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EFFECT OF BUFFALO *(BUBALUS BUBALUS)* CERVICAL MUCUS DURING THE ESTRUS PHASE ON SURVIVAL OF *BRUCELLA MELITENSIS* IN VITRO

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SUMMARY

The role of cervical mucus of buffaloes during the estrus phase in defending against pathogenic infection and protecting the genital tract was studied. In the first experiment, pooled fresh cervical mucus from 5 buffaloes in the estrous phase was collected aseptically, incubated for 30 minutes with five concentrations of colony forming units (cfu) of a known pathogenic strain of Brucella melitensis, Rev 1, (1×10^5, 1×10^6, 1×10^7, 1×10^8, and 1×10^9) and seeded onto 10 plates of brucella agar plates (2 plates for each bacterial concentration). No brucella growth at any cfu concentration was detected in all plates. The second experiment aimed to assaying concentrations of nitric oxide, proteins, and carbohydrates in cervical mucus, throughout 9 hours of normal estrous phase in 5 buffaloes, to find out if there are changes in their secretory pattern and to find out if there are possible correlations between them. Also, the electrophoretic pattern of cervical mucus proteins was assayed. Concentrations of nitric oxide, proteins, and carbohydrates fluctuated throughout the sampling period. Nitric oxide showed significant positive correlation with concentrations of proteins and carbohydrates in cervical mucus ($P < 0.044$ and 0.033 respectively). Electrophoretic pattern of cervical mucus collected at all sampling points was the same for all samples and showed 6 protein bands at 212.5, 130, 91, 70, 52, and 30 KD. Nitric oxide secreted in the cervical mucus of estrus-phase buffaloes has powerful bactericidal activity and constitutes a firm defense mechanism in the genital tract against infection even in case of intracellular pathogens. Also, the fluctuating pattern of the three constituents measured is attributed, in general, to the amount and nature of cellular components of the mucus, which are mainly leukocytes and their associated secretory molecules.

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INTRODUCTION

Brucella species are facultative intracellular pathogens that survive intracellular of their host. It was believed that virulence of these species and the establishment of chronic infections by them is thought to be essentially due to their ability to avoid the killing mechanisms within macrophages (Baldwin and Winter. 1994). The organism may escape from the host defense mechanism, but the development of a disease condition is the outcome of many factors including immune-competence of the host, number of the organisms invaded the host, and finally timing and route of organism entrance. In animals, the transport of fresh and frozen semen to be used for artificial insemination creates a mode of disease transmission between farms (Thacker et al. 1984). The ovarian hormones; estrogens and progesterone in general control uterine defense mechanism. During the estrus phase, when the secretion of estrogens is high, the uterine defense mechanism is upregulated and the genital tract is immunocompetent. Meanwhile, during the luteal phase, when the ovaries secrete large amounts of progesterone, the uterine defense mechanism is downregulated (Ramadan et al., 1997, Ramadan and Hassan, 1999 and Ramadan et al., 2001). During estrus phase the cervix secretes large quantities of cervical mucus that is rich in cellular components, which are mostly leukocytic in nature, beside soluble compounds. The aim of current study was to investigate the role of cervical mucus in the genital tract defense mechanism, especially that cervical mucus exhibited many biological functions and activities during and after matting.

MATERIAL AND METHODS

Collection of Cervical Mucus:

Cervical mucus samples were collected from buffaloes showing clear estrus signs at the Experimental Farm belonging to Animal Reproduction Research Institute, El-Haram, Giza. Samples were collected using sterilized plastic pipetts which were transferred into sterilized tissue culture bottles and kept at -40°C until utilized. Samples were pooled together at the time of use. All samples were taken during the period from December 2000 up to February 2001.

In Vitro Bacteriological Study on Brucella:

Smooth brucella strain (B. melitensis, Rev. 1) was used to test the in vitro bactericidal activity of cervical mucus freshly collected from estrus-phase buffaloes. Recently, cultured brucella were transferred into brucella broth (Difco Laboratories, Detroit, MI, USA) and allowed to grow for 48 hr and harvested by centrifugation, washed twice with saline (0.85%) and suspended in 10 ml RPMI medium. The approximate number of cfu of brucella in medium was evaluated by cfu determination (Antoine et al., 1998). Known amounts of saline containing desired number of cfu, 1×10^5, 1×10^6, 1×10^7, 1×10^8, and 1×10^9 were taken and centrifuged and the pellet was mixed with 10 ml

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of pooled freshly collected cervical mucus, from estrus-phase buffaloes, in sterilized culture tubes and kept at 37°C for 30 minutes. At the end of incubation, the content of each culture tube was seeded onto brucella agar plates and incubated for 3-5 days at 37°C at 5% CO_2, and inspected for the growth of brucella organisms.

Biochemical Analysis of Cervical Mucus:

Ten cervical mucus samples (10 ml cervical mucus) were collected every 45 minutes from 5 buffaloes in the estrus phase to test whether the biochemical parameters (concentrations of protein, carbohydrates, nitric oxide, and electrophoretic analysis) vary during the estrus phase or not. Samples collected from the 5 buffaloes at each sampling point were pooled together and lyophilized.

Protein concentration.

Protein concentration was determined in cervical mucus samples using Bradford method (1976). Briefly, 100 µl from each cervical mucus sample was added to 5 ml of Bradford reagent prepared in the laboratory (50 mg Coomassie Brilliant Blue G-250 dissolved in 50 ml 95% ethanol and 100 ml 85% orthophosphoric acid and diluted with 850 ml distilled water). The absorbance of each sample was read spectrophotometrically at 595 nm using Spectronic spectrophotometer. A standard curve of bovine serum albumin (10, 30, 50, 80, 100, 200 (µg) was generated using the same procedures and a regression analysis equation was generated from a standard curve to calculate the concentration of protein in the samples.

Carbohydrate concentration.

Quantitative determination of carbohydrates in cervical mucus samples was assessed using the phenol-sulfuric acid procedure (Dubois et al., 1956). Briefly, 1 ml of mucus sample was mixed with 1 ml of 5% aqueous phenol solution and 5 ml of concentrated sulfuric acid. The mixture was allowed to stand for 20 minutes to allow color development. The absorbency of the samples and standard (glucose standard curve was generated using 10, 30, 50, 80, 100, and 200 µg of glucose) was measured spectrophotometrically at 470 nm. The regression equation of the standard curve was used to calculate the unknown concentration of carbohydrates in mucus samples.

Nitric oxide concentration.

Measurement of nitric oxide was assessed according to the assay described by Rajaraman et al., (1998). The concentration of nitric oxide in cervical mucus was measured in samples after lyophilization. Exactly 9 ml of cervical mucus from each sampling point was lyophilized and reconstituted in 500 µl distilled water. One hundred (l from each sample was transferred into flat-bottom 96-well ELISA plate and 100 µl of Griess reagent (0.5% sulfanilamide; Sigma Chemical Co.) in 2.5% phosphoric acid (Mereck Co.) and 0.05% N-(1-naphthyl) ethylenediamine dihydrochloride (Sigma Co.). The mixture was incubated at 21°C

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for 10 minutes. Absorbency of the samples and standards was measured at 570 nm using ELISA reader (Dynatech MR7000; Dynatech Laboratories Inc.). Absorbency of test samples was converted to micromolar (µM) of nitrite by comparison with absorbance values of sodium nitrite (Sigma Co.) standard curve within a linear curve fit.

Electrophoretic pattern of cervical mucus.

One-dimensional polyacrylamide-gel electrophoresis, in the presence of 8% w/v SDS (SDS-PAGE), was performed according to Laemmli (1970) for the 10 cervical mucus samples pooled from estrus-phase buffaloes. Gels were stained with Coomassie Brilliant Blue R-250 stain and the developed protein bands were scanned and analyzed using Imaging Densitometer (Model GS-700; Bio-Rad Laboratories, Richmond CA, USA) and Gel-Pro Analyzer software Version 3.0 (Media Cybernetics, Bethesda Maryland, USA).

Statistical Analysis

Data were analyzed using Statistical Analysis System (SAS). Correlation procedures were performed between concentrations of proteins, carbohydrates and nitric oxide to detect any significant correlation between them. Data presented as mean ± SEM.

RESULTS

Bacteriological Examination

None of the inoculated plates with B. melitensis (Rev. 1) or cervical mucus showed brucella growth at different concentrations of cfu used in the present work.

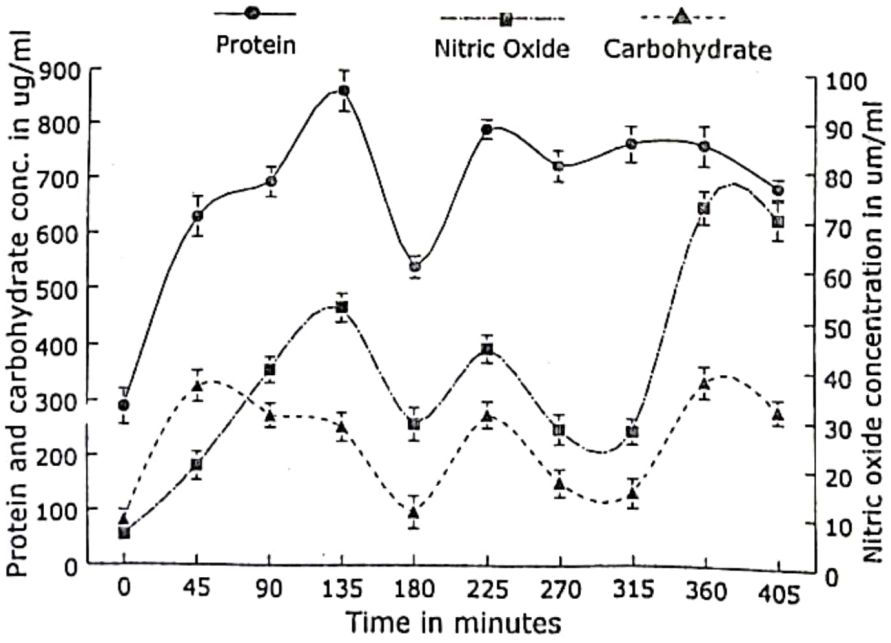

Fig. (1): Concentrations of proteins (µg/ml), nitric oxide (µm/ml), and carbohydrates (µg/ml) in pooled cervical mucus of estrus-phase buffaloes

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Biochemical Analysis

Figure (1) shows the concentrations of protein, carbohydrates, and nitric oxide assayed in cervical mucus of buffaloes collected during estrus-phase. The three parameters measured in this experiment showed fluctuating pattern throughout the sampling period. Moreover, there was a significant positive correlation between concentrations of nitric oxide and protein and carbohydrate as shown in Table (1).

Electrophoretic Analysis

Figure (2) shows the electrophoretic pattern of cervical mucus of estrus-phase buffaloes. Six protein bands were separated on SDS-PAGE. The molecular weights of these bands were 212.5, 130, 91, 70, 52, and 30 KD. There was no difference in protein bands separated from the mucus collected at all sampling points.

Table (1): Gross correlation (r) between concentrations of nitric oxide, proteins and carbohydrates.

	Proteins		Carbohydrates	
Nitric oxide	r	r	r	r
	0.63526	P<0.044	0.67238	0.033

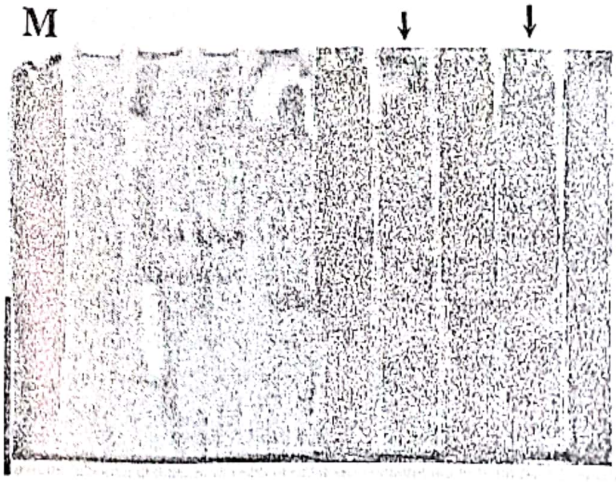

Fig. (2): Photograph of one-dimensional SDS-PAGE electrophoresis of cervical mucus of estrus-phase buffaloes.

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DISCUSSION

In the current study it was proved that the cervical mucus of estrus-phase buffaloes contained lethal amounts of nitric oxide that killed all cfu of B. melitensis in vitro. Indeed, the number of cfu used in the current study was greater than the estimated infective dose of brucella for a single cow, which is 10^6 cfu (Corner, 1983). The average concentration of nitric oxide in 10 ml freshly collected cervical mucus from buffaloes was 388 (M, which is several folds greater than the physiological concentrations of nitric oxide in other body fluids. In a recent study, the direct killing of brucella suis by nitric oxide was demonstrated by incubation of 0.1 and 0.5 mM of sin-1, as a source of nitric oxide radicals, with 10^5 viable brucella suis organisms for 24 hours. All of the brucella organisms were killed within 10 hours of incubation with sin-1 and the concentration of nitric oxide librated from sin-1 was about 75 µM (Antoine et al., 1998).

Nitric oxide, in mammalian cells, is produced by the action of a cytosolic enzyme, inducible nitric oxide synthase (iNOS), which catalyzes the generation of nitric oxide from L-arginine in response to an activation signal. Interferon- γ is one of the activation signals that precede nitric oxide production by macrophages (Green and Nacy. 1993). Estradiol is another signal that induces endothelial nitric oxide synthase (ecNOS), an isoform of iNOS, and upregulates nitric oxide activity in human cervical explants (Gorodeski 2000).

In animals, during estrus cervical mucus is abundant in polymorphonuclear leukocytic cells. Nitric oxide is secreted from immune cells in large quantities during inflammatory reactions. It is high likely that cervical cells also secrete large amounts of nitric oxide during estrus. Moreover, it seems that estrogen may have double actions: it induces cervical cells to produce great amounts of nitric oxide and it upregulates the immune cells to secret more nitric oxide. In vivo, estrogen within the physiological concentrations stimulate the immune cells directly or indirectly through its ability to induce the uterus to secret immunologically active proteins that enhance the activity of lymphocytes and polymorphonuclear leukocytic cells (Ramadan et al., 1997, Ramadan and Hassan, 1999, and. Ramadan, et al., 2001).

The fluctuation in the secretion of nitric oxide, carbohydrates, and proteins observed in the cervical mucus may be correlated with the cellular components of the mucus or may be correlated with other pulsatile hormones secreted during the estrus phase.

In early studies, it was noticed that the use of bulls known to carry brucella organisms in their semen did not induce disease condition in cows when they naturally serviced them. Meanwhile, using semen contaminated with brucella organisms to

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artificially inseminate cows induced brucellosis in most animals. It may be concluded that when brucella organisms introduced at time of service, there was little like hood that an infection will be established especially, when the contaminated semen is introduced by natural service (Hadely and Osborn, 1932). Also, it was reported that abortion could not be produced in heifers naturally mated with bulls having semen contaminated with considerable numbers of brucella organisms, and it was concluded that during estrus phase heifers are slightly susceptible to brucella infection (Thomsen, 1943). Therefore, it was proposed that cervix plays an important role in defending against pathogens, possibly through the secretion of a powerful substance that kills invading pathogens during the time of mating.

Nitric oxide, with a molecular weight of 30 Dalton, is certainly one of the smallest polyvalent molecules that biologically mediate many physiological functions. It is a vasodilator, neurotransmitter, antimicrobial, and play great role in cervical ripening during parturition. Because neurons, blood vessels and immune cells are integral parts of the reproductive organs, it is likely that nitric oxide is an important regulator of the biology and physiology of the reproductive system (Rosselli et al., 1998 and Wieser et al., 1997). Nitric oxide is directly implicated in the immune response regulation (Moncada and Higgs 1993, Moncada et al., 1991 and Schmidt and Walter 1994) in macrophage-mediated cytotoxicity against variety of pathogens, including bacteria, fungi, viruses, helminthes, and protozoa (MacMicking et al., 1997). Moreover, nitric oxide was identified as the effector molecule in killing of a wide range of intracellular pathogens including Toxoplasma gondii (Adams et al.,1990), leishmania spp. (Liew, et al. 1990), Mycobacterium leprae (Adams et al. 1991), Mycobacterium tuberculosis (Denis, M. 1991) and Schistosoma mansoni (James and Glaven 1990). The mechanism of this activity is still unknown, but as a relatively nonpolar uncharged molecule with a small Stokes radius, nitric oxide would be predicted to cross membranes readily. Direct studies indicate that the diffusion of nitric oxide resembles that of oxygen, with the exception that oxygen is more lipophilic (Denicola et al., 1996).

Having such properties, nitric oxide could diffuse through the cell membrane of pathogens and induce many deleterious effects, (1) it can oxidatively damage DNA of the target pathogen, resulting in abasic sites, strand breaks, and a variety of other DNA alterations (Juedes and Wogan 1996). Indeed, nitric oxide can deaminate DNA in vitro (Wink et al., 1991) (2) ribonucleotide reductase enzyme, which is essential for DNA synthesis, is one of nitric oxide targets causing its damage (Lepoivre et al., 1990) (3) nitric oxide has the ability to inhibit some metabolic enzymes or membrane transporters such as guanylyl cyclase and eventually lead to dissipation of transmembrane electrochemical promotive force (Murad 1994) (4) nitric oxide is associated with membrane damage, and

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this action has principally been demonstrated with peroxynitrite, which found to mediate lipid peroxidation of liposomal preparations, via a mechanism that does not require iron (Rubbo et al. 1994) (5) nitric oxide can also induce lipid peroxidation (Halliwell et al. 1992).

Nitric oxide secreted by INF-γ primed-macrophages may practice another lethal action on intracellular pathogen such as brucella organisms; nitric oxide could combine with superoxide anion ($O^{\cdot}$) to generate the deleterious onitric oxide anion that is detrimental to invading pathogen (Augusto, and Giorgio 1996 and Zhu et al., 1992).

In conclusion, the cervix secretes large amount of cervical mucus during the estrus phase and this mucus contains large concentrations of nitric oxide that has lethal effect on infectious pathogens including intracellular bacteria. Introducing the semen directly into the uterus will by-pass the cervix and its powerful defense mechanisms and may cause infection. On the other hand, during natural matting the semen is deposit onto the anterior portion of the cervix and the fate of possible contaminating pathogens will be detrimental.

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