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Lactoferrin: A Novel Strategy for Antivenom Therapy

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ABSTRACT

Snakebites worldwide represent a significant health hazard to humans and animals. Currently antivenom therapeutics is only the cure if given on proper time otherwise leads to death. The objective of this study was to evaluate the immunological effect of lactoferrin (Lf) in production of Naja nigricollis snake antibodies. Camel and cow milk whey were used as a source of 80 kDa Lf in a crude form and separated from milk whey using SDS-PAGE. This investigation was conducted through four experiments. In first experiment, we immunized of horses with N. nigricollis venom and camel milk whey; increased the anti-sera potency to (65 LD_{50}) in comparison to the control group (45 LD_{50}). For second experiment, we immunized of horses with the venom and cow milk whey which led to increase the anti-sera potency to (55.8 LD_{50}) as compared to the control group (42.5 LD_{50}). In the third experiment, using of standard bovine lactoferrin with the venom, the anti-sera potency increased to (75 LD_{50}) as compared to the control group (50 LD_{50}). In fourth experiment, we used of complete Freund's adjuvant with the venom; increase the anti-sera potency to (65 LD_{50}) in compare to the control group (49.2 LD_{50}). The immunological effect of lactoferrin was studied using Lymphocyte Transformation Test (LTT). The LTT mean values were 3.076±0.09 and 2.49±0.05 for the horses immunized with N. nigricollis venom / lactoferrin and the control group (horses injected with N. nigricollis venom without lactoferrin), respectively. This is the first study concluded that milk whey have the adjuvant effect on the antibodies levels and immunostimulatory effect in production of snake antisera from horses due to presence of lactoferrin. This specific property could be targeted as potential strategy for treatment of snake venom.

Keywords: Lactoferrin, *Naja nigricollis*, Antivenom Therapy, Immune effect.

INTRODUCTION

Snakebite, a very painful injury accompanied with different symptoms due to the poison in the bite. It cause redness, swelling, and severe pain at the bitten area, sweating, vomiting which may end up with death if delay in the time of medical intervention. Estimates of global mortality from snake bite have been reported to 100,000 per year approximately (Chippaux, 1998). So far antivenom antibodies have been used as therapeutics to reduce the deaths. For production of therapeutic anti-sera, horses have been used by some organization for production of therapeutic anti-sera for human welfare. Horses are subjected to process of active immunization by toxins, toxoid and different venoms for the production of antitoxins and antivenom (Chippaux and Goyffon, 1998). Employing adjuvants as a strategy to improve vaccine efficacy is one of major research focus worldwide. The primary purpose of an adjuvant

is to enhance the immune responses to a particular antigen of interest which may act through three basic mechanisms (León G *et al.*, 2011). The first mechanism is to sustain the release of the antigen by functioning as a depot; long-term exposure to the antigen should increase the length of time the immune system is presented with the antigen for processing as well as the duration of the antibody response (da Silva WD and Tambourgi DV, 2011). The second is the interaction of the adjuvant with immune cells (Teena Mohan *et al*., 2013). Adjuvants may act as non-specific mediators of immune cells function by stimulating or modulating immune cells. Adjuvants may also enhance macrophage phagocytic activity after binding the antigen as a particulate (a carrier / vehicle function) (Lipman *et al*., 1992).

Recent studies revealed that lactoferrin (Lf) has a wide range of effects on the immune system. It is an important component of the nonspecific immune system, which has many physiological roles, including regulation of iron metabolism and protection against microbial infection. It also plays an important role in regulation of immune function, stimulation of nonspecific immune responses (innate), and modulation of the inflammatory response (Shan *et al*., 2007). Lf has been implicated in immunoregulatory functions, as a modulator of vaccine function, and also containing chemopreventive activity (Choi *et al*., 2008). Addition of Lf as an adjuvant to the BCG vaccine led to an up regulation of the delayed type hypersensitivity response. It increases the host protective response during infection of mice with virulent *Mycobacterium tuberculosis* and decreases deleterious pulmonary pathology (Wilk *et al*., 2007). The aim was to investigate lactoferrin adjuvant activities for production of *Naja nigricollis* antivenin which is not well studied so far.

MATERIALS AND METHODS

Electrophoresis of cow and camel milk whey and fractions containing lactoferrin

Cow & camel milk whey were obtained by using ultra speed centrifuge at 15000×g at 4°C for 30 min accordingly modified Elaraby method (Elaraby, 2009). Followed ultracentrifugation, milk serum was freeze-dried using freeze-drying apparatus (Free Zone®-Model 77500-USA) to get whey in a powder form which contain lactoferrin in its normal habitat, and stored at 2-8 °C.

Milk whey was characterized using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in comparison to standard bovine Lf (Sigma) using Laemmeli method (Laemmeli, 1970). Samples were denatured by diluting in 2- mercaptoethanol (Sigma) sample buffer and boiled for 5 minutes at 95°C, followed by 5 minutes centrifugation at maximum speed. Denatured samples were separated in 12% SDS gels and run at 80 volts. Gels were stained with 1% coomassie blue R-250 (Sigma), then destained at room temperature in 5% methanol and 7.5% acetic acid with shaking. Destaining was done until the proteins appeared blue on a clear background. The gels were scanned and the different fractions were quantified using Bio-Rad GS 700 imaging densitometer molecular analysis software.

Production of antivenom

Animals

Total of 48 adult male horses' local breed, 7-10 years old, weighed about 420 ± 30 kg and of approx. 150 cm high were used for the production of antivenom. These horses were belonging to Center of Laboratory Animals Facilities, Venom and Crude Antisera Production, (Helwan Farm) of the Holding Company for Biological Products and Vaccines (VACSERA).

Also Albino mice 16-18 g body weight, obtained from the laboratory animal unit, Helwan Farm, VACSERA, were used for determination of lethal dose fifty (LD_{50}) of different venoms and also in venom-neutralization assay for titration of the antivenom antibody level in horse sera or serum neutralization test (SNT) according to to World health organization (WHO 1981).

Venom

Naja nigricollis snake was subjected to milking process to obtain venom as shown in figure 1. The extracted venom was freeze-dried using freeze-drying apparatus to get venom in a powder form and stored at -20 ºC (Angulo *et al.*, 1997).

Figure 1: Milking of snake to extract venom

Immunization of horses

The crude lactoferrin separated from cow and camel milk whey was used as adjuvant in compare to complete Freund's adjuvant (Sigma) and standard bovine Lf (Sigma). The horses were immunized subcutaneously with *Naja nigricollis* venom at different time intervals till day 23 with different dose as shown in table 1. At 23rd day serum was collected from the immunized horses to measure the potency of antibodies using neutralization test. Bleeding of horses was done at the day 24th (plasmapheresis) to collect plasma (8-10 liters of plasma). Then horses released into yard for 30 days as a rest period till the next schedule of immunization (Magdesian *et al.*, 1992).

Table 1: Schedule of the immunization of the horses with snake's venom

The snake's venom dose was calculated dose in mg per horses at different time point

Day	Dose (mg) / horse
0	10 (1st immunization dosage)
7	20 (2nd immunization dosage)
14	20 (3rd immunization dosage)
23	Serum sample
24	Plasma collection (plasmapheresis)

Experimental design

Production of antivenom was done in 4 different experiments and in each experiment 2 groups consisted of 6 horses (each group) were investigated. One group was used for injection of venom with adjuvant and second control group was injected with venom without using adjuvant. The immunization schedule was done at three time intervals as day 0, day 7 and day 14 as shown in Table 1. In experiment (1), horses in group 1 were injected with 3 doses of *Naja nigricollis* venom with 0.5 g of camel milk whey powder, as adjuvant, dissolved in normal saline with vigorously mixing. In experiment (2), horses in group 1 were injected with 3 doses of *Naja nigricollis* venom with 0.5 g of cow milk whey powder, as adjuvant, dissolved in normal saline. In experiment (3), horses in group 1 were injected with 3 doses of *Naja nigricollis* venom with 0.5 g of standard bovine lactoferrin powder, as adjuvant, dissolved in normal saline. In experiment (4), horses in group 1 were injected with 3 doses of *Naja nigricollis* venom with 2 ml complete Freund's adjuvant dissolved in normal saline. The lethality neutralization assay was performed in mice as recommended by the World Health Organization to estimate antivenom potency. Venom doses ranging from 2 to 6 median lethal doses (LD_{50}) are appropriate to be used as challenge in this test (Solano *et al.*, 2010).

In vitro lymphocyte transformation test

Lymphocyte proliferation test was performed with modification using MTT (3-(4, 5-dimethyl thiazol-2-yl) 2, 5-diphenyl tetrazolium bromide) assay (Rai-Elbalhaa *et al*., 1985). The heparinized blood samples were collected from horses in experiment 3. The lymphocytes were separated using Ficol gradient at 400 g at 4 ºC for 30 minutesa ccording to McGuckin et al. with some modifications (McGuckin et al., 2008). The interface layer containing lymphocytes was carefully aspirated and placed in sterile tubes containing 2 ml RPMI 1640 medium. Cells were washed 3 times with RPMI 1640 medium by centrifugation at 400xg for 10 min at 4ºC. Then the pellet was resuspended in 1ml of RPMI 1640 medium containing 10% fetal calf serum. 1x10^6 lymphocytes per well were seeded in triplicate in flat-bottom 96-well microtiter plates (Costar) in 150 µl of medium either alone or with 15µg per ml of Phytohemagglutinin (PHA) as a control. The plates were incubated for 3 days under 5% CO_2 at 37°C. After 3 days, 100 µl of supernatant was removed from the wells and 10 µl of MTT solution was added to all the wells. The plate was incubated further for 4 h at 37 °C. The MTT formazon was extracted from the cells using dimethyl-sulphoxide (100 µl / well). Then the OD was measured using an ELISA reader at a wave length of 570 nm. The test was repeated at least two times and mean average values were taken for statistics.

Statistical analysis

All data were expressed as mean ± standard error (SE) and comparisons for significance were tested using an analysis of variance (ANOVA) test. The Statistical Products and Service Solutions (SPSS) 22 program was used for statistical analysis and a difference was considered to be significant at $P < 0.05$. (Borenstein, *et al.* 1997).

RESULTS

Electrophoresis of milk whey samples and fractions containing lactoferrin

Lf was collected and purified from camel and cow milk whey using a cation exchange chromatography on SP-Sepharose. Characterization of cow and camel Lf was done using SDS-PAGE. The results revealed that both the camel and cow Lf was separated around molecular weight of 80 kDa (Figure. 2 and 3).

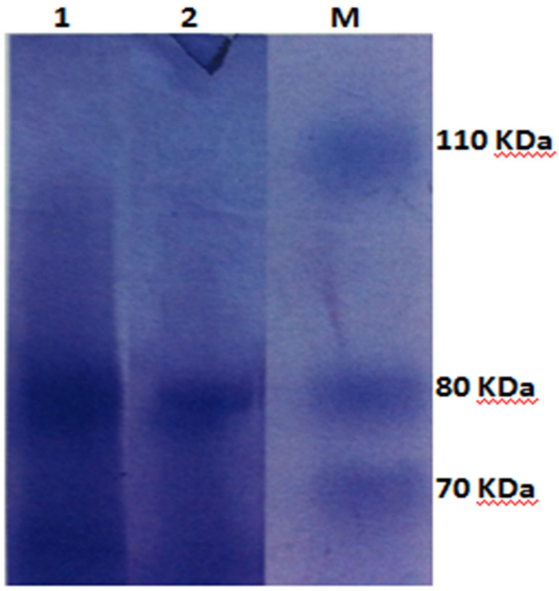

Figure 2: SDS PAGE analysis of camel whey

Lane 1: camel whey, lane 2 standard bovine lactoferrin (Sigma) and lane M: broad range protein ladder (Fermentra SM1841)

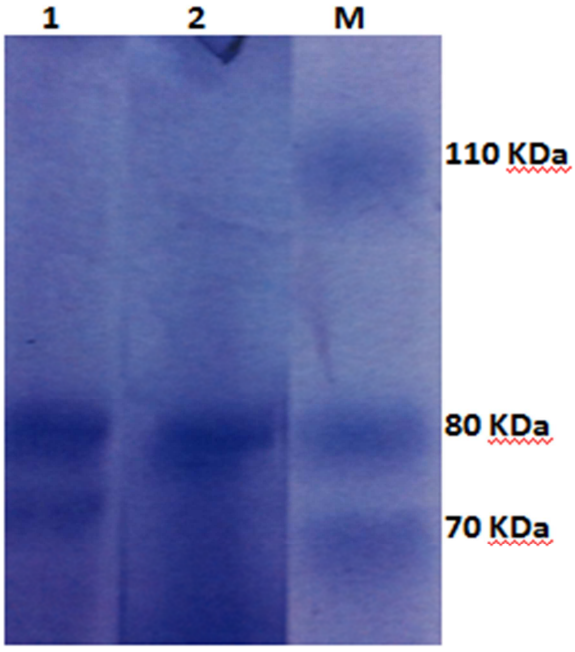

Figure 3: SDS PAGE analysis of cow whey

Lane 1: cow whey, lane 2: standard bovine lactoferrin (Sigma) and lane M: broad range protein ladder (Fermentra SM1841)

Antisera potency using neutralization test (SNT)

The antisera potency in experiment 1, (*N. nigricollis* venom with camel milk whey) increased to 65 LD_{50} in comparison to 45 LD_{50} among the control group. Also, immunization of horses in experiment (2) with the venom and cow milk whey increased the antisera potency to 55.8 LD_{50} compared to the control group (42.5 LD_{50}). In experiment 3, standard bovine Lf was used of with the venom, showed that there was increased antisera potency to 75 LD_{50} in comparison to the control group (50 LD_{50}). In experiment (4), using complete Freund's adjuvant with *N. nigricollis* venom increased the antisera potency to 65 LD_{50} in comparison to the control group (49.2 LD_{50}).

Estimating the Immunological effect of Lactoferrin by using lymphocyte transformation test (LTT)

The immune effect of lactoferrin was studied by lymphocyte transformation test (LTT). The obtained results illustrated that the lymphocyte transformation mean value of PHA was 2.37± 0.06 (Table 2). While the LTT mean values were 3.076±0.09 and 2.49±0.05 among the horses injected with *N. nigricollis* venom with standard bovine lactoferrin and the control group (horses injected with *N. nigricollis* venom without lactoferrin), respectively.

Table 2: results of lymphocyte transformation test (LTT) among the horses immunized with *N. nigricollis* venom and standard bovine lactoferrin

Items	PHA alone	Horses injected with *N. nigricollis* venom + lactoferrin	Horses injected with *N. nigricollis* venom alone
LTT means ±SE	2.37± 0.06	3.076±0.09**	2.49±0.05*

*PHA: Phytohemagglutinin; ** = significant (P < 0.01); * = significant (P < 0.05).*

DISCUSSION

Snake venoms are composed of different peptides, enzymes, toxins and inorganic ions. These different components are responsible for a variety of toxic properties of venoms. Snakebites cause life-threatening symptoms including uncontrolled bleeding and paralysis and treatment with antivenom is potentially lifesaving (Stone *et al*., 2013). Snake antivenom is formulations of immunoglobulin found in plasma of animals immunized with snake venom. Their therapeutic success lies in their ability to mitigate the progress of toxic effects induced by snake venom components, when administered intravenously (León *et al*., 2013). Increasing the potency of anti-sera/immunologin is one of current need for better therapeutics.

Tremendous studies showed that lactoferrin (Lf) has a wide range of effects on the immune system and is an important component of the nonspecific immune system (Tania Siqueiros-Cendón et al 2014). Abundant expression and secretion of lactoferrin, in particular in milk and fluids of the digestive tract, Lf is the first line of defense for any entry point in the body (Legrand *et al.*, 2005; Tania Siqueiros-Cendón et al 2014). Besides its direct effects in host defense on bacteria, fungus and parasites, possible roles in the modulation of the immune response were reported (Legrand *et al.*, 2005). Camel lactoferrin has been shown to have anti-viral activity (EL-Fakharany *et al*., 2013). The present study aimed to improve the antivenom potency of *N. nigricollis* venoms using Lf as adjuvant.

First, camel and cow milk whey were used as a source of lactoferrin in a crude form. The SDS profile analysis reveals that, milk whey samples had a band at 80 kDa (Figure 1). This finding was simliar with Elagamy *et al.* (1996) previous finding of who recorded that the purified lactoferrin from camel's milk was estimated at 79.5 kDa (Elagamy et al. (1996). Lactoferrin is a promising natural adjuvant which is an 80 kDa iron binding protein commonly found in secretory fluids. A promising natural adjuvant candidate is lactoferrin, an 80 kDa iron binding protein commonly found in secretory fluids (Legrand *et al.*, 2004). Lactoferrin is multifunctional glycoprotein, present in milk, and but also found in other biological fluids, such as saliva, tears, bile and pancreatic juice (EL-Fakharany *et al*., 2013).

In our experiments, using of cow or camel milk whey as adjuvant to *N. nigricollis* venom in the immunized horses increased the antisera potency as compared to the control group. The main immunostimulatory component in whey is lactoferrin and this finding was reported and suggested that lactoferrin would have the potential to generate a local environment to promote development of antigen-specific TH1 cellular response from activated presenting cells (Hwang *et al.* 2005). Lactoferrin, acting as an adjunct adjuvant, was able to augment vaccine efficacy (Hwang *et al.* 2005). In addition, whey contain other protein than lactoferrin such as ß-lactoglobulins, α-lactalbumins, serum albumins, immunoglobulins, and proteose-peptones (Mehaia, 2006). These whey proteins help in magnification of inflammatory process around site of injection of venom in horse skin, thus may increases the infiltration of macrophages and other antigen presenting cells which reflects on immune status, both cellular and humoral immune response (Beg *et al.* 1985). Albumins (such as α-lactalbumins, serum albumins) in whey considered as a weak antigen alone, although it has high molecular weight of 14.6 kDa due to its simple structure not complex as reported

by Beg et al. (1985). As a complexity is an important factor to any material to be a good antigen but when using whey powder (which containing albumins) as adjuvant with venoms in hyper immune sera horses, albumins act as a carrier to venom particles (especially in N. nigricollis venom which consider a weak immunogen). These venom particles are considered as a hapten, resulted in magnification of immune response, thus increasing of antibodies titer against venom.

This finding was in accordance with Patterson(1985) who reported that hymenoptera venoms for vaccine immunization of human subjects are prepared by copolymerizing the venom with albumin using glutaraldehyde as the polymerizing reagent (Patterson ,1985). Sufficient albumin is used to produce water-soluble copolymers of high molecular weight above 200 kDa. It is very clear that, there were differences in immunostimulatory effect between both camel and cow whey, as camel whey increased antibodies titer against *N. nigricollis* venom more than cow whey. These results were explained by basically three reasons, first, is the higher content of lactoferrin in camel whey (170 mg/l) than cow (76 mg/l) respectively (Elagamy, 2000). Second reason is the quantity of nitrogen in whey protein is higher in camels' milk whey (106 mg/100 ml) than in cows' milk whey (91 mg/100 ml) (Mehaia, 2006). Third reason is unique structure and physical properties of camel lactoferrin, as it have been significantly more heat resistant than cow lactoferrin (Farah and Atkins 1992; Elagamy, 2000). Thus, it makes camel lactoferrin (in camel whey) more stable during venom-whey mixture preparation and gives more immunostimulatory effect. Another advantage, camel whey is devoid of ß-lactoglobulins protein as β-lacoglobulin is responsible for some of the observed allergies to cow's milk (Chobert *et al.* 1997). These explain the increased post immunization reaction in horse's skin after injection of venom-cow whey mixture as compared with post immunization reaction in horse's skin after injection of venom-camel whey mixture. So, it is evident from the above results that camel whey induced more immunostimulatory effect with minimal post immunization reaction in horses used in production of antisera.

For the antivenom production the vast majority was still produced by traditional technology in horses, immunized with crude venoms. Generally, complete and incomplete Freund's adjuvants were used to promote immune responses (Theakston *et al.* 2003). In last set of experiment, use of complete Freund's adjuvant (CFA) with N. nigricollis venom in horses; increase the antisera potency in comparison to the control group.

Jennings (1995) mentioned that for many years the adjuvant of choice was CFA. It is also clear that standard bovine Lf produced antivenom of high neutralizing activity. The immunostimulatory effect of cow whey and complete Freund's adjuvant was nearly the same, but the horses which injected with CFA exhibited local reactions at site of injection. There were many drawbacks in using complete Freund's adjuvant as discussed by (Raw *et al.* 1991) who mentioned that in order to minimize the local reactions in use of CFA, a multi-emulsion form of CFA was used in Brazil. However, sterile abscess and granuloma could still be formed in about 25% of the horse. Jennings (1995) concluded that CFA had a significant track of frequently producing abscesses, granulomas and tissue sloughs. It contains paraffin oil, killed mycobacteria and mannide monoosleate, and the paraffin oil is not metabolized; it is either expressed through the skin (via a granuloma or abscess) or phagocytized by macrophages. The multiple exposures to CFA will cause severe hypersensitivity reactions, Chippaux and Goyffon (1998) reported that the use of CFA is a very potent adjuvant but can cause serious side effects, i.e. sterile abscess and granuloma and its use in horses has been discouraged (Chippaux and Goyffon (1998).

The present investigation evaluated the immune effect of standard bovine lactoferrin using Lymphocyte Transformation Test (LTT) as shown in Table (2). The LTT mean values were higher among the horses injected with *Naja nigricollis* venom with lactoferrin than the control group (horses injected with *Naja nigricollis* venom without lactoferrin). Effectiveness of the lactoferrin adjuvant comparing primary vaccination versus an immunization schedule with a booster administered at 8 weeks. BCG/lactoferrin vaccinating, given once or twice, demonstrated an improvement in pulmonary disease compared to both the BCG vaccinated and non-immunized groups (Hwang *et al.*, 2011). Also, both BCG/lactoferrin vaccinated group exhibited increase production of IFN-γ compared to the non-immunized group and decreased production of IL-10 compared to the group vaccinated with only BCG (Hwang *et al.*, 2011).

CONCLUSION

In conclusion study revealed that, whey is not only milk by product having a high nutritive value, but also shows adjuvant effect on the antibodies levels. This effect is due to presence of lactoferrin and other whey proteins which give immunostimulatory effect in production of snake antisera from horses. This specific property could be targeted as potential strategy for treatment of snake venom. Furthermore, investigations are needed on separation and purification of lactoferrin especially camel lactoferrin as it is not available commercially and studying its role in immunological areas.

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