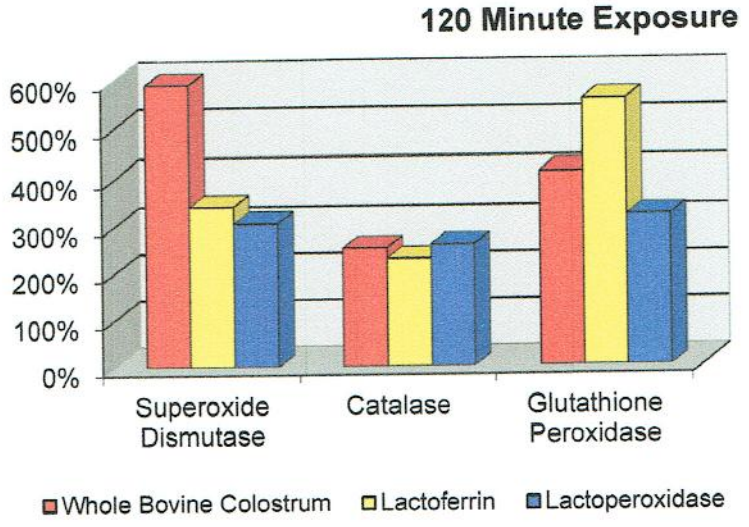
FILE: CHART-oxidant activity 120 min 4-03.xls

	Whole Bovine Colostrum	Lactoferrin	Lactoperoxidase
Superoxide Dismutase	591%	342%	307%
Catalase	252%	230%	259%
Glutathione Peroxidase	412%	557%	321%



Antioxidant Properties of Bovine Colostrum and Its main bioactive Constituents Lactoferrin and Lactoperoxidase

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ABSTRACT

Bovine colostrum and its metabolites bioactive lactoferrin and lactoperoxidase have been shown to prevent oxidant-induced injury of endothelial cells. The present study determined the effects of a dietary bovine colostrum on the generation of hydrogen peroxide (H_2O_2) and superoxide anion (O^{-}_2) and the activity of three antioxidant enzymes in a bovine pulmonary artery endothelial cell line (PAEC). All of the examined colostrum fraction forms exhibited a clear and potent antioxidant activity by increasing significantly the activity of all antioxidant enzymes in a dose and time-dependent fashion. The results suggested that dietary bovine colostrum and its metabolites may be effective antioxidants in preventing or treating endothelial cell injury associated with reactive oxygen species (ROS).

INTRODUCTION

Oxygen free radicals have been implicated in various pathological processes associated with oxidative stress such as ischemia, inflammatory diseases, diabetes, and atherosclerosis in addition to some brain aging and immune disorders (Akaneya et al.; 1995 and Haulica et al.; 2000). The intracellular and released antioxidant enzymes like Superoxide dismutase (SOD), Catalase (CAT), and Glutathione peroxidase (GPX) can potentially scavenge oxidants or prevent their conversion into more toxic species (Leff et al.; 1991) and therefore protect the incidence of carcinogenesis which is developed as a result of cellular oxidizing stress (Bonnefoy et al.; 2002 and Calderon et al.; 2002). Oxidative damage, in particular DNA-damage caused by free radicals generated either by xenobiotics or endogenously, is nowadays thought to be a key molecular mechanism associated with male reproductive health hazard (Ong et al.; 2002).

Bovine colostrum and its metabolite lactoferrin have demonstrated to prevent many forms of human cancer and cardiovascular disorders in addition to retarding the aging process (Covington et al.; 2000). Moreover, dietary bovine colostrum and its bioactive constituents lactoferrin and lactoperoxidase can exert a lot of many other health benefits for human including stimulation of body muscle growth and development (Ballard et al.; 1996 and Antonio et al.; 2001), Stimulation and enhancement general immune responses due to its high content of natural immune factors (Kelleher & Lonnerdal 2001 and Coombes et al.; 2002) and restriction of neoplastic transformation due to the pronounced anti-mutagenic activity (Parodi 1998

&1999).

Dietary intervention with food supplementation with bovine colostrum or its metabolite lactoferrin or lactoperoxidase could offer pronounced protection against several forms of microbial infections in humans (Arthington et al.; 2000 and Qulgley et al.; 2001). Several clinical and epidemiological studies have previously shown that bovine colostrum could offer a lot of growth factors that harmonize the cellular growth and differentiation (Mero et al.; 1997, Baumrucker & Blum 1993 and Chen & Li 1990) and has been mentioned for its good implication in dietary management and intervention in humans due to its powerful health benefits (Collier et al.; 1991 and Levy 1998). Other recent clinical studies have indicated that bovine colostrum and lactoferrin inhibited aflatoxin-induced hepatocarcinoma and other forms of immunity-deficient diseases (Berkhout et al.; 2002 and Nozaki et al.; 2002).

The present research aims to assess a dietary role of crude extract of dietary bovine colostrum and its bioactive metabolite lactoferrin and lactoperoxidase fractions in the generation of hydrogen peroxide (H_2O_2) and superoxide anion (O^{-}_2) as well as the activities of three antioxidant enzymes in bovine pulmonary artery endothelial cells.

MATERIAL AND METHODS

Reagents:

Immuno-Dynamic Inc., (Perry – Iowa, USA), provided bovine colostrum in sterile liquid form as a gift. Whereas, lactoferrin and lactoperoxidase were supplied by DMV-International, Veghel – Holland as 98% purity. All the antioxidant enzyme kits and related reagents were supplied by Sigma Chemical Co. (St. Louis, MO). Other solutions were prepared according to the described methods of AOAC (1998).

Cell line and experimental design:

Bovine pulmonary artery endothelial cell line (PAEC) was obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were subculture weekly at 37 °C in a humidified atmosphere (5% CO_2) and after having grown to confluence, cells were incubated with liquid bovine colostrum or each of its fractions in 24-well plates for 18 h. Following removal of colostrum or each of its fractions, cells grown in 24-well plates were used for determination of hydrogen peroxide and the protein content in cell lysate was determined by the method of Lowry et al., (1951).

Hydrogen Peroxide Measurement:

Hydrogen peroxide was measured by a fluorometric assay using 2,7-dichlorofluorescein diacetate (DCFH-DA) as a probe (Wan, et al.; 1993). The assay is

based on the oxidation of non-fluorescent DCFH-DA to fluorescent 2,7-dichlorofluorescein (DCF) by hydrogen peroxide. The fluorescence intensity was measured at 485 nm excitation and 530 nm emission using a 7620 Microplate Fluorometer (Cambridge Technology, Watertown, MA) according to the method of (Scott et al., 1988). Fluorescence intensity was measured up to 30 minutes at 5 minutes intervals.

Assay of superoxide anion generation:

The superoxide anion production in PAEC was detected using hydroethidine bromide (HEB) which is oxidized to ethidine bromide (EB) by the superoxide anion (Carter et al., 1994). Fluorescence intensity was measured after 60 and 180 min, respectively, at 540 nm excitation and 620-nm emission using a 7620 Microplate fluorometer.

Enzyme activity determination:

Enzyme activities were determined by measuring the optical density using a DU 650 Spectrophotometer (Beckman Instruments, Fullerton, CA). Superoxide dismutase (SOD) activity was measured according to the method of Oyanagui (1984). The optical density was measured at 550 nm and the enzyme activity was calculated according to a SOD standard curve.

Glutathione peroxidase (GPX) activity was measured according to the method of Paglia and Valentine (1967). Measurements were taken in 0.1M phosphate buffer (pH 7.6) at 37 °C. The activity was calculated according to a GPX standard curve.

Catalase (CAT) activity was measured according to the assay of Beers and Sizer (1952) with slight modification. The reaction was carried out in 0.05 M phosphate buffer solution (pH 7.0) at 37 °C. Hydrogen peroxide (0.3 ml, 0.01 M) and 50 µl of cell lysate were added to the cuvette and the absorbency at 240 nm was recorded for 3 min. The enzyme activity was calculated according to a CAT standard curve.

Ascorbate equivalent assay

It was carried out by applying the technique of Foyer et al., (1995). Trichloroacetic acid (5%, w/v) was added to the chlorophyll fraction and then well-mixed and stored at 4 °C for 30 min. This mixture was centrifuged at 21,000 rpm (4 °C, 30 min.) and the supernatant obtained was diluted until a 50% (v/v) with 2.0 M sodium dihydrogen phosphate and then injected into a HPLC Phenomenex Octadecylsilane column (Peckmann Inc, USA) and eluted with an isocratic mobile phase of 0.2 M sodium dihydrogen phosphate at 1 ml/min flow rate. Ascorbate was detected in the elute using an UV Spectrophotometer set at 254 nm. Ascorbate standard was compared to elute measurements at 3.2 min retention time and flow rate 1.8mL/min.

Statistical analyses:

Results were expressed as the mean ± SE. Data were analyzed using one-way analysis of variance (ANOVA) followed by the Tukey's multiple range test for significant difference. Statistical significance was defined as $p < 0.05$. All statistical procedures were performed by means of the Stat. graphics version 5.0 (Snedecor and Cochran 1976)

RESULTS

The effect of bovine colostrum and its functional bioactive constituents lactoferrin and lactoperoxidase on the activity of antioxidant enzymes (superoxide dismutase, catalase and glutathione) at exposure time 60 minutes of PAEC to oxidant stress was listed in Figure 1. It could be observed that all of bovine colostrum concentration used and the levels of 0.2, 0.5 and 1.0 mg/ml of both lactoferrin and lactoperoxidase are significantly higher ($P<0.05$) than control treatment in respect of activation of all measured antioxidant enzymes. The same observation was also noticed when these measurements were assessed at 120 minutes exposure time (Figure 2). There was clear and significant ($P<0.05$) difference in the measured titer of all examined antioxidant enzymes as affected by dose and exposure time of each tested components. Higher concentrations of each examined fraction gave higher antioxidant activities. Regarding to superoxide dismutase activity, whole bovine colostrum was the most potent stimulator for this antioxidant enzyme followed by both lactoferrin and lactoperoxidase which shown relatively similar responses. Whereas, whole bovine colostrum and lactoperoxidase were recorded with the same relatively higher effect rather than lactoferrin toward catalase activity. Whole bovine colostrum was the most potent stimulant for glutathione peroxidase followed by lactoperoxidase then lactoferrin when the exposure time was 60 minutes. While, at exposure time 120 minutes, levels of 0.5 and 1.0 mg/ml of lactoferrin were gave most higher activities rather than any other concentrations of the other two examined fractions (whole bovine colostrum and lactoperoxidase).

Dietary bovine colostrum and all its examined metabolite fractions lactoferrin and lactoperoxidase exhibited a concentration-dependent inhibition of hydrogen peroxide generation. PAEC were incubated with different concentrations of each of examined forms (0.1, 0.2, 0.5, and 1.0 mg/ml) up to 30 minutes through 5 minutes intervals. Both dose and time-dependent suppression of hydrogen peroxide generation was observed (Figure 3). Relative fluorescence units reflecting hydrogen peroxide production by whole liquid bovine colostrum, lactoferrin or lactoperoxidase-treated cell groups were significantly lower than that untreated control cells through an also dose-dependant fashion. All examined components show a significant ($P<0.05$) inhibition for hydrogen peroxide generation. Whole bovine colostrum was the most potent fraction followed by lactoferrin then lactoperoxidase toward inhibition of hydrogen peroxide generation.

At 5-min measurement, there were no significant differences in superoxide

levels between cells pretreated with any of the examined fraction forms and the untreated control cells. After a 10 min or longer period, a concentration-dependent decrease of superoxide generation was noted in cells pretreated with any of whole bovine colostrum, lactoferrin or lactoperoxidase.

The inhibitory effect of whole bovine colostrum, lactoferrin and lactoperoxidase on the generation of superoxide anion was assessed also (Figure 4). It could be observed that all of the examined forms had clear and significant inhibitory effect against superoxide anion generation through both dose and time-dependant fashion. Whole bovine colostrum was observed to has higher inhibitory effect against superoxide generation followed by lactoferrin and lactoperoxidase.

It could be observed that whole liquid colostrum and lactoferrin were highly antioxidant fractions yielding higher ascorbate scores than the lactoperoxidase through a dose-dependant fashion also (Table 5).

DISCUSSION

It has been previously shown that lactoferrin protected against inflammatory and arthritis (Kumar et al.; 2002). The present study demonstrated that bovine colostrum and its metabolite fraction lactoferrin and lactoperoxidase affected hydrogen peroxide and superoxide anion generation in PAEC model. In the absence of oxidant stress, hydrogen peroxide generation in PAEC was modest. Examined colostrum or its metabolite fraction did exhibit a variable inhibition effect after 1–2 h incubation at 37 °C. Significant inhibitory effect occurred after a 2-h incubation and was higher than that exhibited after 1-h. This may mean that under normal condition PAEC are capable of scavenging hydrogen peroxide produced constitutively. However, when the cells were exposed to HPX or XO as oxidative stress factors, a significant inhibition of DCF fluorescence, reflecting hydrogen peroxide generation, was demonstrated at each bovine colostrum form and level. Moreover, the inhibition was related to the concentration of bovine colostrum, lactoferrin or lactoperoxidase and incubation period (Tables 3 and 4). This may indicate that the suppression associated with the antioxidant activity of bovine colostrum was also concentration- and time-dependent.

The antioxidant enzymes superoxide dismutase (SOD), Glutathione peroxidase (GPX) and Catalase (CAT) play an important role in maintaining physiological levels of superoxide and hydrogen peroxide (Johnson 2002 and Yagi et al.; 2002). Superoxide dismutases are a group of enzymes that rapidly catalyze the conversion of superoxide anion to hydrogen peroxide and oxygen. Glutathione peroxidases are major enzymes that inactivate a variety of organic peroxides and thus control the cellular peroxide levels. They also reduce hydrogen peroxide to water and generate oxidized Glutathione. Catalase converts hydrogen peroxide to oxygen and water

(Park & Lil, 1994). Several studies have indicated that treatment of endothelial cells with SOD and/or CAT decreased damage caused by free radicals (Leff et al.; 1991, and Backer et al.; 1994).

Our data show that dietary bovine colostrum and lactoferrin increases the activities of SOD, GPX and CAT significantly rather than lactoperoxidase. By inducing the activity of antioxidant enzymes, bovine and its metabolites may hasten dismutation of superoxide anion and decomposition of hydrogen peroxide In this study, the effects of the dietary bovine colostrum on hydrogen peroxide and superoxide anion generation observed may be therefore due to enhanced activities of these antioxidant enzymes. These observed antioxidant properties of bovine colostrum may be referred mainly to its high content of carotenoid fractions (Patton et al.; 1990) which play an important effective role in control of incidence of more than one kind of human cancer. The highest antioxidant activity of bovine colostrum rather than other examined forms of colostrum fractions could be explained as the accumulative effect of colostrum constituents more than individual fraction.

On the other hand, Tsuda et al.; (2002) reported that lactoferrin could serve as a strong antimutagenic agent through a catalytic degradation mechanism of the proximate electrophile in target cells. Moreover, Tenovuo (2002) stated that dietary lactoferrin could possess anticarcinogenic properties through the strong binding with most of the carcinogens and therefore retard their systemic absorption.

Our results are addressed in parallel to what has been reported by Chierici et al.; (1992). It is highly suggested to extend the food supplementation with lactoferrin due to its highly noticed nutritional properties and since it is well absorbed through the human intestine (Guillen et al.; 2002) ensuring therefore its reaching into all body tissues via blood circulation. In addition, supplementation of human food with bovine colostrum or lactoferrin serve to detoxify human body through the strong interaction between lactoferrin and gastrointestinal pathogens which may present in food or human gastrointestinal tract (Park et al.; 2002 and Ajello et al.; 2002). Therefore, supplementation of human foods with bovine colostrum may provide with many health benefits, which are more relevant than an antioxidant activity by itself. It could offer a pronounced protection against many of severe human diseases and microbial infections like hepatitis virus C (Iwasa et al.; 2002) in addition to offer host defense through regulation of production of tumor necrosis factor alpha (Ward et al.; 2002).

Supplementation of human foods with bovine colostrum or lactoferrin could offer also pronounced enhancement for mineral absorption specially iron and calcium (Rossi et al.; 2002). Tomita et al.; (2002) reported that both lactoferrin and its pepsin hydrolysate (lactoperoxidase) are commonly used as a safe supplement for infant formula due to its antimicrobial activity and modulation of the immune system. (Weinberg, 2001).

In summary, this study has shown that dietary bovine colostrum and its metabolites inhibited hydrogen peroxide and superoxide anion generation and augmented the SOD, CAT and GPX activities. These results suggest that dietary bovine colostrum and its bioactive metabolites may be effective antioxidants in preventing endothelial cell damage and therefore play a significant role in the tissue defense against free radical-mediated disorders.

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Table (1). Effect of bovine colostrum, lactoferrin and lactoperoxidase on the activity of antioxidant enzymes at **60-min** exposure time

Tested Fraction	Concentration mg/ml	Antioxidant Enzymes (U/mg protein)		
		Superoxide dismutase	Catalase	Glutathione Peroxidase
Control		0.55 ± 0.11	33.36 ± 2.89	16.54 ± 3.55
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Whole Bovine Colostrum	0.1	0.83 ± 0.07*	42.44 ± 3.11*	23.11 ± 2.12*
	0.2	1.55 ± 0.34*	59.32 ± 4.56*	39.44 ± 2.42*
	0.5	1.72 ± 0.34*	65.66 ± 5.72*	42.44 ± 3.75*
	1.0	2.28 ± 0.98*	74.28 ± 3.82*	51.25 ± 4.17*
Lactoferrin	0.1	0.71 ± 0.22	36.12 ± 5.26	19.46 ± 3.52
	0.2	0.92 ± 0.39*	41.47 ± 2.44*	24.37 ± 4.38*
	0.5	1.36 ± 0.19*	59.84 ± 4.61*	31.22 ± 5.55*
	1.0	1.79 ± 0.57*	66.33 ± 2.91*	42.45 ± 3.42*
Lactoperoxidase	0.1	0.69 ± 0.44	43.14 ± 4.62*	21.36 ± 3.62*
	0.2	0.78 ± 0.38*	50.19 ± 5.33*	26.59 ± 2.56*
	0.5	1.08 ± 0.22*	56.24 ± 4.62*	35.63 ± 4.48*
	1.0	1.28 ± 0.14*	67.33 ± 4.13*	49.85 ± 3.87*

Data are presented as mean ± SE
Asterisk denotes significant difference compared with control without any treatment ($P<0.05$)

Table (2). Effect of bovine colostrum, lactoferrin and lactoperoxidase on the activity of antioxidant enzymes at **120-min** exposure time

Tested Fraction	Concentration mg/ml	Antioxidant Enzymes (U/mg protein)		
		Superoxide dismutase	Catalase	Glutathione Peroxidase
Control		0.55 ± 0.11	33.36 ± 2.89	16.54 ± 3.55
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Whole Bovine Colostrum	0.1	0.96 ± 0.25*	52.24 ± 2.51*	29.71 ± 3.59*
	0.2	1.87 ± 0.65*	69.32 ± 6.66*	42.64 ± 3.49*
	0.5	2.34 ± 0.44*	75.32 ± 5.72*	52.61 ± 4.55*
	1.0	3.25 ± 0.42*	84.19 ± 3.82*	68.15 ± 3.55*
Lactoferrin	0.1	0.82 ± 0.51	42.73 ± 3.32	22.66 ± 2.12
	0.2	1.19 ± 0.17*	52.50 ± 3.22*	31.28 ± 3.44*
	0.5	1.56 ± 0.26*	67.33 ± 6.45*	81.50 ± 4.15*
	1.0	1.88 ± 0.33*	76.75 ± 4.11*	92.15 ± 5.40*
Lactoperoxidase	0.1	0.99 ± 0.38	52.14 ± 3.11*	29.50 ± 5.60*
	0.2	1.13 ± 0.10*	64.19 ± 4.45*	36.59 ± 4.22*
	0.5	1.28 ± 0.56*	78.24 ± 5.60*	42.11 ± 5.20*
	1.0	1.68 ± 0.35*	86.33 ± 6.58*	53.15 ± 5.25*

Data are presented as mean ± SE
Asterisk denotes significant difference compared with control without any treatment ($P<0.05$)

Table (3). Effect of bovine colostrum, lactoferrin and lactoperoxidase on kinetic of H_2O_2 generation

Tested Fraction	Concentration mg/ml	Relative Fluorescence Units / 5-min Intervals						
		0 min	5 min	10 min	15 min	20 min	25 min	30 min
Control		0	26	59	118	240	438	576
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Whole Bovine Colostrum	0.1	0	16*	32*	57*	165*	322*	455*
	0.2	0	15*	28*	43*	133*	265*	364*
	0.5	0	13*	24*	35*	111*	185*	293*
	1.0	0	11*	19*	28*	98*	115*	148*
Lactoferrin	0.1	0	20	38*	78*	195*	355*	488*
	0.2	0	17*	31*	64*	165*	315*	433*
	0.5	0	15*	27*	53*	126*	265*	365*
	1.0	0	13*	20*	33*	125*	210*	288*
Lactoperoxidase	0.1	0	32	54	92	225	385*	518*
	0.2	0	17*	40*	72*	171*	342*	463*
	0.5	0	15*	33*	64*	144*	220*	388*
	1.0	0	13*	25*	48*	158*	183*	328*

Data are presented as mean ± SE
Asterisk denotes significant difference compared with control samples measured at the same time intervals ($P<0.05$).

Table (4). Effect of bovine colostrum, lactoferrin and lactoperoxidase on kinetic of O^{-}_{2} generation

Tested Fraction	Concentration mg/ml	Relative Fluorescence Units / 5-min Intervals						
		0 min	5 min	10 min	15 min	20 min	25 min	30 min
Control		0	56	166	279	348	415	489
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Whole Bovine Colostrum	0.1	0	51	138	205*	288*	310*	400*
	0.2	0	48	126*	183*	253*	275*	344*
	0.5	0	45	115*	165*	111*	185*	293*
	1.0	0	38*	108*	152*	98*	115*	238*
Lactoferrin	0.1	0	50	154	255*	291*	365*	468*
	0.2	0	47	136*	218*	275*	342*	411*
	0.5	0	41*	128*	194*	246*	288*	345*
	1.0	0	13*	117*	182*	248*	288*	322*
Lactoperoxidase	0.1	0	52	158	262*	298	369*	478
	0.2	0	48	144*	247*	272*	355*	463*
	0.5	0	45*	130*	234*	256*	294*	388*
	1.0	0	40*	128*	228*	248*	280*	348*

Data are presented as mean ± SE
Asterisk denotes significant difference compared with control samples measured at the same time intervals ($P<0.05$).