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Salivary Biomass Assessed by Bioluminescence ATP Assay Related to (Bacterial and Somatic) Cell Counts

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The present work aimed (1) to evaluate ATP content in saliva by the bioluminescent luciferin–luciferase method, (2) to evaluate the relationships between ATP content, bacterial count and epithelial cell numbers in saliva, (3) to study the effect of two different antiseptics (peroxidase system producing hypothiocyanite and chlorhexidine) on the salivary biomass. In 45 young adults, the salivary ATP content ranged from 8 to 1515 nm. Salivary ATP content was significantly and directly correlated to bacterial count and epithelial cell numbers (Spearman–Rank correlation, $P \leq 0.001$). Regression analysis allowed the inference of a mean epithelial cell and bacterial ATP content of 152.7 fg and 8.3 fg per cell, respectively. The salivary ATP content decreased significantly to 38.8 ± 12.3 per cent (mean \pm SEM, $N = 6$) of its initial value after a 30-min incubation in the presence of a peroxidase system producing hypothiocyanite (OSCN⁻). Chlorhexidine (CHX) reduced salivary ATP content to 52.0 ± 16.7 per cent. OSCN⁻ did not affect the transformed logarithm of bacterial count but CHX reduced it from 7.02 ± 0.26 to 0.52 ± 0.33 . No effect of OSCN⁻ was seen on the ratio of epithelial cell viability while CHX reduced it from 46.7 ± 5.1 to 3.9 ± 1.1 per cent. It is concluded that the combination of the evaluations of the ATP content and cell numbers in saliva can provide reliable data about the effects of oral antiseptics on salivary biomass. Copyright © 2000 John Wiley & Sons, Ltd.

KEY WORDS — ATP; bioluminescence; chlorhexidine; peroxidases; saliva

INTRODUCTION

Adenosine triphosphate (ATP) measurement in cell extracts is useful in metabolic studies as an indicator of energy status,¹ or as an indicator of biomass in a biological sample.^{1,2} ATP measurement was indeed developed to estimate somatic or bacterial cell number in biological samples, to ensure microbiological quality in water and food, and to detect bacterial contamination in specific areas of medicine or the food industry.³ It can also be used as an indicator of intracellular regulation.⁴ The bioluminescent luciferin–luciferase method^{5,6} has largely been used in order to evaluate the ATP content in living cells from numerous biological media including dental plaque^{7,8} but not in saliva

where desquamated somatic cells and bacteria are both present.

The present work, therefore, was (1) to evaluate ATP content in saliva by the bioluminescent luciferin–luciferase method, (2) to evaluate the relationship between ATP content, bacterial count and epithelial cell numbers in saliva, (3) to study the effect of two different antiseptics on the salivary biomass: hypothiocyanite produced by a peroxidase system and chlorhexidine.

MATERIALS AND METHODS

Saliva Collection

Resting whole saliva, collected on ice at least 90 min after the last meal was used to study the salivary parameters in 45 adult volunteers aged 20 to 30 years (22 males, 23 females). The effects of the peroxidase system and chlorhexidine were studied

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in six other donors (three males and three females aged 25 to 45 years, non-smokers, free of any medication for a period of at least 1 month before sampling) who agreed to start the saliva collection 90 min after a standard breakfast (50 g bread, 30 g jam and 300 ml water) and 30 min after brushing teeth for 3 min with water only (no toothpaste).

Chemicals

ATP Bioluminescence Assay kit CLS II was obtained from Boehringer-Mannheim (Mannheim, Germany). Glucose oxidase (GOD) was obtained from Sigma Chemicals (St Louis, MO, USA). One unit of GOD produced enough H_2O_2 to oxidize 9.3 μ moles of ABTS (2,2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid) per min in the presence of lactoperoxidase used in this experimental procedure. Lactoperoxidase (Biopole, Belgium) was purified from bovine milk; the absorbance_{412nm}/absorbance_{280nm} ratio showed a purity index of 0.60; the specific activity was 600 ABTS units mg^{-1} of protein at 37°C. One ABTS unit corresponded to the amount of enzyme catalysing the oxidation of 1 mmole of the ABTS substrate per min.⁹ Chlorhexidine digluconate 0.2 per cent (w/v) (Corsodyl®) was purchased from SmithKline Beecham Consumer Brands SA (Genval, Belgium).

Saliva Incubations

Saliva aliquots (1.2 ml) were incubated for 30 min at 37°C in the presence of three separate solutions (0.8 ml) containing (a) glucose in buffer (0.1 M phosphate buffer pH 7) for the controls, (b) glucose, glucose oxidase, thiocyanate (SCN^-), and lactoperoxidase producing $OSCN^-$; and (c) chlorhexidine. The final concentrations were: glucose 44 mM, glucose oxidase 1.27 U ml^{-1} , SCN^- 1.2 mM, lactoperoxidase 48 ABTS units ml^{-1} , chlorhexidine 0.02 per cent (w/v). Under the experimental conditions, the lactoperoxidase system produced $OSCN^-$ which oxidized 277 ± 12 (mean \pm SEM, $N = 6$) nmoles ml^{-1} cysteine after 30 min.¹⁰

ATP Measurement

Samples of saliva (0.5 ml) were immediately frozen after incubation by immersing the assay tube in an acetone-dry ice bath. The samples were stored at $-18^\circ C$ until ATP extraction. Just before ATP measurement, the saliva was thawed in the presence

of 5 μ l 10 mM vanadate (ATPase inhibitor) and 5 μ l 0.01 per cent (w/v) benzalkonium chloride, then sonicated for 5 s (20 W). Salivary extract was diluted from 5- to 200-fold in imidazole-HCl buffer (100 mM, pH 7.75); 0.5 ml of the diluted extract was then mixed with 0.5 ml luciferin-luciferase reagent; ATP content was determined by the luciferin-luciferase bioluminescence method on an LKB Wallace 250 luminometer (LKB-Pharmacia, Uppsala, Sweden). The main characteristics of the luciferin-luciferase method are the following: linearity of ATP concentration between 1 and 9 nM; within-day precision for repeated assay ($N = 6$), coefficient of variation ≤ 5.7 per cent.

Bacterial Count

After incubation, saliva was dispersed and diluted in 155 mM NaCl from 10- to 10⁶-fold, and 50 μ l of each diluted salivary sample was transferred in triplicate to Columbia agar Petri dishes supplemented with 5 per cent (v/v) defibrinated sheep blood. The bacterial colonies were counted after a 24 h aerobic incubation at 37°C. Results were expressed as the number of Colony Forming Units (CFU) per ml of saliva.

Epithelial Cell Viability

After incubation, saliva samples were diluted in trypan blue (4 mg ml^{-1}). Living (excluding trypan blue) and dead (trapping trypan blue) epithelial cells were both immediately counted on a Thoma's cell.¹¹ The percentage of viable cells was calculated therefrom.

Filtration Procedures

Serial filtration (glass fibre filter followed by Minisart 5 μ m and 0.45 μ m) were performed at a constant flow of 1 $ml\ min^{-1}$ on eight different saliva collections. This procedure was chosen to minimize cell lysis.

Statistics

Mean values were always indicated with SEM. Statistical analysis was carried out using Prism® version 2.0 from GraphPad Software (San Diego, USA) for mean, standard deviation, Kolmogorov-Smirnov normality test, Spearman-Rank correlation coefficients, repeated measures ANOVA completed by a Dunnett's or by a Bonferroni's

Comparison Test. SPSS (Statistical Package for the Social Sciences) version 8.0 (Chicago, USA) was used for regression analysis.

RESULTS

Evaluation of the ATP Measurement in Saliva by the Luciferin-luciferase Method

A 20-fold dilution of saliva was shown to be sufficient to avoid luciferin-luciferase inhibition by phosphate, thiocyanate and oxidants present in the peroxidase system; serial dilution of six different saliva samples provided proportional ATP values (data not shown). The recovery of the internal standard was 97 ± 2 per cent ($N = 7$); storing saliva at -18°C ensured stability of ATP beyond 4 weeks. Figure 1 shows the protective effect of vanadate on salivary ATP by ATPase inhibition. Salivary ATP completely disappeared after addition of adenosine 5'-triphosphatase (EC 3.6.1.3) meaning that there was no other substrate in saliva to interfere with the ATP measurements. Careful serial filtration through glass fibre filter followed by Minisart $5 \mu\text{m}$ and $0.45 \mu\text{m}$ filters (stopping cells

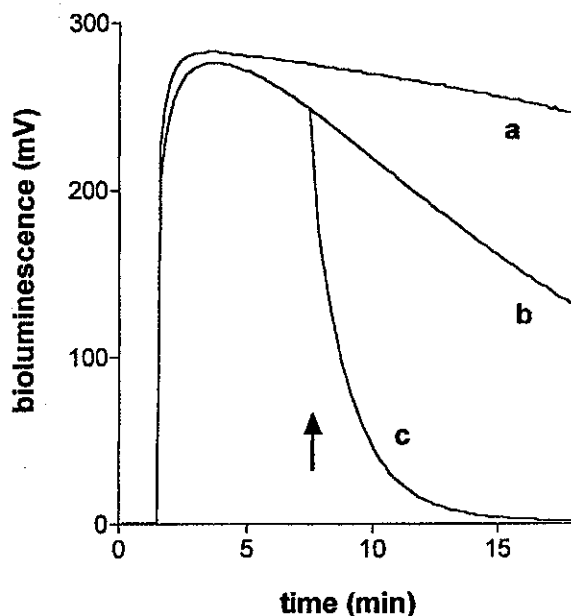


Figure 1. Luciferin-luciferase method for measuring ATP in saliva: bioluminescence curve (expressed as mV) related to the time (a) in the presence of 0.1 mM vanadate, (b) in the absence of vanadate and (c) in the absence of vanadate with addition (indicated by an arrow) of 0.1 U ATPase. The graph is representative of six independent experiments.

and bacteria) at a constant flow of 1 ml min^{-1} retained 85.8 ± 4.4 per cent ($N = 8$) of the ATP confirming that ATP in the total saliva is mostly cellular or bacterial.

Table 1. Range and median values of salivary parameters in 45 subjects aged from 20 to 30 years (22 males, 23 females). Only salivary secretion rate values presented a Gaussian distribution.

	Range	Median
Salivary secretion rate (ml h^{-1})	2.1–60.0	20.2
ATP (pmoles ml^{-1})	8–1515	97
Bacterial count ($\text{CFU} \times 10^6 \text{ ml}^{-1}$)	0.6–20.6	5.1
Epithelial cells ($\text{number} \times 10^3 \text{ ml}^{-1}$)	20–2500	170
Living cells ($\text{Number} \times 10^3 \text{ ml}^{-1}$)	0–1450	110

Usual Values in Saliva

Table 1 illustrates the range and median values of salivary parameters. In 45 young people, the salivary secretion rate ranged from 2.1 to 60.0 ml h^{-1} (median, 20.2 ; mean \pm SEM, $23.2 \pm 1.7 \text{ ml h}^{-1}$). The salivary ATP content ranged from 8 to $1515 \text{ pmoles ml}^{-1}$ saliva (median, $97 \text{ pmoles ml}^{-1}$), the bacterial count ranged from 0.6 to $20.6 \times 10^6 \text{ CFU ml}^{-1}$ (median, $5.1 \times 10^6 \text{ CFU ml}^{-1}$) and the salivary epithelial cell count ranged from 20 to $2500 \times 10^3 \text{ cells ml}^{-1}$ saliva (median, $170 \times 10^3 \text{ cells ml}^{-1}$). Using the trypan blue exclusion test (varying between 0 and 92 per cent) viability was determined by calculating the numbers of living and dead cells. Living cell numbers ranged from 0 to $1450 \times 10^3 \text{ cells ml}^{-1}$ (median, $110 \times 10^3 \text{ cells ml}^{-1}$). Only salivary secretion rate values presented a Gaussian distribution and succeeded in passing the Kolmogorov-Smirnov normality test.

ATP as a Marker of the Salivary Biomass

Table 2 indicates the Spearman-Rank correlation coefficients between the salivary parameters stud-

Table 2. Spearman-Rank correlation coefficients (r) between the salivary parameters studied in the control group ($N = 45$).

	r
Bacterial count versus ATP content	0.4437
Epithelial cells versus ATP content	0.5190
Living cells versus ATP content	0.5000
Salivary secretion rate versus ATP content	-0.2809

ied. Bacterial count, epithelial cell numbers and salivary ATP content were directly correlated. The strongest correlations were found between epithelial cells and ATP content for both total epithelial cells ($r = 0.5190$, $P < 0.001$) and live epithelial cells ($r = 0.5000$, $P < 0.001$). Salivary flow rate only weakly correlated with ATP content ($r = -0.2809$, $P = 0.0481$) and did not correlate with the bacterial count ($r = -0.1077$, $P = 0.4568$). Regression analysis inferred $z = ax + by + C$ as a mathematical model describing the relationship between ATP and cells in saliva, where z is the ATP content (pmoles ml^{-1}), x the number of cells ml^{-1} and y the number of CFU ml^{-1} , with $r = 0.7080$, $a = 0.185 \pm 0.007$ pmoles per 10^3 epithelial cells, $b = 16.3 \pm 0.7$ pmoles per 10^6 CFU and $C = -9.323 \pm 5.8$. Taking into account the living cells only, the mathematical parameters became $r = 0.7000$, $a = 0.301 \pm 0.013$ pmoles per 10^3 epithelial cells, $b = 16.3 \pm 0.7$ pmoles per 10^6 CFU and $C = -8.1 \pm 5.9$. The inferred mean ATP content per epithelial cell and per bacterium were 0.301 and 0.016 fmoles corresponding to 152.7 and 8.3 fg respectively.

Effect of Peroxidase System and Chlorhexidine on Salivary Biomass

The effects of peroxidase systems and chlorhexidine on salivary biomass are described in Figure 2. Salivary ATP content significantly decreased after 30 min incubation in the presence of peroxidase system or chlorhexidine. After OSCN⁻ incubation, ATP fell to 38.8 ± 12.3 per cent of its initial value (repeated measures ANOVA completed by a Dunnett's comparison post-test, $P < 0.01$). Chlorhexidine reduced salivary ATP content to 52.0 ± 16.7 per cent of its initial value ($P < 0.01$). OSCN⁻ did not affect the bacterial count in saliva. On the other hand, chlorhexidine reduced it drastically from 10.5×10^6 to less than 10 CFU ml^{-1} ($P < 0.01$). No effect of OSCN⁻ was shown on epithelial cell viability, although chlorhexidine reduced it from 46.7 ± 5.1 to 3.9 ± 1.1 per cent ($P < 0.01$). Serial filtration of salivary samples resulted in less than 30 per cent of the residual ATP after OSCN⁻ incubation in the soluble fraction but more than 80 per cent after CHX incubation (Figure 3): the difference is significant (ANOVA completed by a Bonferroni's Multiple Comparison Test, $P < 0.001$).

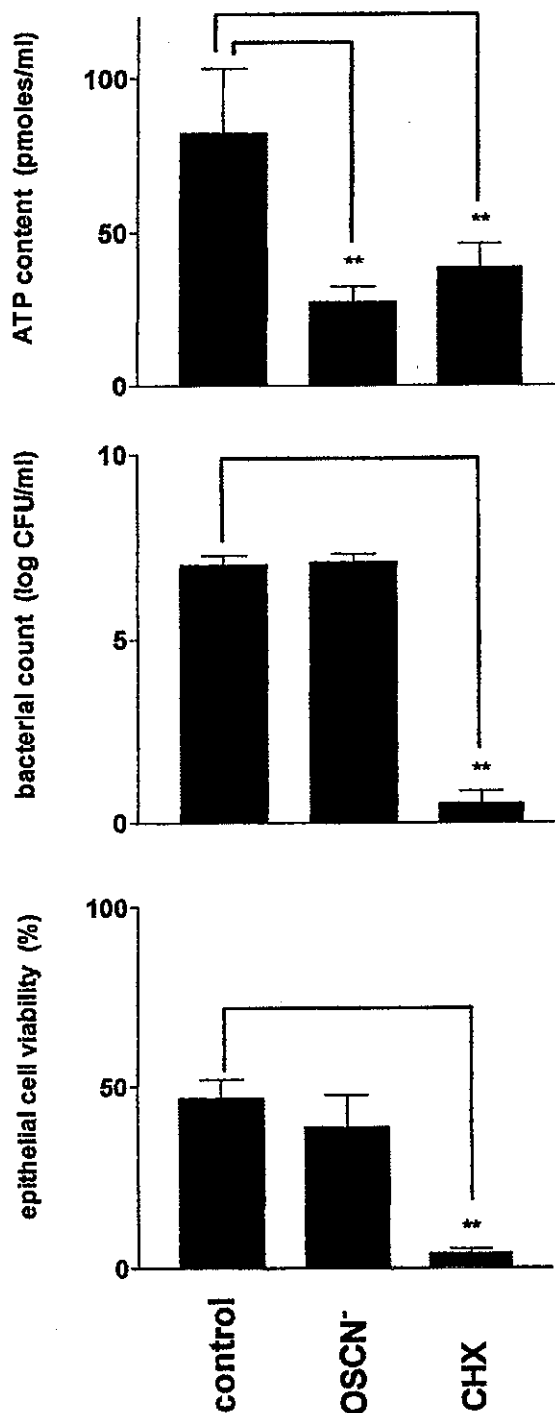


Figure 2. Effect of peroxidase systems and chlorhexidine on ATP content, bacterial count and epithelial cell viability. The saliva ($N = 6$) was incubated at 37°C for 30 min in the presence of an OSCN⁻ generating system or chlorhexidine.

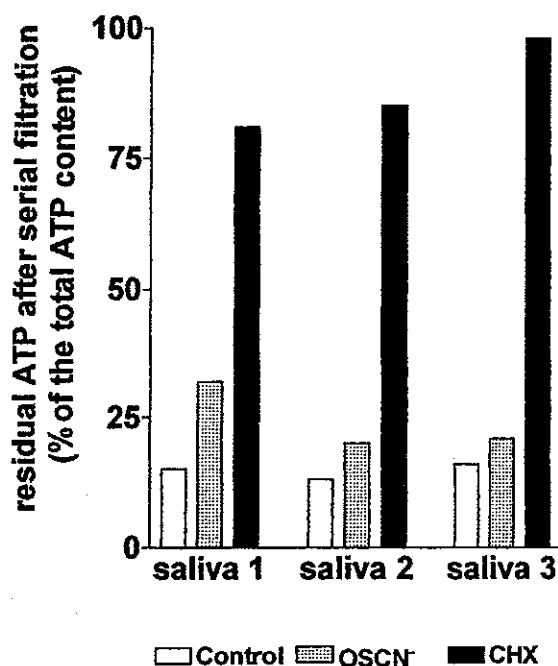


Figure 3. Residual ATP content after serial filtration (glass fibre filter followed by Minisart 5 μm and 0.45 μm) in three different salivary samples each treated with buffer (control), hypochlorite (OSCN⁻) and chlorhexidine (CHX) for 30 min.

DISCUSSION

Salivary ATP Measured by the Luciferin-luciferase Method

ATP measurement by the luciferin-luciferase method has been known for a long time but has not yet been used in saliva. Linearity of serial salivary sample dilutions and the recovery (97 ± 2 per cent) of an internal ATP standard in saliva showed the reliability of the method. The absence of any bioluminescence after ATPase incubation in saliva demonstrated that no other substance interfered with the luciferin-luciferase method and only salivary ATP content was being determined. Moreover, careful serial filtration of six salivary samples at a constant flow of 1 ml min^{-1} (glass fibre filter followed by Minisart 5 μm and 0.45 μm) showed that 85.8 ± 4.4 per cent of the ATP was in the saliva fraction; the 14.2 per cent that passed through the serial filters corresponded to free ATP outside cells or bacteria. It could be argued that this free ATP is either released from dead cells and bacteria

before filtration or that it is the result of an artefact produced by the filtration process itself.

Salivary ATP Related to (Bacterial and Somatic) Cell Counts

The relationship between the ATP content and the number of cells (epithelial cells and bacteria) present in the saliva confirmed that the origin of ATP was cellular. Indeed, analysis of the Spearman-Rank correlations effectively showed a statistical dependency of saliva ATP content on the bacterial count or the epithelial cell number. Regression analysis inferred a mean epithelial cell and bacteria ATP content of 152.7 fg and 8.3 fg per cell respectively, compatible with literature values ranging up to 10,000 fg per cell and from 0.28 to 89 fg per microorganism depending on species.^{2,3,12-14}

Use of Salivary ATP with Cell Counts to Test the Action of Oral Antiseptics

The three parameters (ATP content, bacterial count and epithelial cell viability) of the salivary biomass were used to gather information on the effect of two oral antiseptic compounds: OSCN⁻ produced by a lactoperoxidase system compared to chlorhexidine. The peroxidase system was shown to be active *in vitro* on some oral bacterial strains¹⁵ but failed to affect salivary bacterial count.^{16,17} Chlorhexidine has been largely used until now in oral antiseptics.¹⁸ Lactoperoxidase-generated OSCN⁻ only decreased salivary ATP but did not influence bacterial count. Chlorhexidine showed a strong bactericidal effect characterized by a bacterial count near zero with a decreased ATP content. These results indicate that the lactoperoxidase system has a bacteriostatic effect rather than bactericidal activity on salivary bacteria. Indeed, the bacteriostatic effect suggested by the decrease in ATP without variation of the bacterial count could be reversed after plating the incubated saliva on culture medium.

Nevertheless, a decrease in ATP is not necessarily due to an effect of antiseptic compounds on bacteria alone: ATP loss could indeed originate from epithelial cell damage. While chlorhexidine dramatically decreased epithelial cell viability, OSCN⁻ did not influence the trypan blue exclusion test.

The outcome of the serial filtration may provide relevant information concerning the effect of each

antiseptic used in this study. Indeed, the increase of free salivary ATP after chlorhexidine exposure suggests lysis of the cells by the agent tested in the experimental conditions.

In conclusion, the coupling of ATP content measurement and (bacterial and somatic) cell counts in saliva can provide a clear picture of the salivary changes after exposure to oral antiseptics.

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