

Effects of pasteurization of colostrum on subsequent serum lactoferrin concentration and neutrophil superoxide production in calves

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Objective—To determine the effects of pasteurization of colostrum on serum lactoferrin concentration and neutrophil oxidative function by comparing values from calves given pasteurized (76 C, 15 minutes) colostrum versus calves given fresh frozen colostrum.

Animals—8 Holstein bull calves were used to study the effects of pasteurization of colostrum on the absorption of lactoferrin and neutrophil oxidative burst. Three additional calves were used to study the effect of exogenous lactoferrin on neutrophil oxidative burst.

Methods—Calves were fed fresh frozen or heat pasteurized colostrum (76 C for 15 minutes) via esophageal feeder within 4 hours of birth. Neutrophils were isolated from whole blood samples. Neutrophil oxidative burst was induced by phorbol ester (300 ng/ml) stimulation of cells (1×10^6 cells) at 37 C. Serum lactoferrin concentrations were compared, using immunoblot analysis. Serum IgG concentrations were determined by radial immunoassay. Comparisons were made between the use of the 2 types of colostrum in calves by measuring subsequent serum IgG and lactoferrin concentrations and neutrophil superoxide production.

Results—Serum IgG and lactoferrin concentrations increased more in calves receiving fresh frozen colostrum. Neutrophil superoxide production was higher in neutrophils prepared from calves receiving fresh frozen colostrum. Colostral lactoferrin addition to neutrophil incubations resulted in increased oxidative burst.

Conclusions and Clinical Relevance—Compared with calves given fresh frozen colostrum, calves given pasteurized colostrum had decreased serum IgG and lactoferrin concentrations and neutrophil superoxide production 24 hours after administration. These results suggest that pasteurizing bovine colostrum at 76 C for 15 minutes has substantial effects on passive transfer of proteins and neutrophil function. (*Am J Vet Res* 2000;61:1019–1025)

Susceptibility of newborn calves to infection is dependent on a number of factors including absorption of colostral antibodies, development of the cellular immune response, and function of the innate immune response of calves during the perinatal period.

Received Sep 3, 1999.

Accepted Oct 22, 1999.

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Supported by grants from the Department of Veterinary Medicine and Surgery Committee on Research, and USDA Animal Health Formula Funds, College of Veterinary Medicine, University of Missouri-Columbia 65211.

Timely ingestion and absorption of colostral immunoglobulin is a critical determinant of neonatal calf health. Calves are born without appreciable concentrations of the serum immunoglobulins needed to protect against pathogenic bacteria, viruses, and protozoa.¹ Beneficial effects of passive transfer of colostral immunoglobulin extend beyond neonatal health persisting into the juvenile and adult periods.^{2,3} Calves with failure of passive transfer have significantly increased relative risks for mortality that persist at least until 10 weeks of age, compared with calves without failure of passive transfer.³ Furthermore, cows that survive failure of passive transfer as calves have lower milk production and shorter longevity in the herd.⁴

Results of recent work indicate colostral substances other than immunoglobulins may be beneficial to calves via mechanisms involving neutrophil function and host acquired immune function.⁵ Growth factors, cytokines, and as yet undefined factors have been isolated from colostrum, and their effects on the innate as well as active immune response have been demonstrated.⁵ Lactoferrin, a component of mammary and lacrimal secretions, seminal and synovial fluids, plasma, and neutrophil granules, is an important immunoregulatory protein with multiple functions. Lactoferrin is capable of binding iron,⁶ binding the lipid A portion of endotoxin,^{7,8} inhibiting bacterial colonization of the gastrointestinal tract,⁹ and preventing lipopolysaccharide (LPS) binding to monocyte or macrophages, thereby preventing expression of cellular cytokines.⁷ Lactoferrin in colostrum, along with other proteins such as complement, may play important roles in the neonates innate immunity.¹⁰ Colostral lactoferrin is absorbed by the gastrointestinal tract enterocytes via interaction with specific receptors, and the presence of lactoferrin enhances neutrophil oxidative burst.^{11,12}

Pasteurization of colostrum has been successfully used to limit the spread of chronic debilitating diseases, such as caprine arthritis and encephalitis virus in goats.^{13,14} Several diseases of cattle are potentially spread by the ingestion of colostrum. Examples of such diseases include bovine leukosis and paratuberculosis (Johne's disease).^{15,16} In one study, 22% of latently infected cows shed *Mycobacterium paratuberculosis* in their colostrum.¹⁶ Pasteurization of colostrum reduces the likelihood of culturing *M paratuberculosis* from colostrum under experimental conditions.¹⁷ Optimal programs to prevent and eradicate these diseases generally include the provision that calves are fed colostrum derived from cows of known negative disease status. In some herds, inadequate numbers of bovine leukosis virus negative cows are available to provide adequate

colostrum for calves. In herds with endemic Johne's disease, the low sensitivity of available Johne's diagnostic tests makes it difficult to identify all infected cows and remove them from the pool of potential colostrum donors. Under these circumstances, pasteurization or heat treatment of colostrum may provide a mechanism whereby calves are provided protection against neonatal disease without creating undue exposure and potential for infection by chronic, economically relevant diseases. Sterilization of the milk and colostrum fed to calves is a logical and reasonable strategy to prevent transmission of infectious microorganisms. The potential disadvantages of heat-treating colostrum include loss of immunoglobulins and other factors in colostrum required for normal function of the immune system.¹⁸ Pasteurization at 63 C causes only negligible decreases in colostrum IgG concentration. To our knowledge, absorption and persistence of IgG in calf serum after ingestion of pasteurized colostrum (63 C) has not been reported. A harsher pasteurization protocol was used in the study reported here than in previous studies. This decision was made, because pasteurization at 63 C was not capable of eliminating viable *M paratuberculosis* in 2 of 18 experimentally inoculated colostrum samples.¹⁷ Likewise, other factors in colostrum may be important to the optimum function of the neonate immune response; therefore, pasteurization may substantially alter the passive transfer of immunoglobulins and other proteins or peptides with additive effects on the immune response.

The goals of our study were to determine the effect of pasteurization of colostrum at 76 C on subsequent serum concentrations of lactoferrin in calves and neutrophil oxidative metabolism. Successful use of pasteurization in disease eradication requires optimizing conditions that prevent the transfer of infectious agents from cow to calf while preventing the destruction of proteins required for transferring appropriate immunity.

Materials and Methods

Animals—Eight Holstein bull calves were included in our study, using the following criteria: attendance of the birth, removal from the dam prior to standing and nursing, and the administration of either pasteurized colostrum or fresh frozen colostrum from a multiparous Holstein cow. All enrolled calves were housed in individual calf hutches. Calves were assigned systematically in an alternating manner to 1 of the 2 experimental groups. The 2 aliquots of colostrum that were collected from each cow were administered to calves resulting from sequential births. Calves were fed 4 L of colostrum product using an oroesophageal feeder between 2 and 8 hours of age. Immediately prior to administration, colostrum was thawed and warmed to 25 C using a water bath. Each calf had blood withdrawn prior to colostrum administration of colostrum and 24 hours later for determination of passive transfer by measuring total protein and IgG concentrations, using radial immunodiffusion. All calves were housed individually in pens within the nursery. Calves were fed milk replacer (2 L, twice daily) and allowed access to fresh water, hay, and calf starter ad libitum. Three additional calves that received frozen fresh colostrum (4 L by orogastric feeder) were used to study the effects of exogenous lactoferrin on the neutrophil superoxide induced by phorbol esters.

Colostrum collection and processing—Colostrum was obtained from 5 mature Holstein cows within 8 hours of par-

turition. Six liters of colostrum was collected from each study cow. Colostrum was divided into two 3-L aliquots. One liter of water was added to each 3-L aliquot. From each cow, one 4-L colostrum and water aliquot was frozen. The second 4-L colostrum and water aliquot was heated to 76 C, using a commercially available home pasteurization unit,^a mixed for 15 seconds using a household blender, decanted into 1-L aliquots and immediately frozen.

Blood sample collection—Blood samples were obtained by jugular venipuncture from each calf immediately prior to colostrum administration and 24 hours after administration of colostrum. Serum was harvested after centrifugation. Serum was stored frozen at -20 C until the determination of serum IgG and lactoferrin concentrations. Citrate anticoagulated blood was used to harvest neutrophils for the determination of superoxide production. Blood was drawn by jugular venipuncture into sterile acid-citrate-dextrose (ACD) containing evacuated tubes on day 14 of life.

Serum IgG concentrations—Serum IgG concentrations were determined using adaptations of described techniques.¹⁹ Briefly, radial immunodiffusion kits were obtained from a commercial vendor.^b Three μ l serum from each calf and 3 μ l of each standard were added to the wells. The diameter of the observed zone of precipitation was recorded after 24 hours of incubation at 23 C. Serum IgG concentrations were determined by comparing zone diameters with a standard curve included with each kit.

Neutrophil isolation—Blood samples were transferred to polypropylene centrifuge tubes and the blood cells separated from the plasma by centrifugation at 1,000 \times g for 10 minutes at 23 C. The plasma and buffy coat and approximately 5 mm of the upper portion of the RBC layer were removed, and the remaining RBC fraction was subjected to osmotic lysis of the RBC by addition of 1 ml of sterile, pyrogen-free ultra-pure water for each 4.5 ml of RBC, in 30-second cycles. At the end of each cycle, 1 ml of a 2.7% solution of sodium chloride, 1.34 mM phosphate buffer containing 1% bovine serum albumin was added for every 2 ml of the distilled water to return the osmolality to isotonic.²⁰ Lysed cells were removed by centrifugation at 400 \times g for 10 minutes at 23 C, and the cycle repeated until nearly all of the RBC were gone and the supernatant was clear. Cells were resuspended in 2 ml of calcium and magnesium-free Hanks buffered salt solution and placed on ice. Cell count was determined by dilution of an aliquot of the cell suspension of 1:9 with RPMI-1640^c medium and 20 μ l placed into a hemocytometer. Viability of the purified cells was determined by trypan blue exclusion, using a 0.1% stock solution of trypan blue dissolved in phosphate-buffered saline (0.9% NaCl) solution. Cell isolations were used only if viability was \geq 90%. Differential cytologic examinations were performed on some of the isolated cells by placing 10 μ l of the suspension into 90 μ l of RPMI-1640 solution and placing the suspension onto pre-cleaned glass slides. Cell counts with > 10% mononuclear cells were not used in the determination of neutrophil oxidative burst.

Neutrophil superoxide production—Superoxide production was studied by placing the volume of cell suspension equivalent to 1×10^6 viable neutrophils into sterile, pyrogen free-capped polypropylene test tubes that contained a volume of Hanks buffered salt solution supplemented with 1% fetal bovine serum to give a final volume of 754 μ l. Cells were incubated in a cell culture incubator kept at 37 C, with a 95% O₂/5% CO₂ atmosphere for 20 minutes. At the end of this incubation period, 20 units of superoxide dismutase^d were added to the blank cell incubation, and 4 μ l of horse heart

cytochrome c^e was added to all of the test tube incubations. In incubations where exogenous lactoferrin was added to the tubes, a volume of stock lactoferrin in PBS solution (1 mg/ml) was substituted for the buffer solution added to each well to make the final concentrations of 100, 265, and 530 μ g lactoferrin/ml of media, respectively. Cells were stimulated by addition of 300 ng of phorbol myristate acetate^f dissolved in Hanks buffered salt solution. Stimulated cells were incubated for 20 minutes in the cell culture incubator. At the end of the incubation period, the cells were immediately placed into centrifuge rotors precooled to 4 C and centrifuged at 1,000 \times g for 10 minutes. After the cells were pelleted, the supernatant was removed by pipette and placed into disposable cuvettes for determination of cytochrome c reduction.²¹

Neutrophil cytochrome c reduction by NADPH-oxidase—Supernatants of each incubation were placed into cuvettes and scanned in an UV/Vis spectrophotometer. The spectrophotometer was preprogrammed to analyze each sample by scanning from 530 nm to 570 nm to determine the absorbance of reduced horse heart cytochrome c at 550 nm. Maximum absorbance at 550 nm was subtracted from the baseline absorbance at 530 nm, and the corrected absorbance was determined. Each absorbance value was multiplied by 47.7 to determine the nanomoles of superoxide reduced/ 1×10^6 cells.²¹ Neutrophils from each calf and each concentration of lactoferrin were analyzed in triplicate.

Polyacrylamide gel electrophoresis and western blotting of serum lactoferrin—Frozen serum samples were thawed and 5 μ l used for the determination of total protein, using established methods.²² The volume of serum required for 12.5 μ g of total protein was determined in each sample. Samples were denatured with sodium dodecyl-sulfate (SDS)-sample application buffer containing 10% 2-mercaptoethanol and heated for 3 minutes at 95 C. Proteins were separated by SDS-polyacrylamide gel electrophoresis.^{23,24} Briefly, samples were placed into individual wells of 12% Tris-glycine SDS-PAGE gel and subjected to electrophoresis at 80 volts for 1 hour. After separation, the protein was transferred to nitrocellulose overnight at 13 volts. The blot was blocked by incubation with 5% nonfat dry milk in Tris-buffered saline (pH 7.3), 0.05% Tween 20 for 30 minutes, and the blot was incubated with rabbit-anti-bovine lactoferrin polyclonal antibodies^g (final dilution 1:1,000) overnight at 4 C. Rabbit anti-goat horseradish peroxidase conjugate^h was used as the secondary antibody (final dilution 1:2,000) and incubated at 23 C for 30 minutes. After washing, the bound protein of interest was viewed, using a chemiluminescent substrate following the manufacturer's recommendation.ⁱ The membrane was exposed to chemiluminescence film^j for 3 and 5 seconds. Positive controls included varying concentrations of bovine lactoferrin.^k Qualitative analysis of plasma proteins were performed using a computer program.^l Known quantities of colostrum lactoferrin were loaded into each well of a 12% Tris-Glycine-SDS, electrophoresed, blotted, and immunoblotted as described. Band density was calibrated by determining the mean gray value of each band and comparing the area under the curve (optical density) with the known quantity of lactoferrin protein loaded onto each lane. The line of regression was determined for comparison with calf plasma protein concentration. Calculation of the quantity of lactoferrin in each lane of the blot containing calf serum proteins was performed by comparing the optical density of the lactoferrin band using the calculated slope and intercept of the standard regression line. Concentration of lactoferrin was estimated by back calculating to the volume of serum from which this band was obtained.

Statistical analysis—Mean and SD of serum IgG concentrations were reported prior to and at 24 hours after

colostrum administration. Mean serum IgG concentrations were compared between the 2 experimental groups using a paired *t*-test. Serum concentration of lactoferrin from each calf receiving heat-treated colostrum was compared with the serum lactoferrin concentration of calves that received fresh frozen colostrum derived from the same cow. Differences were considered significant when *P* values were < 0.05.

Superoxide production by neutrophils from triplicate incubations of calves receiving heat-treated or fresh frozen colostrum was summed and the mean value (nmol of cytochrome c reduced/ 10^6 cells) determined. Mean values for each calf receiving heat-treated or fresh frozen colostrum were compared, using the paired *t*-test. Effects of varying concentrations of bovine colostrum lactoferrin on neutrophil oxidative burst were compared, using a 1-way ANOVA with significant differences determined using the Tukey test. Significance was assigned when *P* < 0.05.

Calf serum lactoferrin estimated by image analysis from each calf receiving heat-treated or fresh frozen colostrum were summed and the mean value (lactoferrin, mg/ml) compared using the *t*-test. Because serum obtained before the administration of colostrum did not contain measurable quantities of lactoferrin, difference in the lactoferrin concentrations after administration of colostrum was compared between groups using the paired *t*-test. Significance was assigned when *P* values were < 0.05.

Results

Serum IgG concentrations of calves receiving fresh frozen or pasteurized colostrum were significantly altered by pasteurization. Day 0 serum IgG concentrations were < 100 mg/dl in all calves. Mean serum IgG concentrations in calves receiving fresh frozen colostrum were greater than in calves receiving pasteurized frozen colostrum at 24 hours, although the differences were not significant (*P* = 0.08). Serum immunoglobulin concentrations in the calves receiving fresh frozen colostrum were 1,946 \pm 670 mg/dl (range, 1,165 to 2,504 mg/dl), whereas calves receiving the pasteurized colostrum were 795 \pm 878 mg/dl (range, 133 to 2,058 mg/dl; *P* = 0.08, compared with calves receiving fresh frozen colostrum). All calves receiving fresh frozen colostrum had serum IgG concentrations that exceeded 1,000 mg/dl. However, only 1 of the 4 calves receiving pasteurized frozen colostrum exceeded 1,000 mg/dl. Phorbol ester stimulated horse heart cytochrome c reduction by neutrophils from calves receiving fresh frozen colostrum (15 \pm 9.9 nmol/million cells) was significantly greater than calves receiving pasteurized frozen colostrum (4.9 \pm 1.5 nmol/million cells; *P* < 0.001).

Pasteurization resulted in significant differences of the serum concentrations of lactoferrin achieved 24 hours after administration of colostrum (Fig 1). Calves receiving pasteurized colostrum had lower serum concentrations of lactoferrin (0.42 \pm 0.32 mg/ml; range 0.1 to 0.85 mg/ml; approx 5.6×10^{-9} M) 24 hours after administration of colostrum, compared with calves receiving fresh frozen colostrum (1.97 \pm 1.0 mg/ml; range 0.7 to 3.4 mg/ml; approx 2.6×10^{-8} M; *P* = 0.033).

On electrophoresis, lactoferrin in serum of calves was observed to migrate at similar rates to that of the lactoferrin standard protein (Fig 1). Lactoferrin in serum of calves prior to administration of colostrum was not measurable. Standards prepared from affinity purified bovine lactoferrin were run under identical

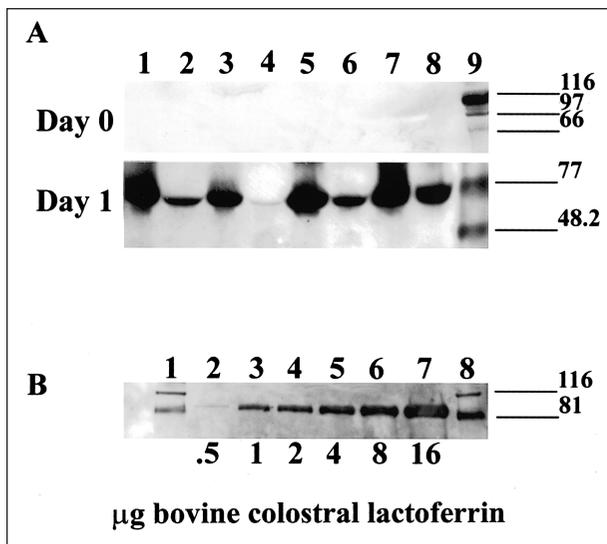


Figure 1—(A) Electrophoresis of lactoferrin in serum of calves given fresh frozen colostrum (lanes 1, 3, 5, and 7) and in calves given pasteurized colostrum (lanes 2, 4, 6, and 8). Lactoferrin is recognized by use of a polyclonal antibody to bovine lactoferrin and enhanced chemiluminescence. Lane 9 consists of molecular weight marker proteins. Day 0 indicates the serum from each calf before the administration of colostrum. Day 1 indicates serum obtained from each calf 24 hours after the administration of 4 L of colostrum. (B) Affinity purified bovine colostrum lactoferrin separated in an identical manner as calf serum samples on 12% SDS-PAGE gels. In lanes 2 through 7, protein was added to each well (ie, 0.5, 1, 2, 4, 8, and 16 μg of bovine colostrum lactoferrin). Lanes 1 and 8 are protein molecular weight markers.

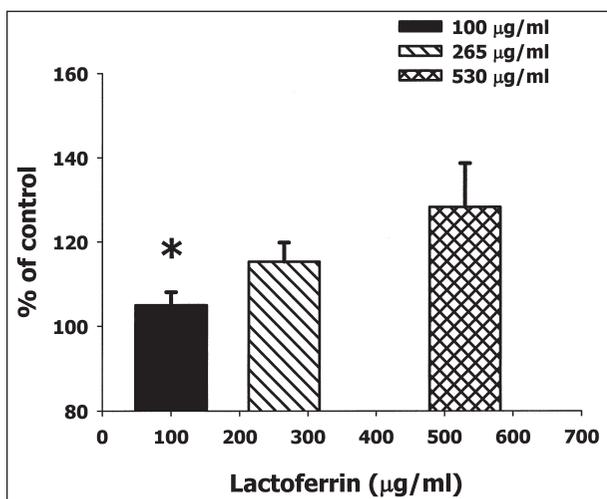


Figure 2—Effects of exogenous lactoferrin on oxidative burst of neutrophils from neonatal calves as a percentage of control superoxide production. Final concentrations of 100, 265, and 530 μg of bovine lactoferrin/ml of PBS solution were used, and the cells were incubated for 20 minutes with cytochrome c and phorbol esters. Each bar indicates the mean \pm 1 SD of the 3 calves examined. Each concentration for each calf was performed in triplicate. * $P < 0.05$ compared with 530 μg of lactoferrin/ml.

conditions for estimation of serum lactoferrin concentration in these calves.

Effects of exogenous lactoferrin on the neutrophil superoxide burst were determined in a manner similar to that described. Addition of lactoferrin increased the quantity of reduced cytochrome c in a concentration-dependent manner (Fig 2). However, less than 2-fold

increases in the cytochrome c reduction were observed for each of the concentrations of lactoferrin studied, and variability in the response of the triplicate incubations is evident.

Discussion

Absorption of colostrum IgG and lactoferrin by calves receiving pasteurized frozen colostrum was diminished in comparison with calves receiving the same colostrum frozen fresh. The pasteurization protocol in our study resulted in variable absorption of IgG by calves. On day 1 after colostrum administration, calves receiving pasteurized colostrum had serum IgG concentrations roughly 40% of that observed in matched controls. Likewise, serum lactoferrin did not increase in calves receiving pasteurized frozen colostrum compared with calves receiving the same colostrum frozen fresh. The matched fresh frozen and pasteurized frozen colostrum samples were used to eliminate any inherent variability in colostrum composition. In our study, the poor absorption of IgG and lactoferrin, as determined by low plasma concentrations of each at 24 hours, indicates that pasteurization at 76 C either destroyed or altered colostrum proteins enough to prevent their absorption from the gastrointestinal tract.

Reduced serum lactoferrin concentration in calves receiving pasteurized colostrum is of additional concern. Lactoferrin, a glycoprotein of approximately 77 to 80 kd, has the capacity to bind iron avidly.^{7,8} Lactoferrin has bactericidal properties, presumably by sequestering iron, thereby preventing access to this metal ion by microbes.^{7,8} This protein is found in mammary and lacrimal secretions, seminal, plasma, and synovial fluids, and stored in neutrophil granules. Neutrophil lactoferrin is released during the process of degranulation.²⁵ This protein augments chemotaxis and superoxide and hydroxyl radical production in human neutrophils.²⁵ In the absence of this protein, superoxide reduction and chemotaxis are reduced by half.^{12,25} This effect was demonstrated when soluble, particulate, or receptor mediated stimuli were used to activate the oxidase of the neutrophil.^{13,25} Effect of lactoferrin on superoxide has been proposed to be caused by alteration in neutrophil signal transduction pathways.²⁵ This was not examined in our study, however, because lactoferrin concentrations in tears, nasal secretions, and seminal fluids are $> 100 \mu\text{g/ml}$, it may be of importance in the resolution of infections in tissues.²⁶ Reduced colostrum lactoferrin absorption by calves fed pasteurized frozen colostrum may have resulted in reduced neutrophil superoxide production via altered neutrophil signal transduction or via as yet undefined mechanisms. In our study, increased lactoferrin concentrations resulted in increased superoxide production that was dependent on concentration of this protein; however, it did not result in greater than 2-fold increase in the oxidative burst at apparently supraphysiologic concentrations we tested. In sheep, lactoferrin does not alter neutrophil chemotaxis, although the addition of this protein to neutrophil incubations results in a dose-dependent enhancement of superoxide production.¹² Results of our study are similar to that described for adult sheep, in which the maximum increase in superoxide production

was approximately 140% of control.¹² One of the limitations of our study was that we did not perform these experiments with higher concentrations of lactoferrin, and we did not do so in colostrum-deprived calves. This may be of importance, because the healthy 2-week-old calves we used were presumably lactoferrin replete. Further examination of this phenomenon in colostrum-deprived calves is necessary to more fully understand the role of lactoferrin in neonatal immune function. Although the methods used in our study were as close to that reported previously as possible, these methods do not address the effects of added protein to cell stability, nor do they determine the effects of priming on neutrophil oxidative metabolism.¹² In similar fashion, lactoferrin alters lymphocyte proliferation,¹² reduces LPS binding to monocyte or macrophages and reduces serum tumor necrosis factor alpha activity in stressed animals.^{7,27} Calves that receive heat-treated colostrum may have reduced serum immunoglobulin, lactoferrin, and neutrophil antimicrobial activity. This may negate the beneficial effects of reduction of infectious agents in colostrum. Although we believe that there is benefit to neonates in receiving this protein in colostrum, as in humans and pigs, the precise effects of these proteins on neutrophil function needs further attention.^{7,9,11}

In summary, pasteurization of colostrum at 76 °C will commonly cause failure of passive transfer of immunoglobulins. Likewise, other proteins in colostrum may play important roles in the host defense of the neonate. In our study, failure to absorb colostrum lactoferrin was associated with reduced neutrophil oxidative burst in vitro. The process of monitoring colostrum immunoglobulin absorption, although important, may not be adequate by itself to determine the host defense of the newborn. Sterilization procedures that result in low absorption of colostrum proteins should not be used to prevent the colostrum transmission of disease. Further studies are needed to determine suitable methods to reduce transmission of infectious agents while optimizing absorption and persistence of immunoglobulins and immunomodulatory proteins in colostrum.

^aPres-Vac Home Pasteurizer, the Schlueter Co, Janesville, Wis.

^bBovine IgG SRID kit, VMRD, Pullman, Wash.

^cRPMI-1640 Medium, GIBCO Life Technologies, Rockville, Md.

^dSuperoxide Dismutase, Sigma Chemical Co, St Louis, Mo.

^eHorse heart cytochrome c, superoxide dismutase free, Sigma Chemical Co, St Louis, Mo.

^fPMA Phorbol 12-, 13 myristate acetate, Sigma Chemical Co, St Louis, Mo.

^gBethyl laboratories, goat anti-bovine lactoferrin (affinity purified), Montgomery, Tex.

^hRabbit anti-goat IgG H + L, Kirkegaard Perry Laboratories, Gaithersburg Md.

ⁱRenaissance western blot plus, Chemiluminescent Reagent, New England Nuclear Life Sciences Products Inc, Boston, Mass.

^jHyperfilm ECL, Amersham Life Science, Buckinghamshire, UK.

^kBovine lactoferrin, Sigma Chemical Co, St Louis, Mo.

^lScion Image, version 3 beta, 1998, Scion Corp, Frederick Md. This software is available free online at www.ScionCorp.com. It is based on the NIH Image version for the Macintosh computer.

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