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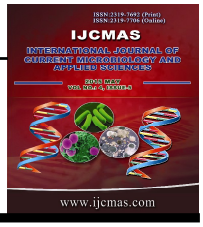

Original Research Article

Immunomodulatory Effect of Lung Surfactant on Buffalo Monocytes and Polymorphs in vitro

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A B S T R A C T

The innate immunological effects of buffalo lung surfactant were investigated through performance of some cellular immunity assays. There was a remarkable decrease in CFU growth number and increase in the percentage of killed *Escherichia coli*, with slight increase in the phagocytosed bacteria, accompanied with an increase of surfactant concentration. Also, there was a significant increase in nitric oxide and super oxide reactive molecules produced by macrophages. These results revealed that buffalo pulmonary surfactant exhibit both immunostimulant and bactericidal activities.

K e y w o r d s

Buffalo pulmonary surfactant, Macrophage, Phagocytosis, Bactericidal,

Introduction

Innate immunity plays an important role in defense mechanism against pathogens in respiratory tract, and includes many constituents such as phagocytes; “macrophages and neutrophils”, and surfactant which line alveoli (Whitsett, and Alenghat 2015). Lung surfactant is a mixture of lipids and proteins that covers alveolar surfaces. This bioactive material keeps alveoli from collapsing at expiration by reducing surface tension at the air–liquid interface (Lawson, and Reid. 2000). Surfactant proteins (SPs) are important constituents of lung surfactant and are synthesized and secreted by alveolar type II cells. SPs belong to the collectin subgroup of C-type lectin super family. Four specific proteins have been described in surfactant and are designated surfactant protein A (SP-A), SP-B, SP-C and SP-D. These proteins have been shown to play pivotal roles in the regulation of surfactant lipid metabolism, the lipid membrane organization and host defense in the lung (Whitsett and Weaver, 2002).The innate immune system protects against invasion of microorganisms as a first line of defense and stimulates the clonal responses of adaptive immunity (Akira *et*

al., 2006). Macrophages are considered the cellular element which achieve defense through both phagocytic and killing activities by production of bactericidal nitric oxide and superoxide molecules (Alvarez-Domínguez, *et al*. 2000). Nitric oxide acts as signaling molecule or as neurotransmitter when produced in low quantities, but when produced in higher quantities for extended period, it can kill microorganisms and tumor cells. Another potent bactericidal radical, is superoxide anion produced through respiratory burst mechanism (Martinez *et al*. 2000). Only a few studies have investigated interactions between bovine phagocytes and surfactant with concern to cattle not buffalo. Our study investigated the immunomodulatory effect of buffalo calf lung surfactant and its possible mechanisms in direct actions on microbes, neutrophils, and macrophage phagocytosis.

Materials and Methods

Preparation of surfactant

Apparently normal lung of apparently healthy buffalo calf was excised and lavaged to total lung capacity with phosphate buffer saline (PBS), pH 7.2 containing 100 mg streptomycin per ml. The lavage was centrifuged at 200 xg for 30 minutes at 4ºC to remove alveolar macrophages. Buffalo lung lavage fluid was then centrifuged at 9150 xg for 30 minutes at 4ºC to pellet the surfactant. Then the surfactant was resuspended in PBS, pH 7.2 then lypholized (Rauprich *et al*. 2000).

Neutrophils isolation

A total of 40 ml of blood sample were collected on heparin (10 IU/ml) using sterile syringe from the jugular vein of healthy buffalo calf and transferred to sterile tissue culture tubes. The blood sample was centrifuged at 3000 rpm for 10 minutes. The buffy coat was aspirated and layered on neutrophil isolation medium 1:3, in tissue culture tubes, then centrifuged at 280 xg for 25 minutes at room temperature. The pellet containing neutrophils and RBCs was transferred in 50 ml sterile tube. A total of 20 ml sterile cold distilled water were added and let to stand for exactly 45 seconds to lyse RBCs. Isotonicity was restored by the addition of 10 ml of sterilized normal saline, and then the content was centrifuged at 3000 rpm for 10 minutes. The pellet was washed 3 times by Hanks balanced salt solution (HBSS) and resuspended in 1ml and kept at 4ºC until used (Hogan *et al*. 1990).

Monocytes derived macrophages

The interphase layer that contained mononuclear cells was aseptically aspirated and centrifuged at 3000 rpm for 5 minutes. Then the collected cells were washed 3 times using RPMI-1640 medium without fetal calf serum. The cells were seeded into tissue culture plate wells and incubated at 37 ºC in a humidified 5% CO_2 atmosphere for 3 hours. After that the non-adherent cells were removed by 3 times washing with RPMI-1640 medium without fetal calf serum. The adherent cells were fed with RPMI-1640 medium containing 10% fetal calf serum, and traces of mercaptoethanol and incubated at 37 ºC in a humidified 5% CO_2 atmosphere for 5 days (Ding *et al*. 1988).

Preparation of bacterial suspension

Twenty four hours broth culture of *Esherichia coli* was adjusted to 52.5% transmission as each 1ml contained $50x10^6$ CFU. The broth containing the bacteria was centrifuged at 3000 rpm for 10 minutes and the pellet was washed twice with HBSS. The bacteria were opsonized with 1ml of 10% homologous serum (obtained from 5 serum

samples collected from 5 different buffalo calves) for 30 minutes at 37 ºC with gentle shaking. After opsonization, the bacteria were centrifuged, washed once and suspended in 1ml HBSS (Silva *et al* 1988).

Bactericidal assay

The test was done in sterile eppendorf tubes as the first group contained 150µl of HBSS. The other groups contained 50µl of different surfactant concentrations with 100µl of HBSS as each group consisted of 4 tubes. Fifty µl of prepared bacterial cell suspension were added to all tubes and incubated at 37 ºC for 2 hrs, at the end of incubation period the tubes were centrifuged at 3000 rpm for 5 minutes. Then 10 µl of the supernatant were diffused on MacConkey agar plates, and incubated at 37 ºC for 24 hrs (Rauprich et al. 2004).

Phagocytic activity assay

Smears of the isolated phagocytic cells and bacteria by a ratio of 1:10 were prepared on clean glass slides, and stained with Giemsa stain as control. The test slides contained phagocytic cells, bacteria and two different concentrations of surfactant treatment (80 and 200 µg). A total number of 200 phagocytic cells were randomly counted for each slide. Cells that phagocytosed at least one bacterial cell were recorded positive reacted cells. Phagocytic activity = no of +ve reacted treated cells / no of +ve reacted control cells (Winnicka et al. 2000).

Nitric oxide production assay

Nitric Oxide (NO) production was assayed according to the method described by Ramadan *et al*. (2001), with some modification (Hakim 2002). This method is based on the Griess reaction, which quantifies the nitrite content of the macrophage supernatant, since NO is an unstable molecule and degrades to nitrite and nitrate.

After incubation of macrophages the medium was aspirated from the plates and 50 µl of HBSS were added to each well together with 50 µl of heat killed opsonized *E. coli* in triplicate manner as standard wells. In the other wells, 50 µl of 6 different surfactant concentrations (20µg, 40µg, 80µg, 150µg, 200µg, and 300µg) were added in triplicate manner. The plates were incubated for 2 hours at 38.5ºC in a humidified 5% CO_2 tension. At the end of incubation, 100µl of the supernatants from each well was transferred into another plate and 100µl of Griess reagent were added and the mixtures were incubated at 21ºC for 10 minutes, then read at 570nm using ELISA reader. The Optical density of the each well was determined and converted to micro moles of nitrite by comparison with the absorbance values of sodium nitrite standard curve within a linear curve fit.

Opsonization of zymosan

A total of 5 mg of zymosan powder (Sigma USA) were washed twice with HBSS and added to 2 ml of 10% homologous serum of 5 different buffalo calves and incubated at 56 ºC for 30 minutes with continuous shaking and washed once with HBSS. The pellet was resuspended in 1ml HBSS and refrigerated until use (Bogdana *et al*. 2000).

Super oxide anion production assay:

The assay was performed in triplicate, a working solution was prepared (cytochrome-C 400mg: opsonized zymosan 100mg). Each well received a mixture of 50µl of the cell suspension in HBSS then 100µl of working solution. Add superoxide dismutase (4units/well) to the mixture and served as a

blank reference wells. Other wells received different surfactant concentrations beside the mixture as test wells (T-well) another well received one surfactant concentration added to the cell suspension and cytochrome-C without zymosan (S-well). The wells that received only the mixture were served as control wells (C-well). The plate was incubated at 37ºC in a humidified 5% CO_2 tension for 2 hours, then read at 570nm using ELISA reader (An *et al*. 2000), with some modification (Hakim 2002). The super oxide anion production was calculated as followed

Blank well - test well X 15.87*

As 15.87 is a coefficient calculation based on the quantity of solution per well and dimension of the well.

Statistical analysis

The data were compared by using T- test and analysis of variance. Results were expressed as the means ± standard deviations, and the differences were considered significant at $P < 0.05$.

Results and Discussion

The obtained results in table (1) showed remarkable decrease in CFU growth number and increase in % of killed *E. coli* accompanied with an increase of surfactant concentration. These results were agreed with that of van Rozendaal *et al*. (2000) who mentioned that incubation of *Candida albicans* with SP-D resulted in profound fungal growth inhibition and decreased hyphal outgrowth, in addition to bind *C. albicans*, resulting in agglutination of the microorganisms. Furthermore, SP-D did not agglutinate encapsulated *Klebsiella pneumoniae* but selectively agglutinates spontaneous, unencapsulated phase variants, such as *Klebsiella* strain K50-3OF, through interactions with their lipopolysaccharides (Kostina *et al*. 2005).

The obtained results in table (2) showed slight increasing in phagocytosed *E. coli* accompanied with increasing of surfactant concentration, agreed with that of Ofek *et al*. (2001) who pre coated K50-3OF with SP-D resulting in enhancement of the phagocytosis and killing of these organisms by rat alveolar macrophages in cell culture. Also, Geunes-Boyer *et al*. (2009) who stated that SP-D enhanced the phagocytosis of cap59Delta cells *Cryptococcus neoformans* by approximately fourfold in vitro. Kudo *et al*. (2004) provided compelling evidence that SP-A and SP-D enhance mannose receptor-mediated phagocytosis of *Mycobacterium avium* by macrophages, as SP-A serves as an opsonin and stimulates the uptake of bacteria playing an important role in mediating the phagocytic response of neutrophils to IgG-opsonized particles (Wofford and Wright 2007).

Table.1 Effect of different surfactant concentrations on the growth of *E. coli* on MacConkey agar plates

Incubation mixture	CFU	% of killed *E. coli*
HBSS (Test buffer)	62.5 ± 1.04	0.0 %
80 µg of surfactant	44 ± 1.068	30%
150 µg of surfactant	33.5 ± 1.5	47%
300 µg of surfactant	25.75 ± 1.65	59%

% of killed E. coli = 1- [(CFU Incubation mixture / CFU Test buffer)] X 100

Table.2 Effect of two different surfactant concentrations on phagocytic activity assay

Surfactant concentrations	No of cells which phagocytose the bacteria	Phagocytic index
0	88 ± 3.7	-
80 µg	114 ± 4.72	1.29
200 µg	131 ± 3.21	1.48

Table.3 Effect of different surfactant concentrations on nitric oxide liberation from macrophages in vitro

Surfactant concentrations	Absorbance	Concentration of produced nitric oxide in µmol.
0 µg	0.39 ± 0.04	18.78
20 µg	0.56 ± 0.04	24.88
40 µg	0.63 ± 0.06	27.24
80 µg	0.68 ± 0.06	28.89
150 µg	0.75 ± 0.06	31.18
200 µg	0.80 ± 0.06	32.82
300 µg	0.88 ± 0.05	35.43

Table.4 Effect of different surfactant concentrations on super oxide anion liberation from macrophages in vitro

well	Surfactant concentrations	Absorbance	Blank Absorbance	Final concentration of superoxide anion
C	0 µg	0.757 ± 0.09	0.837± 0.08	1.27
T	80 µg	0. 743 ± 0.09		1.49
	150 µg	0. 738 ± 0.03		1.57
	200 µg	0. 677 ± 0.06		2.53
S	300 µg	0.469 ± 0.02		5.84

The obtained results in table (3) showed that there was a significant increasing in nitric oxide production accompanied with increasing of surfactant concentration and those agreed with that of Ofek *et al*. (2001) who mentioned that SP-D enhanced the NO response to K50-3OF LPS adsorbed to Latex beads. Also, Stamme, *et al*. (2000) stated SP-A enhanced production of NO and iNOS in cells stimulated with IFN-gamma or INF-gamma plus LPS.

The obtained results in Table (4) showed that there was a significant increasing in super oxide anion production accompanied with increasing of surfactant concentration. Shepherd and Lopez (2001) found that direct interactions of SP-A and SP-D with macrophages resulted in modulation of phagocytosis or the production of reactive oxygen species. Kramer and Speer (2003) Surfactant proteins (SP) A and D play a key role in pulmonary host defense through production of reactive oxygen species. In the opposite

manner, Wofford and Wright (2007) stated that SP-A did not affect reactive oxygen production.

Surfactant was found to play a key role in pulmonary host defense through different mechanisms as; it directly interacted with macrophages and neutrophils enhancing the phagocytosis of *Escherichia coli*. Also, Surfactant proteins contributed to bacterial clearance and stimulated macrophage killing activity via increasing the production of nitric oxide and super oxide reactive molecules.

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