play an important role in genital tract protection against infection. Moreover, in most buffaloes, the retained fetal membranes without systemic involvement had no major effect on the postpartum reproductive performance.

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