

Decrease in salivary lactoferrin output in chronically intoxicated alcohol-dependent patients

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Abstract: Salivary lactoferrin is a glycoprotein involved in the elimination of pathogens and the prevention of massive overgrowth of microorganisms that affect oral and general health. A high concentration of lactoferrin in saliva is often considered to be a marker of damage to the salivary glands, gingivitis, or leakage through inflamed or damaged oral mucosa, infiltrated particularly by neutrophils. We conducted a study to determine the effect of chronic alcohol intoxication on salivary lactoferrin concentration and output. The study included 30 volunteers consisting of ten non-smoking male patients after chronic alcohol intoxication (group A), and 20 control non-smoking male social drinkers (group C) with no history of alcohol abuse. Resting whole saliva was collected 24 to 48 hours after a chronic alcohol intoxication period. Lactoferrin was assessed by enzyme-linked immunosorbent assay. For all participants, the DMFT index (decayed, missing, or filled teeth), gingival index (GI) and papilla bleeding index (PBI) were assessed. The differences between groups were evaluated using the Mann–Whitney U test. We noticed significantly decreased salivary flow (SF) in alcohol dependent patients after chronic alcohol intoxication (A), compared to the control group (C). Although there was no significant difference in salivary lactoferrin concentration between the alcohol dependent group A and the control group C, we found significantly decreased lactoferrin output in group A compared to group C. We found a significant correlation between the amount of daily alcohol use and a decrease in lactoferrin output. There was a significant increase in GI and a tendency of PBI to increase in group A compared to group C. We demonstrated that chronic alcohol intoxication decreases SF and lactoferrin output. The decreased lactoferrin output in persons chronically intoxicated by alcohol may be the result of lactoferrin exhaustion during drinking (due to its alcohol-related lower

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biosynthesis or higher catabolism) or to decreased function of neutrophils affected by the ethanol. The poorer periodontal state in alcohol dependent persons compared to controls may be a result of lower salivary flow and decreased protection of the oral cavity by lactoferrin. (*Folia Histochemica et Cytobiologica* 2012, Vol. 50, No. 2, 248–254)

Key words: alcohol, ethanol, saliva, lactoferrin

Introduction

Almost 4% of global deaths, 4.6% of diseases and injuries, and 30% of health care costs, are attributed to alcohol abuse [1]. Alcohol drinking is the world's third largest risk factor for disease and disability, and in middle-income countries it is the greatest risk factor [2]. About 2% of the world-wide population is dependent on alcohol [3]. Ethanol is directly toxic to the mucosa of the oral cavity, mouth, throat, esophagus and stomach. Acute local mucosa damage is reversible and proportional to the concentration of alcohol. Ethanol immediately diffuses into saliva and oral tissues, reaching in saliva and oral tissues higher concentrations than in plasma [4–9]. Acetaldehyde, the main metabolite of ethanol, also damages oral tissues [10]. However, oral tissue damage may also be the result of a direct action of ethanol, reactive oxygen species (ROS) generated by ethanol and its metabolites, and fatty acid ethyl esters (FAEEs). Oral mucosa is the portal of entry for many pathogens. The saliva and system of innate and acquired immunity of the oral mucosa eliminate the majority of pathogens and prevent massive overgrowth of microorganisms. Salivary proteins participate not only in protection of the oral cavity, but also in protection of the upper gastrointestinal and respiratory tracts [9–12].

Lactoferrin is a multifunctional glycoprotein of the transferrin family that participates in the innate immune response. It is secreted by the intercalated duct cells of salivary glands [7, 13]. Lactoferrin is found on the surface of mucous membranes, in the granules of polymorphonuclear leukocytes, neutrophils, and in biological fluids such as milk, saliva, tears and seminal fluid [13]. An increase in lactoferrin extracellular concentration is often regarded as an indicator of neutrophil activation [14, 15]. A high lactoferrin concentration in saliva is often considered a marker of damage to the salivary glands, gingivitis, or leakage through inflamed or damaged oral mucosa, infiltrated by neutrophils [16]. As iron cations are an essential nutrient for certain types of bacteria, two binding sites for iron cations in lactoferrin provide its bacteriostatic effect by limiting availability of iron for bacteria such as *Streptococcus mutans* [17, 18]. Through direct interaction with the bacterial porins, lactoferrin exerts its bactericidal effect [13, 15]. Lactoferrin

interacts with endotoxin (LPS, lipopolysaccharide) of the Gram-negative bacterial membrane, thus releasing the LPS from the membrane [13]. The consequence of subsequent LPS binding by the lactoferrin is reduced synthesis of LPS-mediated tumor necrosis factor (TNF- α) that protects against septicemia [19]. Lactoferrin also participates in the inhibition of bacterial adhesion to tooth surfaces, aggregation of bacteria, and the activation of phagocytic cells. Lactoferrin has also antifungal, antiviral (prevents infection by binding to the host cell), antiparasitic (reduces the integrity of the parasite membrane and its interaction with host tissues, binds parasite nutrients i.e. iron cations), as well as immunomodulatory action (increases production of IL-1, IL-2, and increases activity of monocytes/macrophages, granulocytes and NK cells) and anti-inflammatory properties [7, 17, 20]. Lactoferrin is partially digested in the gastrointestinal tract by pepsin and trypsin to lactoferricin, a cationic peptide that exhibits antimicrobial activity in the lower gastrointestinal tract [21]. This would support the hypothesis that salivary proteins besides the oral cavity are also involved in protection downstream along the gastrointestinal tract.

It has been reported that there is a reduction in the stimulated release of lactoferrin after acute *in vitro* effect of ethanol on neutrophils [22], a tendency towards an increase in serum lactoferrin concentration in alcohol dependent patients [14], and no effect of acute alcohol intoxication on salivary lactoferrin concentration [7]. The objective of the present study was to determine for the first time the effect of chronic alcohol consumption on the concentration and output of salivary lactoferrin.

Material and methods

Subjects. The study included 30 volunteers comprising ten male alcohol-dependent non-smoking patients (group 'A', standing for alcohol) admitted to the Detoxification Unit of the Psychiatric Hospital in Choroszcz, Poland, after chronic alcohol intoxication (mean age: 49 years; range: 39–57) (100–200 g/day of alcohol), and 20 control male social drinkers (group 'C', standing for control) with no history of alcohol abuse or smoking (mean age: 42 years; range: 30–53). Alcohol dependent individuals in group A met the criteria for alcohol dependence according to ICD-10 (the average

time of dependence was 12 ± 8 years). The length of alcohol intoxication ranged from three to 30 days (mean ~ 8). Material from alcohol dependent persons admitted to the Detoxification Unit was collected on the second day of the abstinence period.

Ethical issues. This study was approved by the Bioethical Committee of the Medical University of Białystok, Poland (R-I-003/289/2005). Informed written consent was obtained from all the participants after explanation of the nature, purpose, and potential risks of the study.

Data and sample collection. A check-up of the oral cavity was done by one qualified dentist in artificial light, using a dental mirror and a probe. Following the World Health Organization criteria, the level of dental caries in subjects was determined using the DMFT index (decayed, missing or filled teeth; [7]). Gingival status was assessed using the gingival index (GI; Löe and Silness [23]) and papilla bleeding index (PBI; [24]). The DMFT, GI, and PBI indices of our subjects were: in alcohol dependent participants (20.6 ± 3.5 , 1.58 ± 1.04 , 0.93 ± 0.65 , respectively), and in controls (18.8 ± 5.70 , 0.30 ± 0.47 , 0.35 ± 0.48 , respectively). The subjects were instructed to refrain from food and beverages, except water, for two hours before the collection of saliva. All salivary samples (3 ml of resting whole saliva) were collected to plastic tubes placed on ice by the spitting method, under standardized conditions [25, 26], between 8.00 am and 9.00 am, to minimize the influence of circadian rhythms. The saliva samples were centrifuged at $3,000 \times g$ for 20 minutes at 4°C , to remove cells and debris. The resulting supernatants were divided into $200 \mu\text{l}$ portions, frozen and kept at -80°C , until analyzed. Salivary flow (SF) was calculated by dividing the volume of saliva by the time of its collection. In all samples, the lactoferrin concentration was assayed in duplicate, and the means of the duplicate results were used as final values.

Lactoferrin assay. An enzyme-linked immunosorbent assay (ELISA) method was used to determine the salivary lactoferrin concentration, as previously described [7]. In brief, MaxiSorp™ microplates (Nunc Brand cat. no 449824, Roskilde, Denmark) were coated with rabbit anti human lactoferrin (primary) antibody 1:500 (Sigma, L3262, St Louis, MO, USA) and blocked with 2% bovine serum albumin (BSA; Sigma, A7030). Lactoferrin from human milk (Sigma, L0520) was applied as a standard. Saliva samples [diluted 1:10 and 1:20 in phosphate-buffered saline (PBS) containing 0.75% BSA and 0.1% Tween 20], $50 \mu\text{l}/\text{well}$], were applied onto blocked plates. Then secondary antibodies 1:15,000 (rabbit anti-human lactoferrin antibody; polyclonal antibody biotinylated, ICN, 673581, Costa Mesa, CA, USA) were applied. The microplates with secondary antibodies were incubated with horseradish peroxidase (HRP) conju-

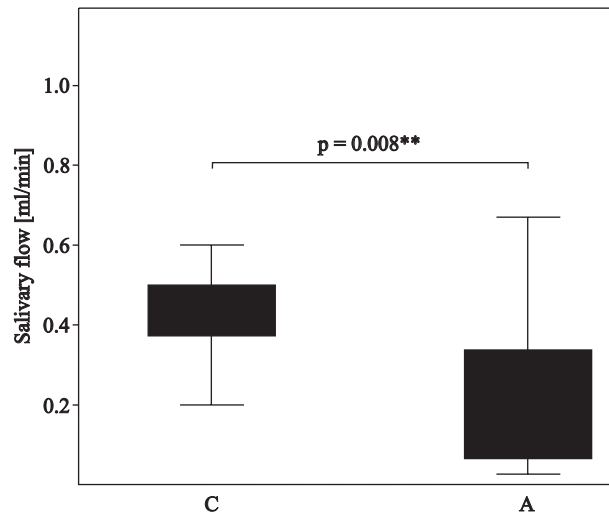


Figure 1. Salivary flow (SF) in control social drinkers (C) and alcohol dependent persons (A)

gated with streptavidin (Dako, P0397, Glostrup, Denmark) 1:2,000 in PBS. Streptavidin has a high affinity for biotin. HRP bound to streptavidin is ready for color development after addition of the substrate, o-phenylenediamine (OPD). HRP reaction was stopped by adding 0.5 M H_2SO_4 , $50 \mu\text{l}/\text{well}$. Color development was measured at 450 nm (Elx800™, Bio-Tek Instruments, Inc., Winooski, VT, USA) and was proportional to the quantity of lactoferrin in the sample. As negative controls, two wells were incubated for 30 minutes with PBS instead of sample/standard, two wells were processed without primary antibody, and two without secondary antibody. Results were analyzed by the KC Junior program. Bio-Tek software applications include KC4, IBM, and KC Junior for MS-DOS (all manual and automated readers).

Statistical analysis. Statistical analysis was performed using Statistica version 9.0 (Statsoft, Krakow, Poland). Results are expressed as means \pm SD. The differences between groups were evaluated using the Mann-Whitney U test. Statistical significance was assumed at $p < 0.05$.

Results

Salivary flow (SF) was significantly lower in chronically intoxicated alcohol dependent patients (0.23 ± 0.24) than in the control social drinking group (0.43 ± 0.10 ml/min) ($p = 0.008^{**}$) (Figure 1). No significant differences were found between the concentration of lactoferrin in the saliva of alcohol dependent patients (4.53 ± 2.56 mg/l) and the control social drinkers (4.48 ± 2.92 mg/l) ($p = 0.929$). Salivary lactoferrin output was significantly decreased in

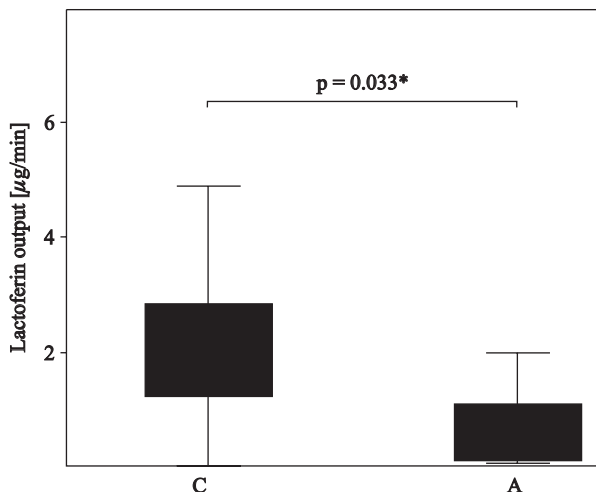


Figure 2. Lactoferrin output in control social drinkers (C) and alcohol dependent persons (A)

Table 1. Effect of chronic ethanol intoxication (A) on the DMFT (decayed, missing or filled teeth) index, GI (gingival index) and PBI (papilla bleeding index), compared to the control social drinkers (C)

Variable	C	A	p
DMFT	18.8 ± 5.7	20.6 ± 3.5	0.355
GI	0.30 ± 0.47	1.58 ± 1.04	0.029*
PBI	0.35 ± 0.48	0.93 ± 0.65	0.081

Values are expressed as the mean ± SD; p < 0.05 considered statistically significant: *p < 0.05, **p < 0.01, ***p < 0.001; p: C to A group

alcohol dependent persons chronically intoxicated by ethanol ($0.74 \pm 0.74 \mu\text{g}/\text{min}$) compared to the control group ($1.93 \pm 1.35 \mu\text{g}/\text{min}$) ($p = 0.033^*$) (Figure 2). Gingival index (GI) was significantly higher in alcohol dependent persons (1.58 ± 1.04) than in the control group (0.30 ± 0.47) ($p = 0.029^*$) (Table 1). Papilla bleeding index (PBI) had a high tendency to increase in alcohol dependent persons (0.93 ± 0.65) compared to the control group (0.35 ± 0.48) ($p = 0.081$). There was a weak tendency of DMFT

Table 3. Spearman’s rank correlation coefficient between salivary flow (SF) and the DMFT (decayed, missing or filled teeth) index, GI (gingival index) and PBI (papilla bleeding index), in alcohol dependent subjects

Variable	DMFT	GI	PBI
SF [ml/min]	$r^2 = -0.098$ $p = 0.630$	$r^2 = -0.423$ $p = 0.038^*$	$r^2 = -0.419$ $p = 0.041^*$

p < 0.05 considered statistically significant: *p < 0.05, **p < 0.01, ***p < 0.001

index (decayed, missing or filled teeth) to increase in alcohol dependent persons (20.6 ± 3.5) than in the control group (18.8 ± 5.7) ($p = 0.355$). There was a significant correlation between the amount of daily alcohol consumption and the output of salivary lactoferrin ($r^2 = 0.819$, $p = 0.045^*$) (Table 2). There were no significant correlations between the duration of alcohol consumption and salivary lactoferrin output/concentration. We also did not find any correlations between amount/duration of alcohol intoxication, SF, lactoferrin concentration/output, and the DMFT, GI, and PBI indices, in group A. Significant inverse correlations were found between SF and PBI ($r^2 = -0.419$, $p = 0.041$), as well as between SF and GI ($r^2 = -0.423$, $p = 0.038$) (Table 3).

Discussion

After chronic ethanol consumption, the following have been reported: fat accumulation, acinar cell swelling, salivary gland atrophy, reduced salivary flow rate, markedly decreased salivary protein and glycoprotein levels/outputs (e.g. immunoglobulin A) [6–8, 27, 28]. However, little is yet known as to the possible effects of chronic ethanol intoxication on the level of salivary lactoferrin. Previous studies concerning alcohol’s effect on lactoferrin have reported a reduction in stimulated release of lactoferrin after acute action *in vitro* of ethanol on neutrophils [22], a tendency to increase in serum lactoferrin concentration in alcohol dependent patients [14], and no effect of

Table 2. Spearman’s rank correlation coefficient between amount/duration of alcohol intoxication and lactoferrin concentration/output, in alcohol dependent subjects

Variable	Amount of alcohol per day [g]	Duration of alcohol intoxication (days)
Lactoferrin concentration [mg/l]	$r^2 = 0.698$ $p = 0.122$	$r^2 = 0.206$ $p = 0.696$
Lactoferrin output [$\mu\text{g}/\text{min}$]	$r^2 = 0.819$ $p = 0.045^*$	$r^2 = 0.205$ $p = 0.695$

p < 0.05 considered statistically significant: *p < 0.05, **p < 0.01, ***p < 0.001

acute alcohol intoxication on the salivary lactoferrin concentration [7].

Although we did not find a significant difference between the concentration of lactoferrin in the saliva of alcohol dependent persons and in that of control social drinkers, salivary lactoferrin output was significantly decreased in alcohol dependent persons compared to the social drinkers ($p = 0.033$) (Figure 2). Since alcohol abuse has been found to decrease some glycoprotein biosynthesis, modification and transport, and to increase other glycoprotein secretion, elimination and catabolism [6], the decreased lactoferrin output may also be the result of subsequent lactoferrin exhaustion during a long drinking period. As we noted a high correlation between the amount of daily alcohol consumption and salivary lactoferrin output (Table 2), ethanol may exert a directly toxic effect on lactoferrin secretion. It should be mentioned that salivary lactoferrin is secreted not only by intercalated duct cells of salivary gland tissue [7, 13], but may also be released by activated neutrophils in damaged salivary glands, in gingivitis, or may be the result of blood leakage through inflamed or damaged oral mucosa [16, 29]. It has been reported that six hours after acute ethanol (40%) consumption, damage had been caused to the oral mucosa epithelium with degeneration of the epithelial cells and leukocyte infiltration; after 12 hours, irreversible cytolysis or swelling of the intermediate layer cells of the oral mucosa developed, whereas 24–48 hours after acute alcohol consumption, disseminated ulcerations developed in the mouth [7, 30, 31]. After induced oral tissue damage (e.g. by alcohol), one of the first lines of defense against pathogens is the tissue recruitment of neutrophils [32]. It has been shown that alcohol intoxication generally inhibits the recruitment and activity of neutrophils and macrophages as well as their hydrogen peroxide production [33, 34]. This is consistent with the results of our study, as we noted significantly decreased salivary lactoferrin output in chronically intoxicated alcohol dependent persons.

Since lactoferrin is a marker of neutrophil activity, its reduced salivary output may suggest a deleterious effect of chronic alcohol intoxication on neutrophil activity, even if neutrophil infiltration occurred. Our study is not consistent with the one by Nilsson et al. [22] who observed no changes in the spontaneous secretion of lactoferrin by neutrophils acutely treated *in vitro* by ethanol. This could however be due to low levels of ethanol (1%) or too short a duration of alcohol action (10 minutes) to change the cellular metabolism of lactoferrin (our participants drank 5–40% alcohol for 3–30 days). The lack of changes in lactoferrin concentration has been previously de-

scribed after acute alcohol intoxication in saliva [7], and after chronic alcohol intoxication in serum [14].

Our results of decreased lactoferrin output, compared to Thome et al. [14], suggest that the salivary glands and oral cavity may be much more sensitive to chronic alcohol intoxication than serum. There is nothing surprising in that, since ethanol diffuses rapidly into saliva during drinking, and immediately after drinking ethanol salivary concentration is temporarily much higher than in plasma [9], suggesting that ethanol easily penetrates to the salivary glands. After alcohol intake, the level of acetaldehyde in saliva strikingly exceeds the level in systemic blood. From saliva, acetaldehyde, ethanol, and other metabolites of alcohol and toxins such as reactive oxygen species (ROS) or fatty acid ethyl esters (FAEEs), easily reach all local tissues [5–9]. The lack of lactoferrin concentration decrease in our earlier acute alcohol intoxication study [7] might be the result of the lack of salivary flow data. After chronic alcohol intoxication, we also found no changes in lactoferrin concentration. But when compared to the SF data, we found decreased lactoferrin output. Salivary flow is the main factor affecting salivary composition, which varies in accordance with the type, intensity, and duration of the stimulus [17]. As an increase in SF generally is accompanied by an increase in the concentration of total protein, sodium, calcium, chloride, bicarbonate, and pH level in the saliva [17], the reduced SF level in our study might also reduce the output of lactoferrin.

In our study, SF was significantly decreased in persons chronically intoxicated by alcohol, compared to control social drinkers (Figure 1). Our findings agree with the alcohol-induced SF reduction observed in experimental studies [35]. Our results are also consistent with the study reported by Dutta et al. [36], in which chronic alcohol intoxication resulted in a reduction in the stimulated SF from parotid glands. The SF reduction in patients suffering from alcohol dependence may be due to morphological changes of the salivary glands, since in these persons enlarged or atrophied salivary glands associated with accumulation of fat in the secretory cells, reduction of the weight of the salivary glands, and inflammatory infiltration of the parenchymal space around salivary ducts, have all been reported [7, 27, 37–39]. In alcohol abusers, ethanol replaces water from hydrogen bonds of hydrophilic domains of proteins, which increases the fluidity of parenchymal cell membranes of salivary glands. Replacement of water by ethanol impairs the activation of cAMP in the membrane, Ca^{2+} mobilization, and finally impairs the secretory process [5–9]. The decrease in SF may also be the result of alcohol withdrawal syndrome stress. Alcohol

withdrawal syndrome stress is characterized by a significant central and peripheral sympathetic hyperactivity, hypercortisolemia, reduced food intake and fluid diet (which reduces mastication that stimulates SF), and reduced number of muscarinic receptors as well as reduced secretion/action of paracrine gastrointestinal hormones and transmitters such as somatostatin, PP peptide, and cholecystokinin [5, 9, 40–42].

The association between heavy alcohol drinking and poor oral hygiene is widely known [43]. Dental and oral hygiene are often neglected by persons addicted to alcohol [8, 9, 44, 45]. It has also been found that persons addicted to alcohol with poor dental status have a doubled concentration of acetaldehyde in their saliva compared to those addicted to alcohol but who take care of their oral hygiene [43]. In alcohol abusers, reduced salivary flow (SF) may lead to inflammatory states of the oral cavity, periodontal diseases, or infections [46]. Although we found only a weak tendency to increase in DMFT index (decayed, missing or filled teeth) in alcohol dependent persons than in the control social drinkers, we found a significantly higher gingival index (GI) and a strong tendency of papilla bleeding index (PBI) to be higher in alcohol dependent persons than in the control social drinkers (Table 1). This suggests a significantly poorer periodontal state in alcohol dependent persons than in social drinkers, which may be the result of decreased salivary flow in alcoholics as significant inverse correlations were found between SF and PBI and between SF and GI (Table 3).

We have here demonstrated decreased salivary flow and lactoferrin output after chronic alcohol intoxication. Lower lactoferrin output in persons after chronic alcohol intoxication may be the result of lactoferrin exhaustion (due to its alcohol-induced lower biosynthesis or higher catabolism), or to decreased function of neutrophils affected by the ethanol. A poorer periodontal state in alcohol dependent persons than in social drinkers may be a result of lower salivary flow and decreased lactoferrin protection of the oral cavity.

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