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ANÁLISE DOS NÍVEIS DE ENDOTELINA-1 NA SALIVA DE PACIENTES PORTADORES DE CARCINOMA ESPINOCELULAR BUCAL

Porto Alegre
2010

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Tese apresentada à
Faculdade de Odontologia da Pontifícia Universidade
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Orientadora: Professora Doutora Maria Martha Campos

Co-orientadora: Professora Doutora Liliane Soares Yurgel

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Tese apresentada como parte dos requisitos para obtenção do Título de Doutor pelo Programa de Pós-Graduação da Faculdade de Odontologia da Pontifícia Universidade Católica do Rio Grande do Sul.

Aprovada em _____ de _____ de 2010.

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Doutor Pedro António Gonzalez Hernández

Dedico esta Tese à minha família,

que com seu amor insuperável,

alicerçou minha chegada até aqui.

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"Toda tarefa, por mais nobre que seja, está destinada a enfrentar problemas e obstáculos. É importante avaliar por completo a finalidade a que nos propomos e quais são os fatores que determinam a nossa conduta. É importante que a pessoa seja verdadeira, honesta e sensata. Suas ações devem ser tão boas para os outros quanto para si própria."

Dalai Lama

RESUMO

Objetivos: Verificar se a Endotelina-1 (ET-1) salivar pode representar um biomarcador eficiente para avaliação de risco e prognóstico do carcinoma espinocelular bucal.

Metodologia: Neste estudo, foi realizada coleta de saliva de 20 pacientes saudáveis, 15 pacientes portadores de leucoplasia oral (diagnóstico histopatológico de hiperceratose a displasia severa), 14 pacientes que já tiveram carcinoma espinocelular bucal (CEB) e estavam em um período pós-tratamento e livres de doença por, no mínimo cinco anos e, 20 pacientes portadores de CEB. A ET-1 foi quantificada na saliva através do método ELISA.

Resultados: Não houve diferença estatisticamente significativa na expressão de ET-1 entre os grupos estudados, mesmo quando pacientes com idade superior a 65 anos ou hipertensos foram excluídos da análise. Da mesma forma, quando a amostra foi separada por sexo, também não houve diferença significativa.

Conclusões: A ET-1 salivar não se mostrou um biomarcador eficiente do carcinoma espinocelular bucal.

DESCRITORES[1]: Endotelina-1, câncer bucal, carcinoma espinocelular, leucoplasia, peptídeos, saliva.

[1] DeCS – Descritores em Ciências da Saúde, disponível em http://decs.bvs.br

ABSTRACT

SALIVARY ANALYSIS OF ENDOTHELIN-1 IN ORAL SQUAMOUS CELL CARCINOMA

Objectives: The present study aimed to verify if the salivary Endothelin-1 (ET-1) expression might represent an efficient biomarker to evaluate risk and prognosis from oral squamous cell carcinoma (OSCC).

Methods: For the present study, saliva obtained from 20 healthy patients, 15 patients with oral leukoplakia (histopathologic diagnosis of hyperceratosis or severe dysplasia), 14 patients that had OSCC and were free of disease for more than 5 years, and from 20 patients with OSCC were collected. The ET-1 levels were quantified by means of ELISA.

Results: No statistical differences were observed in the salivary ET-1 levels among the studied groups, even when patients with age superior to 65 years or with arterial hypertension were removed from the sample analysis. Likewise, when the sample was divided by sex, no statistical significance was observed among the four groups included in the study.

Conclusions: Taking into account the present results, salivary ET-1 expression was not found to represent an efficient biomarker of either OSCC or oral leukoplakia.

DESCRIPTORS[2]: Endothelin-1, oral cancer, squamous cell carcinoma, oral leukoplakia, peptides, saliva.

[2] MeSH – Medical Subject Headings, available at: www.nlm.nih.gov/mesh

LISTA DE ILUSTRAÇÕES

ARTIGO DE REVISÃO

ARTIGO EXPERIMENTAL

LISTA DE ABREVIATURAS

CEB	Carcinoma espinocelular bucal
ELISA	Enzyme Linked Immuno Sorbent Assay
ET-1	Endothelin-1 / Endotelina-1
ET-2	Endothelin-2
ET-3	Endothelin-3
ET_AR	Endothelin Receptor A
ET_BR	Endothelin Receptor B
ECE	Endothelin-converting enzyme
ETs	Endothelins
OSCC	Oral squamous cell carcinoma
RT-PCR	Reverse Transcription-Polymerase Chain Reaction

SUMÁRIO

Introdução

1 INTRODUÇÃO

O carcinoma espinocelular bucal (CEB) é um dos tipos mais frequentes de câncer atualmente. Em sua patogênese, estão envolvidos fatores exógenos como, por exemplo, o abuso de álcool e de fumo, além de alterações genéticas. Essas alterações conduzem a uma desregulação protéica, afetando os ciclos de divisão e diferenciação celular, o reconhecimento imunológico, a invasão tecidual e as metástases[1], estando essas também refletidas em graus variáveis nas lesões cancerizáveis[2].

O termo leucoplasia pode ser usado para designar placas brancas de risco questionável para o câncer, excluindo outras lesões brancas que reconhecidamente não apresentam tal risco[3]. As alterações histopatológicas correspondentes à leucoplasia podem variar de hiperceratose e acantose a vários graus de displasia. Além disso, as lesões leucoplásicas podem evoluir para CEB, especialmente aquelas com alterações displásicas[4].

Mutações genéticas ocorridas durante a carcinogênese e invasão do CEB podem ser observadas por meio de marcadores celulares ou mudanças moleculares que são associadas a estágios específicos da doença. A expressão e o padrão do biomarcador podem estar relacionados com o risco de transformação carcinogênica em lesões benignas e com a progressão da doença nos tumores[5].

A detecção precoce do câncer é a alternativa mais efetiva na redução da morbidade e da mortalidade causada por essa doença. Assim, é necessário que novas ferramentas sejam desenvolvidas para aprimorar o diagnóstico precoce. Tem sido sugerido que a identificação de biomarcadores em fluidos biológicos para predizer o desenvolvimento do câncer em estágios iniciais ou em lesões cancerizáveis tem uma aplicabilidade clínica significativa[6].

Em 1985, Hickey et al.[7] detectaram um fator causador de contrações na musculatura lisa, produzido pelo endotélio. Três anos mais tarde, essa substância foi isolada por Yanagisawa et al.[8] (1988) em uma cultura de células endoteliais de artérias de porcos, sendo então denominada endotelina-1 (ET-1), em alusão à sua origem celular.

A ET-1 é produzida principalmente pelas células endoteliais, sendo encontrada em vários fluidos corporais, tais como saliva, leite, urina, fluido cerebroespinhal e plasma[9]. Esse biomarcador tem sua expressão aumentada em um grande número de neoplasias, como

carcinoma de próstata, de pulmão, de mama, hepatocelular, coloretal, assim como no carcinoma de boca. Também afeta múltiplos sistemas biológicos e está envolvida na patogênese de outras doenças, como hipertensão arterial, hipertrofia vascular e do miocárdio[10], doença renal[11], doenças do trato gastrointestinal alto[12], dentre outras.

Pickering et al.[13] (2007) analisaram os níveis de endotelina-1 na saliva de 8 pacientes com diagnóstico de CEB, previamente ao tratamento, por meio do método ELISA e de 8 pacientes saudáveis. Também avaliaram 8 fragmentos de biópsia de CEB e 3 amostras de epitélio normal por ELISA, 10 fragmentos de CEB por RT-PCR e um fragmento por imunoistoquímica com anticorpo primário monoclonal de rato para ET-1. Comparando os resultados de todos os grupos estudados, houve um aumento significante dos níveis de ET-1 nos pacientes com câncer. Embora os autores esperassem um aumento dos níveis salivares de ET-1 resultante da sua secreção pela porção exposta do tumor na cavidade bucal, não foi encontrada correlação entre o tamanho da superfície do tumor e a concentração salivar de ET-1.

A análise salivar é um método promissor para o diagnóstico de muitas doenças, visto que a saliva é um fluido facilmente disponível para coleta. A identificação e a análise de um biomarcador salivar poderiam trazer benefícios para o diagnóstico e controle de pacientes com CEB, com história prévia dessa doença ou, ainda, para pacientes com lesões cancerizáveis e/ou expostos a fatores de risco para o câncer bucal.

O presente estudo compreende dois trabalhos apresentados sob a forma de artigos científicos. O primeiro artigo representa uma revisão da literatura sobre a relação entre a ET-1 e o câncer, com ênfase no CEB. O segundo artigo descreve a parte experimental do estudo, que teve como objetivo verificar os níveis de de ET-1 na saliva de pacientes portadores de CEB antes do tratamento anti-neoplásico, em pacientes que já tiveram CEB e estavam em um período pós-tratamento e livres de doença por, no mínimo 5 anos, em pacientes portadores de leucoplasia bucal com diagnóstico histopatológico de hiperceratose à displasia leve à severa e, comparar os valores encontrados para ET-1 nesses diferentes grupos de pacientes com os valores encontrados para essa substância nos pacientes saudáveis e não expostos a fatores de risco para o câncer bucal aqui considerados (álcool e tabaco).

Objetivos

2 OBJETIVOS

Objetivo geral

Verificar se a ET-1 salivar pode representar um biomarcador eficiente para avaliação de risco e prognóstico do carcionoma espinocelular bucal.

Objetivos específicos

a. Verificar os níveis de ET-1 na saliva de pacientes portadores de CEB antes do tratamento anti-neoplásico.

b. Verificar os níveis de da ET-1 em pacientes que já tiveram CEB e estavam em um período pós-tratamento e livres de doença por, no mínimo 5 anos.

c. Verificar os níveis de da ET-1 na saliva de pacientes portadores de leucoplasia com diagnóstico histopatológico de hiperceratose a displasia severa.

d. Comparar os valores encontrados para ET-1 salivar nos pacientes portadores de CEB com os valores encontrados para essa substância nos pacientes saudáveis e não expostos aos fatores de risco para o câncer bucal (aqui considerados álcool e tabaco), bem como com os outros grupos incluídos no estudo.

Artigo de Revisão

3 ARTIGO DE REVISÃO

O artigo a seguir intitula-se **“Endothelins and their receptors as biological markers for oral cancer”** e foi formatado de acordo com as normas do periódico *Oral Oncology* (Fator de impacto 2,928, Qualis A1 internacional).

Title:

Endothelins and their receptors as biological markers for oral cancer

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Abstract:

Oral squamous cell carcinoma (OSCC) is one of the most common malignancies recognized nowadays, and represents a public health problem. Its early detection is the best alternative to provide a good quality of life for the patients. During the last years, several studies have identified potential biomarkers of OSCC progression and prognosis. Endothelins and their receptors are involved in several pathophysiological conditions and in various cancer types. The present review article discusses whether the endothelin system would represent a biomarker for OSCC.

Keywords: oral cancer, endothelin-1, peptides, prognostic factor.

Abbreviations: OSCC, Oral squamous cell carcinoma; ET-1, Endothelin-1; ET-2, Endothelin-2; ET-3, Endothelin-3; ETs, Endothelins; ECE, Endothelin-converting enzyme; ET_AR, Endothelin Receptor A; ET_BR, Endothelin Receptor B.

Oral Squamous Cell Carcinoma

Oral squamous cell carcinoma (OSCC) represents a serious public health problem, and its early detection and prevention remains the most effective solution to avoid patient mutilation or death. The treatment comprises surgery, in combination to radiotherapy, and less frequently chemotherapy.

Malignant changes in oral mucosa are likely caused by mutations of genes implicated in cellular growth regulation, leading to increased cell proliferation, abnormal keratinization, epithelial dysplasia, increased cell motility and angiogenesis[1,2]. Oral cancer is linked to genetic and epigenetic alterations that lead to protein deregulation, compromising cell division, differentiation, immune recognition, tissue invasion and metastasis. During the last years, several studies have identified potential biomarkers of OSCC progression and prognosis[2, 3, 4, 5]. However, additional studies on this regard are still needed.

Endothelins

In 1985, Hickey et al.[6] detected an endothelium-derived factor with contractile effects on the smooth muscle. In 1988, this substance was isolated by Yanagisawa et al.[7] from cultured pig arterial endothelial cells and was called endothelin-1 (ET-1). This is a peptide composed by 21 amino acids, 2 disulphide bonds, between amino acids 1 and 15, and 3 and 11. Endothelin is generated by cleavage of the pre-pro-endothelin precursor, by means of specific endopeptidases, resulting in big endothelin (with 39 amino acids). Big endothelin is then converted to endothelin by of the action of an endothelin-converting enzyme (ECE)[7].

Endothelins comprise a family of three small peptides: ET-1, ET-2 (with similar structure of ET-1) and ET-3[7, 8]. ET-1 is expressed primarily by endothelial cells, ET-2 in kidney and intestine, whereas ET-3 is found mainly in the brain[8]. ET-1 is the principal isoform in the cardiovascular system, and remains the most potent and long-lasting constrictor of human vessels discovered until now[9, 10]. ET-1 is mainly produced by endothelial cells, vascular smooth muscle cells, and to a lesser extent by astrocytes and neurons, Sertoli cells, mesangial cells, and hepatocytes[11].

Endothelins exert their effects by binding to cell-surface receptors, namely ET-A (ET_AR) and ET-B (ET_BR). Both receptors belong to the G-protein-coupled receptor super-family[8, 10, 12, 13]. ET-A receptor binds ET-1 with 10-times greater affinity than ET-3, whereas ET-B

receptor binds all three endothelins with similar affinity. Most ET-1 effects are mediated via interaction with ET-A receptor[14].

ET-peptides are mainly involved in regulation of the vascular system, but also in the genesis of cardiovascular diseases, such as hypertension, cardiac hypertrophy, and remodeling in the case of heart failure[11, 15, 16]. Besides, they play a seminal role in the atherogenic process [16, 17], in pulmonary hypertension, and acute or chronic renal failure [15, 16, 18]. Interestingly, ET-1 stimulates nociceptors and sensitizes them to painful stimuli[19,20].

Not many studies have evaluated relevance of endothelins in the oral cavity milieu. Ansai et al. (2002)[21] analyzed the expression of ET-1 in gingival epithelial cells from adult patients with periodontitis. They have identified an increase of ET-1 expression in samples obtained from infected individuals. Similar results were observed by Yamamoto et al. (2003)[22] in inflamed gingival tissues obtained from patients with periodontal disease. Therefore, endothelins and their receptors are likely implicated in several relevant diseases, including those of the oral cavity, representing possible rational targets for developing new therapeutic alternatives.

Endothelin-1 and Cancer

All components of the endothelin system appear to contribute to tumor growth and progression in a number of cancer types, such as prostatic, ovarian, renal, pulmonary, colorectal, cervical, breast, bladder, endometrial carcinoma, brain tumor, melanoma and OSCC [13, 23, 24,]. Some relevant literature evidence on this matter is presented below.

Expression of endothelins and their receptors is associated with high grade, aggressive breast tumors, as well invasion and metastasis. There are increased systemic levels of endothelins in patients with lymph node metastases. The mechanisms by which endothelins induce an invasive phenotype include the interaction between the tumor cells, the infiltrating macrophages and the breast tumor microenvironment. This complex interaction leads to modulation of matrix metalloproteinase (MMP) activity, cytokine expression, immune infiltrate activation, apoptosis and expression of endothelins themselves[25, 26]. Alanen et al. (2000)[27] found a significant elevation of ET-1 immunopositivity in breast carcinomas compared to normal tissues. Guise et al. (2003)[14] identified three breast cancer lines that cause osteoblastic metastases in female nude mice, and provided evidence that tumor-produced ET-1 mediates the osteoblastic response. They observed that ET-1stimulates osteoblast

proliferation and new bone formation, and these effects were blocked by selective ET_AR, but not ET_BR antagonists.

Ishibashi et al. (1993)[28] compared plasma ET-1 and big ET-1 levels between 30 patients with hepatocellular carcinoma, and 29 patients with cirrhosis, using 34 healthy patients as control. The ET-1 and big ET-1 concentrations were significantly higher in patients with hepatocellular carcinoma, than in patients from the control group. From 29 patients with cirrhosis, 4 presented a moderate elevation of big ET-1, in relation to the control group[28].

Noteworthy, Lam et al. (2004)[29] analyzed the presence of salivary immunoreactive ET-1 in patients with upper gastrointestinal diseases, compared with patients with a normal esophagogastroduodenoscopy. The salivary concentrations of ET-1 were significantly higher in patients with gastric ulcers, duodenal ulcers and gastritis, than in patients with normal exams. Significant differences in ET-1 levels were not found between patients with normal esophagogastroduodenoscopy and patients with esophagitis or gastric cancer, as between men and women or among different ages.

More recently, Jiao et al. (2007)[30] investigated the effects of ET-1 in the invasion of esophageal cancer, and evaluated cathepsin B activity and expression: ET-1 may enhance the invasive ability of human esophageal cancer cells, and this is correlated with cathepsin B activity, a kind of cysteine protease that participates in the proteolytic degradation of the cellular matrix. The same authors demonstrated a significant elevation of ET-1 production, as well as an association between plasma big ET-1 levels and disease stage, invasion depth, lymph node status, recurrence, and survival rate[31].

ET-1 and ET receptors are also likely involved in lung cancer[32]. Zhang et al. (2008)[33] studied the implication of ET-1 on cell proliferation in human lung adenocarcinoma. They proposed that ET-1 acts as an autocrine growth factor, enhancing cell proliferation via ET_AR activation.

To test the hypothesis that ET-1 is important to lung metastasis, Titus et al. (2005)[34] injected lung metastatic bladder carcinoma cells in mice treated with a selective ET_AR antagonist, Atrasentan. ET_AR antagonism resulted in a reduction of lung metastases, suggesting that this might represent a new therapeutic target to prevent lung metastases.

ET-1 appears also to be involved in prostatic cancer. Cella et al. (2006)[35] performed a meta-analysis about the influence of Atrasentan, a selective ET_AR antagonist, in the hormone-refractory prostate cancer. Data revealed beneficial effects in the life quality of metastatic hormone-refractory prostate cancer patients, under treatment with Atrasentan.

Lahav (2005)[36] studied the relation between ET_BR and melanoma: this study proposed that ET_BR is a good biomarker of melanoma progression, and its specific antagonist BQ788 is able to inhibit the growth of human melanoma cell lines. Lan et al. (2005)[37] demonstrated that elevated ET-1 levels are related to hyperpigmentation in basal cell carcinoma. Finally, Berger et al. (2006)[38] investigated the role of ET-1 axis in the proliferation and/or apoptosis of human melanoma cells lines and the action of Bosentan, a dual $ET_{A/B}$-receptor antagonist. ET-1 is not a growth factor for human melanoma cells, but ET receptor blockade decreases cell proliferation, induces apoptosis, and potentiates the effects of classical anticancer agents.

It has been proposed that ET-1 plays a critical role in ovarian carcinoma progression[23, 39, 40, 41, 42]. For instance, ET-1 and ET_AR are both overexpressed in primary and metastatic ovarian carcinoma, and high levels of ET-1 are detectable in patient ascites, suggesting that ET-1 might promote tumor dissemination. Moreover, ET-1 enhances the secretion of MMPs, disrupts intercellular communications, and stimulates cell migration and invasion in ovarian cancer cells. Besides, ET-1 has the ability to promote epithelial to mesenchimal transition, a metastasis process involving sustained loss of epithelial markers and gain of mesenchimal characteristics[25, 43].

Endothelin-1 and Oral Cancer

There is convincing data indicating the involvement of endothelin system in OSCC. Awano et al. (2006)[44] investigated the expression and distribution of ET-1, its receptors (ET_AR and ET_BR), and the isoforms of its specific converting enzyme (ECE-1a, 1b, 1c) in OSCC. They concluded that ET-1, ET_AR and ET_BR and ECE-1 isoforms are overexpressed in OSCC, and that ET-1 acts as a survival factor to induce proliferation via ET_AR and ET_BR. Furthermore, inhibiting ECE-1 activity either by siRNA or an ECE-specific inhibitor effectively reduced the proliferation of OSCC *in vitro*.

Pickering et al. (2007)[45] analyzed the salivary ET-1 levels in 8 patients with OSCC prior to the treatment, in comparison to 8 healthy patients. The statistical comparison among the studied groups, revealed a significant increase in ET-1 levels in saliva of patients with cancer. An increase in salivary ET-1 levels in oral cancer patients could be expected as a result of secretion by the portion of the carcinoma exposed to the oral cavity. In spite of that, no correlation was found between the tumor surface size and the salivary ET-1 concentration.

As discussed before, ET-1 plays a critical role in a number of painful conditions, as it directly sensitizes nociceptors. Moreover, the administration of ET receptor antagonists might

be effective for pain relief[46, 47]. Pickering et al. (2008)[48] described the effects of ET-1 in pain alterations induced by OSCC and oral melanoma in tumor mouse models. They concluded that elevation of ET-1 concentrations induced by the tumor is a determinant factor for pain magnitude.

Schmidt et al. (2007)[49] demonstrated a significant elevation in the levels of ET-1 in HSC3 cells, a lineage obtained from human OSCC . The intra-tumoral administration of the selective ET_AR antagonist BQ-123 had an analgesic effect similar to that observed with morphine in a mouse model of OSCC. More recently, Quang et al. (2010)[50] investigated whether ET_AR antagonism affects endogenous opioid secretion in mouse OSCC comparing with normal tissue. They used RT-PCR to quantify the ET-1 and ET_AR levels: ET-1 levels in OSCC cells were two-fold increased in comparison to normal oral keratinocytes, whereas the ET_BR levels were significantly lower in cancer. The treatment with ET_AR antagonist BQ-123 induced a greater production of β-endorphin and leu-enkephalin, indicating that analgesic effects of this antagonist are related to the stimulation of endogenous opioids. On the other hand, the same authors[51] demonstrated that ET_BR agonist BQ-3020 induced the secretion of β-endorphin in OSCC cell culture and its intra-tumor injection presented an antinociceptive effect, while treatment with the ET_BR antagonist BQ-788 had no effect. They have also found an elevated expression of ET-1 in OSCC, in comparison to normal oral keratinocytes.

Endothelins as possible targets for cancer treatment

For the endothelin receptors, a series of antagonists with either selectivity for ET_AR or ET_BR, or mixed antagonists are available, and they might well represent promising alternatives for cancer treatment[10]. Strikingly, in clinical studies, ET_AR antagonists can be administered orally and have a favorable tolerability profile[25].

ET_AR activation by ET-1 largely contributes to tumor growth and progression, inducing cell proliferation, survival, angiogenesis and metastatic spread, indicating that ET_AR antagonism might improve cancer treatment[43, 52]. ET-1, by acting via ET_BR, modulates different stages of neovascularization. ET-1 can also modulate tumor angiogenesis indirectly, through induction of vascular endothelial growth factor (VEGF). Activation of ET_AR by ET-1 induces VEGF production by increasing the levels of hypoxia-inducible-factor 1α. Moreover, tumor cells themselves, predominantly those expressing the ET_AR, might form vessel-like channels[53]. Endothelin receptor antagonists can potentially reduce angiogenesis by inhibiting endothelial cell mitogenesis, whereas they might also prevent the production of MMPs from

macrophages, indicating an anti-apoptotic effect for endothelins[54]. Blockade of ET-1 receptors can sensitize human tumor cells to apoptosis[38]. Therefore, it is tempting to infer that blocking ET receptors, especially ET_AR, might be a useful alternative as an adjuvant treatment of OSCC. A scheme regarding the involvement of endothelins and their receptors in OSCC is provided in Figure 1.

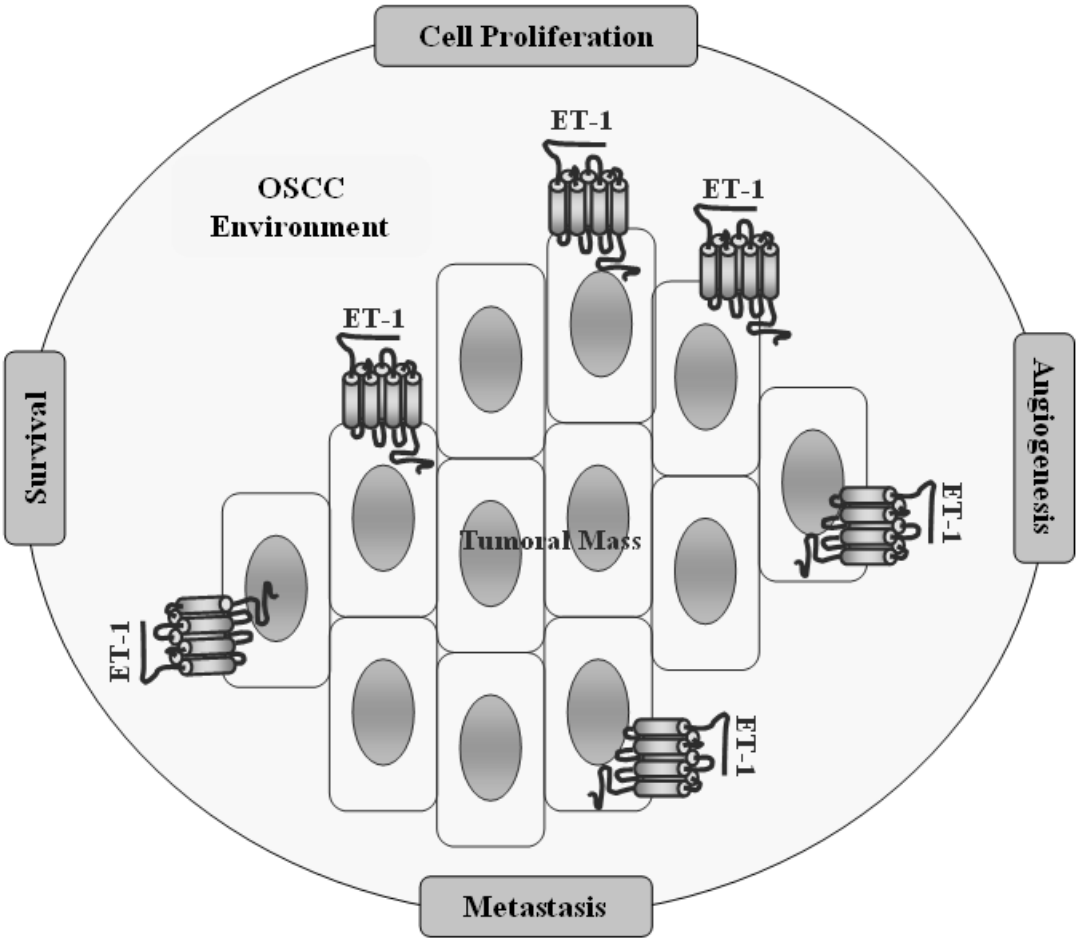

Figure 1. Scheme indicating the possible implication of ET-1 and their receptors in OSCC.

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Artigo Experimental

4 ARTIGO EXPERIMENTAL

O artigo a seguir intitula-se **“Evaluation of Salivary Endothelin-1 Levels in Oral Squamous Cell Carcinoma and Oral Leukoplakia”** e foi formatado de acordo com as normas do periódico *Regulatory Peptides* (Fator de impacto 2,276, Qualis A2 internacional).

Title: Evaluation of Salivary Endothelin-1 Levels in Oral Squamous Cell Carcinoma and Oral Leukoplakia

Abstract

Oral squamous cell carcinoma (OSCC) is the most frequent malignant neoplasia of the oral cavity, which largely compromises the patient's life quality. Therefore, the identification of biomarkers for this kind of cancer is essential to provide a better diagnosis and prognosis for patients. Endothelin-1 is a peptide produced mainly by endothelial cells, and might be found in several body fluids, such as saliva, milk, urine, cerebrospinal fluid and plasma. It has been demonstrated that expression of this peptide is increased in a great number of neoplasias, including oral carcinoma. The identification of salivary biomarkers would be a useful tool for scanning and monitoring patients with risk of developing OSCC, as well to early detect recurrence, or the formation of a new primary tumor. In the present study, we have analyzed the levels of endothelin-1 in saliva obtained from patients with OSCC or oral leukoplakia, in comparison to healthy control patients. This study also evaluated the salivary ET-1 levels in patients with complete remission of OSCC. The results revealed no statistical difference in salivary endothelin-1 levels, neither in OSCC nor in oral leukoplakia, even when conditions such as elderly, sex and hypertension were taken into consideration. Although, ET-1 might display an important role in OSCC, its levels in saliva do not seem to be a good marker of neoplasias grade or malignant transformation.

Keywords: Oral cancer, oral squamous cell carcinoma, leukoplakia, endothelin, saliva.

Abbreviations: OSCC, Oral squamous cell carcinoma; ET-1, Endothelin-1; ET-2, Endothelin-2; ET-3, Endothelin-3; ETs, Endothelins; ECE, Endothelin-converting enzyme; ET_AR, Endothelin Receptor A; ET_BR, Endothelin Receptor B.

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Introduction

Oral squamous cell carcinoma (OSCC) represents a great public health problem and its early detection and prevention are the most effective solution to decrease morbidity associated with treatment and to improve overall long-term survival. Due to the absence or unclear early signs or symptoms for most of head and neck cancers, sensitive and specific biomarkers would be useful for screening high-risk patients[1, 2].

The term leukoplakia is currently used to define white plaques of questionable risk, after excluding other known diseases or disorders that carry no increased risk for cancer[3]. The histopathological alterations corresponding to leukoplakia could vary from hyperkeratosis and acanthosis to many degrees of dysplasia and even carcinoma. Furthermore, leukoplakia lesions might evolve to squamous cell carcinoma, especially those associated to dysplasia[4].

Malignant changes in oral mucosa are likely caused by mutations of genes implicated in cellular growth regulation, leading to increased cell proliferation, abnormal keratinization and epithelial dysplasia. There is also an increase of cell motility and angiogenesis[5]. The transformation suffered by the oral mucosa in the premalignant lesions and OSCC has been correlated to the chronic exposure to agents such as tobacco, betel nut and alcohol[6]. During the last years, several studies have identified potential biomarkers of OSCC progression and prognosis[5]; however, additional studies on this regard are still needed.

Endothelins comprise a family of three small peptides: ET-1, ET-2 and ET-3[7, 8]. Endothelins exert their effects by binding to cell-surface receptors, namely ET-A and ET-B. Both receptors belong to the G-protein-coupled receptor super-family, with seven transmembrane-spanning domains[8, 9, 10, 11, 12]. Noteworthy, most ET-1 effects are mediated via interaction with ET-A receptor[13].

The ET axis displays a series of important physiological roles, including the modulation of vasomotor tone, tissue differentiation and development, cell proliferation and hormone production[14]. It has been well demonstrated that ET-1 promotes growth and progression in a variety of tumors, comprising OSCC[11, 15, 16, 17]. Of high relevance, Awano et al. (2006)[18] investigated the participation of ET-1 and its receptors (ET_AR and ET_BR), and the isoforms of its specific converting enzyme (ECE-1a, 1b, 1c) in cell OSCC proliferation. They concluded that ET-1, ET_AR and ET_BR and ECE-1 isoforms are overexpressed in OSCC, and that ET-1 acts as a survival factor to induce proliferation via ET_AR and ET_BR. Furthermore, inhibiting ECE-1 activity either by siRNA or using an ECE-specific inhibitor effectively reduced proliferation of OSCC[18].

The salivary analysis of ET-1 by ELISA is a simple and non-invasive method that has been used in the control and evaluation of many diseases, and might represent a valuable tool for the study of oral diseases. In this regard, a recent study conducted by Pickering et al. (2007)[19] analyzed the salivary ET-1 levels in 8 patients with oral squamous cell carcinoma prior to the treatment, in comparison to 8 healthy patients, demonstrating a significant increase of ET-1 levels in patients with cancer. In the present study, we have evaluated the salivary levels of endothelin-1 in patients with OSCC, in comparison to patients with oral leukoplakia and patients with complete remission of cancer for more than 5 years, by means of ELISA. Attempts have also been made to correlate the salivary expression of ET-1 to some factors such as elderly, sex and hypertension.

Patients and Methods

Ethical concerns

Patients were recruited from the Stomatology Service of São Lucas Hospital, Pontificia Universidade Católica do Rio Grande do Sul. Informed consent was obtained from all patients included in the study. The experimental protocols were approved by The Institutional Ethics Committee (Protocol number 08/04260).

Distribution of groups

The experimental groups were divided as follows:

Group 1: 20 patients without premalignant lesions or neoplasias, not exposed to alcohol and tobacco, and paired by sex and age with the patients of Group 4.

Group 2: 14 patients with oral leukoplakia - histopathological diagnosis of hyperkeratosis (10 patients), mild to moderate dysplasia (03 patients), and severe dysplasia (01 patient).

Group 3: 15 patients with a previous history of OSCC that had been treated by any modality of treatment (surgery, chemoterapy or radiotherapy), and are free of disease by 5 years or more;

Group 4: 20 patients with histopathological diagnosis of OSCC before the treatment.

Demographic characteristics

The demographic features of the studied population are shown in Table 1. The age of patients included in the study ranged between 32 and 89 years-old and the general male: female ratio was 2:1. Data regarding tumor localization is provided in Table 2. As it can be observed, the most prevalent tumor localizations were the tongue and the floor of the mouth, accounting for 60 % of carcinomas.

Table 1

Demographic distribution of patients within the studied groups

	Group 1 *Control*	**Group 2** *Leukoplakia*	**Group 3** *Cured patients*	**Group 4** *Carcinoma*
Age range	32-78	35-70	51-89	33-79
Mean age	59,1	52,6	69	59,3
Sex ratio (M:F)	2:1	1:2	3:1	2:1
Male	14	5	11	14
Female	6	9	4	6

Table 2

Localization of tumors in patients include in the Group 4

Site	**Patients (*n*)**
Tongue	6
Lower lip	2
Floor	6
Buccal mucosa	3
Gingiva	2
Soft palate	1

Procedures for saliva collection and ET-1 levels determination

For saliva collection, the patients were without feeding or smoking for at least 1 h. Firstly, the patients carried out a mouth wash with water. Next, they were instructed to rest in the seated position for 5 min. Saliva was collected in 5-ml graduated recipients, and

immediately stored at -70°C for posterior analysis. ET-1 levels were quantified by ELISA technique according to manufacture's recommendations (Biomedica Gruppe, Vienna, Austria). The results were expressed as fmol of ET-1 per ml of saliva, as calculated from a standard curve.

Statistical analysis

The results are presented as the mean ± standard error of the mean (s.e.m.) of 14 to 20 patients per group. Statistical comparison of data was performed by one-way analysis of variance (one-way ANOVA) followed by Tukey's test. *P*-values less than 0.05 ($P < 0.05$) were considered as indicative of significance.

Results

Salivary endothelin levels

The salivary levels of ET-1 ranged from 0 to 9.629 fmol/ml, in the group 1; from 0 to 9.453 fmol/ml, in the group 2; between 0.321 and 8.252 fmol/ml, in the group 3, and finally from 0 to 7.554 fmol/ml, in the group 4. We have compared the levels of ET-1 in saliva samples obtained from patients within the groups 1, 2, 3 and 4. The obtained results revealed no statistical difference among the groups, as indicated by One-Way ANOVA followed by Tukey's test ($P > 0.05$) (Figure 1).

To assess whether the salivary expression of ET-1 might vary due to some factors such as elderly, sex and hypertension, new series of statistical analysis were performed considering these variables. This evaluation showed no significant difference among groups 1 to 4, either when patients with hypertension (Figure 2A) or patients with age above 65 years were removed from the sample (Figure 2B) (One-Way ANOVA followed by Tukey's test; $P > 0.05$). Furthermore, no significant difference was observed between males and females (Figure 3) (One-Way ANOVA followed by Tukey's test; $P > 0.05$).

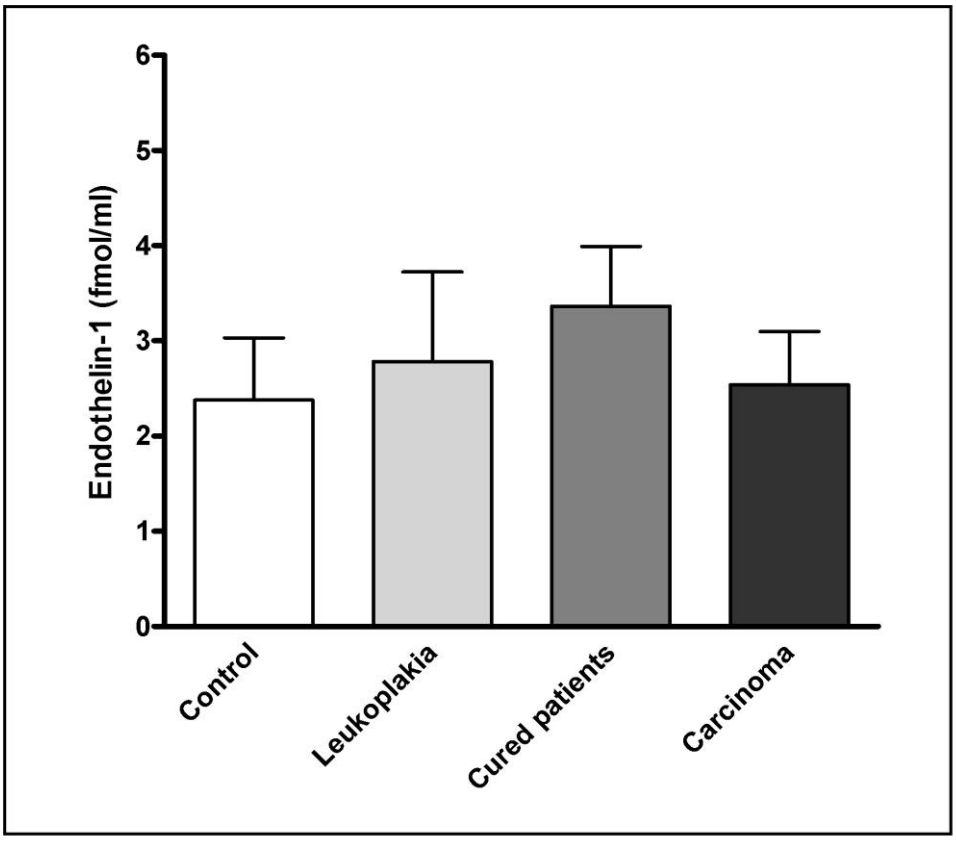

Figure 1: ET-1 in saliva from healthy patients, patients with oral leukoplakia, patients with previous history of OSCC and cured for more than 5 years and patients with OSCC.

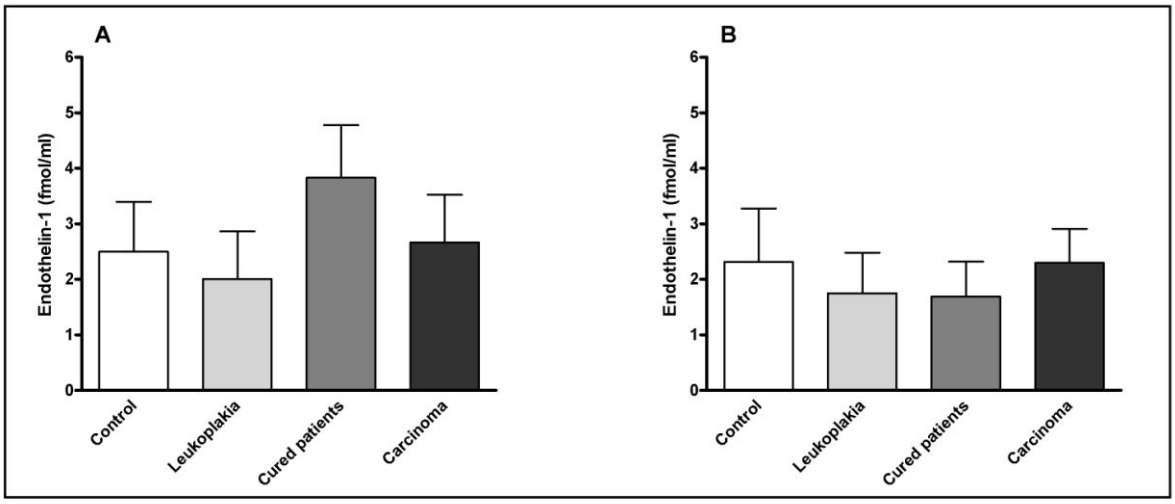

Figure 2: ET-1 salivary levels after excluding patients with hypertension history (A) or with age above 65 years (B) from the Groups 1 to 4.

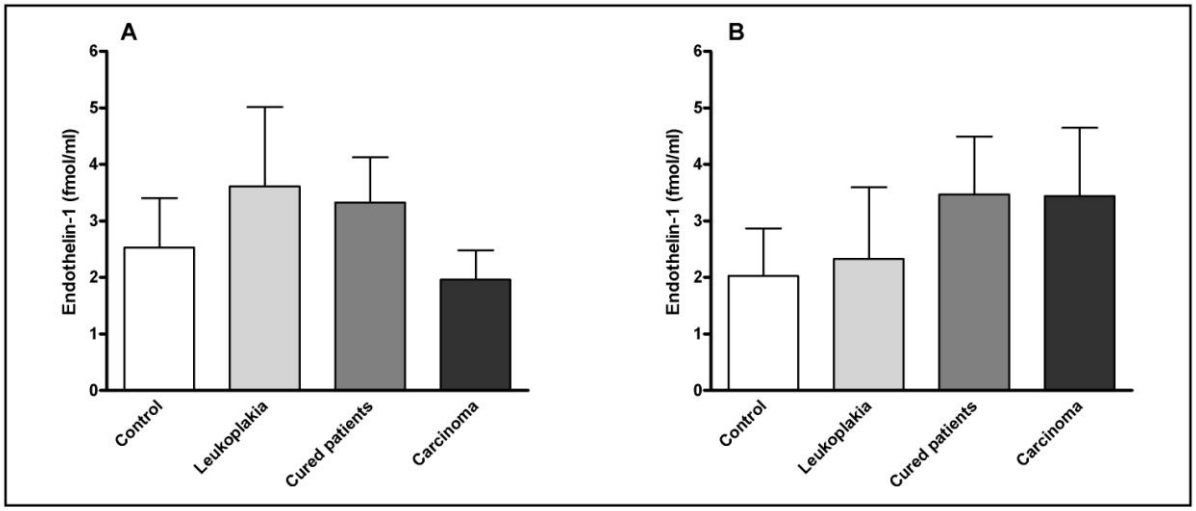

Figure 3: ET-1 salivary levels in males (A) and females (B) from the Groups 1 to 4.

Discussion

Endothelin is a multifunctional peptide with cytokine-like activity that affects almost all aspects of cell function[20]. ET-1 is likely involved in many kinds of tumors[11, 15, 16, 17]. In this way, some authors found an elevation of ET-1 concentration in OSCC[18, 19, 21, 22, 23]. The present study was designed to evaluate the salivary levels of endothelin-1 in patients with OSCC, in comparison to patients with oral leukoplakia or in patients with complete remission of OSCC for more than 5 years, by means of ELISA.

Awano et al. (2006)[18] investigated the expression and distribution of ET-1, its receptors (ET_AR and ET_BR), and the isoforms of its specific converting enzyme (ECE-1a, 1b, 1c) in OSCC. They concluded that ET-1, ET_AR and ET_BR and ECE-1 isoforms are overexpressed in OSCC, and that ET-1 acts as a survival factor to induce proliferation via ET_AR and ET_BR. Furthermore, inhibiting ECE-1 activity either by siRNA or an ECE-specific inhibitor effectively reduced the proliferation of OSCC *in vitro*. In addition, Schmidt et al. (2007)[22] demonstrated a significant elevation in the levels of ET-1 in HSC3 cells, a lineage obtained from human OSCC . These pieces of evidence are clearly indicative of a relevant role for the endothelin system in oral cancer.

Determination of biological markers in saliva constitutes a non-invasive method, which might be an effective modality for diagnosis and for prognosis of oral cancer, as well as for

monitoring post-therapy status[24, 25]. Noteworthy, the salivary analysis of the ET-1 has been already used in the control and evaluation of many diseases. For instance, Xiang et al. (2003)[26] have investigated the physiologic determinants of ET-1 in saliva obtained from healthy and chronic heart failure patients. The ideal storage temperature of saliva was found to be -80ºC. Furthermore, the ET-1 concentration was higher when saliva was collected in seated rest patients, in relation to patients in the supine position, whereas there was no clear indication of diurnal variation in salivary ET-1 concentrations.

In a recent study, Pickering et al. (2007)[19] analyzed the salivary ET-1 levels in 8 patients with OSCC prior to the treatment, in comparison to 8 healthy patients, by using ELISA assay. Data revealed a significant increase in ET-1 levels in saliva of patients with cancer. In our study, we have adopted the conditions recommended by Xiang et al. (2003)[26] for both collecting and storing saliva. However, different from data described by Pickering et al. (2007)[19], we have not found any significant difference between control and OSCC patients. Several different factors might be related to that discrepancy, including the sample size that was composed by 20 patients in each group in the present study. Furthermore, in our study, we have also analyzed the ET-1 salivary levels in 14 patients with oral leukoplakia and in 15 patients with a previous history of OSCC that had been cured. Again, no significant difference was observed among the groups. Additionally, other factors might be pointed out to explain the differences between our results and that from literature data, such as the presence of periodontal disease, traumatic lesions, dietary factors, the tumor stage or even the previous radiotherapy treatment in the group 3.

It is well described that ET-1 is largely implicated in vascular-related alterations such as hypertension and ageing[9, 27]. Therefore, we wondered if the absence of significance in our study could be related to other factors that would influence ET-1 salivary levels. For that reason, we decided to separate patients according to age (65 years and older individuals), sex and hypertension history. Again, there was no significant difference among the groups, discarding the hypothesis of the influence of the above mentioned systemic variations in our results. Corroborating our data, Lam et al. (2004)[28] did not found significant difference in salivary immunoreactive ET-1 among healthy patients and patients with esophagitis or gastric cancer, even when considering sex or age, according to assessment by radioimmunoassay. Therefore, although ET-1 might display an important role in OSCC, its levels in saliva do not seem to be a good marker of neoplasias grade or malignant transformation. Further studies with a greater sample are required to solve the reasons for the different outcomes between previous literature data and our results.

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Discussão Geral

5 DISCUSSÃO GERAL

O carcinoma espinocelular bucal é uma das neoplasias mais prevalentes nos dias atuais e continua sendo causador de mortes e mutilações pelo seu diagnóstico tardio. A prevenção e a detecção precoce desse tipo de neoplasia é a alternativa com melhores resultados quanto à qualidade e à expectativa de vida dos pacientes acometidos por essa doença.

As leucoplasias correspondem a placas brancas de risco questionável para o câncer, em especial às que apresentam alterações displásicas[3, 4]. As transformações ocorridas na mucosa bucal em lesões cancerizáveis e no CEB têm sido correlacionadas à exposição crônica a agentes exógenos, como o tabaco e o álcool[2].

Nos últimos anos, muitos estudos têm identificado potentes biomarcadores para diagnóstico, progressão e prognóstico das lesões ditas cancerizáveis e do CEB. Dessa forma, a ET-1, que é um peptídeo envolvido em muitas funções celulares e presente em um grande número de tumores[14, 15, 16, 17, 18], foi também estudada por alguns pesquisadores no CEB[13, 19, 20, 21, 22, 23]. O presente estudo teve como objetivo avaliar os níveis salivares de endotelina-1 em pacientes com CEB, em pacientes com leucoplasia, em pacientes com cura de CEB por mais de 5 anos e em um grupo de pacientes saudáveis, comparando os valores obtidos entre os grupos, através do método de ELISA.

Awano et al.[19] (2006) investigaram a expressão e a distribuição da ET-1, seus receptores (ET_AR e ET_BR), e isoformas da enzima conversora de endotelina (ECE-1ª, 1b, 1c) em CEB. Como conclusões encontraram um aumento da expressão dessas substâncias no CEB e, também, que a ET-1 atua como um fator de sobrevivência induzindo a proliferação via ET_AR e ET_BR. Além disso, a inibição da atividade da ECE-1, tanto pelo siRNA, quanto pelo inibidor específico de ECE reduziram a proliferação do CEB *in vitro*. Em 2007, Schmidt et al.[21] também demonstraram uma elevação nos níveis de ET-1 em células da linhagem HSC3, obtidas a partir de CEB humano. Da mesma forma, nos estudos de Quang et al. (2010) [22, 23], os níveis de ET-1 estavam aumentados em células oriundas de CEB, em relação a ceratinócitos bucais normais. Esses estudos são indicativos da presença de uma relação entre ET-1 e CEB.

A pesquisa de marcadores salivares constitui um método não-invasivo, podendo ser efetivo no diagnóstico e prognóstico do câncer bucal, assim como para o acompanhamento dos pacientes após o tratamento[24, 25]. A análise salivar da ET-1 já é utilizada no controle e avaliação de algumas doenças. Um exemplo disso é o estudo de Xiang et al.[26] (2003), onde os

determinantes fisiológicos da ET-1 na saliva obtida de um grupo de pacientes saudáveis e de um grupo de pacientes com insuficiência cardíaca crônica foram estudados. De acordo com os autores, fatores como a temperatura de armazenamento da saliva podem alterar a concentração de ET-1, sendo a temperatura ideal na faixa dos -80ºC. Além disso, a maior concentração de ET-1 foi encontrada em pacientes que tiveram sua saliva coletada quando sentados, em relação a pacientes na posição supina, enquanto que não houve uma clara indicação de variação circadiana desse peptídeo na saliva.

Em 2007, Pickering et al.[13] (2007) analisaram os níveis de ET-1 na saliva de 8 pacientes com CEB previamente ao tratamento, em comparação a 8 pacientes saudáveis, por meio do método ELISA. Os dados obtidos revelaram um aumento significante dos níveis de ET-1 salivar nos pacientes com câncer. No presente estudo, foram adotadas as condições recomendadas por Xiang et al.[26] (2003) para coleta e armazenamento de saliva. Entretanto, diferentemente dos resultados descritos por Pickering et al.[13] (2007), no trabalho aqui apresentado não foram encontradas diferenças significativas entre os níveis de ET-1 salivar dos pacientes com câncer e os do grupo controle. Muitos fatores podem estar relacionados a essa diferença, incluindo o tamanho da amostra que foi de 20 pacientes em cada grupo no presente estudo. Adicionalmente, foi analisada a concentração de ET-1 na saliva de 14 pacientes com leucoplasia oral e na de 15 pacientes com história prévia de CEB, curados há mais de cinco anos. Novamente, nenhuma diferença estatisticamente significativa foi observada entre os grupos. Outros fatores poderiam estar relacionados a estas diferenças, tais como a presença de doenças infecciosas, úlceras traumáticas, dieta, estadiamento tumoral, entre outros.

A falta de significância estatística neste estudo poderia estar relacionada a outros fatores capazes de interferir sobre os níveis de ET-1 salivar. Com base nesta suposição, os pacientes foram separados de acordo com a idade (abaixo de 65 anos e acima desta idade), sexo e histórico de hipertensão arterial. Igualmente, não houve diferença significativa entre os grupos, descartando a hipótese de que essas variantes pudessem interferir nos resultados aqui apresentados. Essa estratégia foi adotada, levando-se em consideração as alterações vasculares que ocorrem no envelhecimento e na hipertensão, que podem estar relacionadas com alterações dos níveis de ET-1. Corroborando com os resultados desta pesquisa, no trabalho de Lam et al.[12] (2004) não foi encontrada diferença significativa na imunorreatividade para ET-1 salivar entre pacientes saudáveis e pacientes com esofagite ou câncer gástrico, mesmo quando as variáveis sexo e idade foram consideradas. Neste sentido, embora a ET-1 possa desempenhar um importante papel em CEB, sua expressão na saliva não

parece ser um bom marcador de graduação de neoplasia ou transformação maligna nas leucoplasias. Assim, sugere-se que novas pesquisas, com uma amostra maior, sejam realizadas para solucionar as diferenças entre os resultados do presente estudo e os da literatura pregressa.

Conclusões

6 CONCLUSÕES

- Os níveis de ET-1 salivar em pacientes portadores de CEB antes do tratamento anti-neoplásico não apresentaram diferença estatística significante em relação aos níveis dessa substância no grupo de pacientes saudáveis, bem como em relação aos grupos de paciente com leucoplasia bucal e com cura de CEB há mais de 5 anos.

- Não foi encontrada diferença significante nos níveis de ET-1 salivar de pacientes que já tiveram CEB e estavam em um período pós-tratamento e livres de doença por, no mínimo, 5 anos e os níveis de ET-1 em pacientes saudáveis, bem como não houve diferença quando comparados estes valores com os obtidos para pacientes com leucoplasia bucal.

- A ausência de diferença estatística significante entre os resultados dos grupos de pacientes com leucoplasia bucal, CEB e saudáveis sugere que a análise salivar da ET-1 não seja um biomarcador eficiente para avaliar o risco de malignização de uma lesão cancerizável.

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Apêndices

APÊNDICE A – Termo de Consentimento Livre Esclarecido

Projeto: ANÁLISE DOS NÍVEIS DA ENDOTELINA-1 NA SALIVA

A Endotelina-1 é uma substância encontrada naturalmente em nosso corpo. Porém, em algumas doenças como, por exemplo, pressão alta, doenças do fígado e em algumas lesões de boca, os seus valores se modificam. O seu estudo pode ajudar a trazer novas perspectivas quanto ao tratamento dessas doenças.

Assim, estamos lhe convidando a participar de um estudo, onde no senhor (a) será realizado um exame de boca de rotina e uma pequena quantidade de sua saliva será coletada para análise dessa substância. Pretende-se, avaliar os níveis da Endotelina-1 em pacientes saudáveis, pacientes com lesões brancas e em pacientes com carcinoma ou que já tenham recebido tratamento para essa doença.

O benefício esperado com esta pesquisa é conhecer melhor a forma como essa substância se comporta, a fim de contribuir na prevenção do câncer bucal. Ressalta-se que os riscos para os indivíduos são inexistentes, pois se trata apenas de exame da boca e coleta de saliva e que não haverá custos para os pacientes em questão.

CONSENTIMENTO ESCRITO

Pelo presente termo consentimento informado, eu _____, declaro que fui esclarecido, de forma clara e detalhada, livre de qualquer forma de constrangimento e obrigação, dos objetivos, da justificativa, dos procedimentos a que serei submetido, eventuais desconfortos e benefícios do presente projeto de pesquisa, todos acima citados.

Fui igualmente informado:

- da garantia de receber resposta a qualquer pergunta ou esclarecimento a qualquer dúvida acerca dos procedimentos, benefícios e outros assuntos relacionados com a pesquisa;

- da garantia de informação caso a pesquisa agregue informação potencialmente útil como prognóstico ou fator de risco para a população estudada;

- da liberdade de retirar meu consentimento a qualquer momento, e deixar de participar do estudo, sem que isto traga prejuízo à continuação do meu acompanhamento e tratamento;

- da segurança que não serei identificado, e que se manterá o caráter confidencial das informações relacionadas com a minha privacidade;

- do compromisso de fornecer informação atualizada durante o estudo.

Caso surjam novas perguntas sobre este estudo ou sobre os meus direitos como participante, posso entrar em contato com a professora Dra. Liliane Soares Yurgel no telefone (51) 3320.3538, com a doutoranda Renata da Rocha Hoffmann no telefone (51) 9954.1063 ou com o Comitê de Ética em Pesquisa da PUCRS pelo telefone 3320-3345.

Declaro que recebi cópia do presente termo.

_____ _____

Assinatura do Paciente Assinatura do Pesquisador Responsável

_____

Assinatura do Pesquisador Assistente

Este formulário foi lido para _____ em ___/___/___ pela pesquisadora Renata da Rocha Hoffmann enquanto eu _____ estava presente.

_____

Assinatura da testemunha

Porto Alegre, ____ de _____ de 20___.

APÊNDICE B – Ficha de Coleta de Dados dos Pacientes

N°. da FICHA:..... REGISTRO:..... PACIENTE: .....

1.Identificação:

Nome:_____

Idade: _____ **Sexo:** _____ **Raça:** _____ **Telefone:** _____

2.Descrição da lesão:

3.Biópsia

Local da biópsia na cavidade oral:_____

N° AP	Características Histopatológicas	Data

Diagnóstico histopatológico: _____
Realização de nova biópsia: Não ( ) Sim( ) – Diagnóstico: _____
Tempo de evolução da lesão: _____

4.Presença de outras doenças associadas:

	Não	Sim	Qual?
Neoplasias			
Hipertensão			
Doença cardíaca			
Doença renal			
Doença gastrointestinal			
Estresse			
Doença periodontal			
Outras:			

5.Hábitos

	Quantidade	Tempo
Tabaco		
Ex-tabagista		
Álcool		
Ex-etilista		

Anexos

ANEXO A – Carta de aprovação pela Comissão Científica e de Ética da Faculdade de Odontologia – PUCRS

Comissão Científica e de Ética
Faculdade da Odontologia da PUCRS

Porto Alegre 13 de junho de 2008

O Projeto de: Tese

Protocolado sob nº:	0023/08
Intitulado:	Análise dos níveis da endotelina-1 na saliva de pacientes com carcinoma espinocelular bucal
Pesquisador Responsável:	Profa. Dra. Liliane Soares Yurgel
Pesquisadores Associados	Renata da Rocha Hoffmann
Nível:	Doutorado

Foi ***aprovado*** pela Comissão Científica e de Ética da Faculdade de Odontologia da PUCRS em *23 de abril de 2008.*

Este projeto deverá ser imediatamente encaminhado ao CEP/PUCRS

Prof. Dr. Eraldo Luiz Batista Júnior
Presidente da Comissão Científica e de Ética da
Faculdade de Odontologia da PUCRS

Av. Ipiranga, 6681, Prédio 06 sala 209
Porto Alegre /RS – Brasil – Cx. Postal:1429
90619-900
Fone/Fax: (51) 3320-3538
e-mail: odontologia-pg@pucrs.br

ANEXO B – Carta de aprovação pelo Comitê de Ética em Pesquisa – PUCRS

Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
COMITÊ DE ÉTICA EM PESQUISA

OF.CEP-1090/08 Porto Alegre, 03 de outubro de 2008.

Senhora Pesquisadora,

O Comitê de Ética em Pesquisa da PUCRS apreciou e aprovou seu protocolo de pesquisa registro CEP 08/04260 intitulado: **"Análise dos níveis da endotelina-1 na saliva de pacientes com carcinoma espinocelular bucal"**.

Salientamos que sua investigação está autorizada a partir desta data.

Os relatórios do andamento do protocolo devem ser encaminhados a este CEP.

Atenciosamente,

Prof. Dr. José Roberto Goldim
Coordenador do CEP-PUCRS

Ilma. Sra.
Profa. Dr. Liliane Soares Yurgel
Faculdade de Odontologia
N/Universidade

PUCRS
Campus Central
Av. Ipiranga, 6690 – 3ºandar – CEP: 90610-000
Sala 314 – Fone Fax: (51) 3320-3345
E-mail: cep@pucrs.br
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ANEXO C – Carta de Aceitação do Artigo de Revisão pelo Periódico *Oral Oncology*

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Article title: Endothelins and their receptors as biological markers for oral cancer
Reference: OO2212
Journal title: Oral Oncology
Corresponding author: Dr. Maria Martha Campos
First author: Dr. Renata R. Hoffmann
Online publication complete: 24-JUL-2010
DOI information: 10.1016/j.oraloncology.2010.06.015

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Article title: Evaluation of salivary Endothelin-1 levels in Oral Squamous Cell Carcinoma and Oral Leukoplakia
Reference: REGPEP4169
Journal title: Regulatory Peptides
Corresponding author: Dr. Maria M. Campos
First author: Dr. Renata R. Hoffmann
Received at Editorial Office: 2-JUN-2010
Article revised: 2-JUL-2010
Article accepted for publication: 11-AUG-2010
Accepted manuscript (unedited version) available online: 18-AUG-2010
DOI information: 10.1016/j.regpep.2010.08.006

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ANEXO E – Especificações Técnicas da Endotelina-1

ENDOTHELIN (1-21)

ENZYME IMMUNOASSAY FOR THE QUANTITATIVE DETERMINATION OF HUMAN ENDOTHELIN (1-21) IN SERUM, EDTA PLASMA, URINE, SALIVA AND CELL CULTURE SUPERNATANTS.
CAT. NO. BI-20052. 12 X 8 TESTS

ENZYMIMMUNOASSAY ZUR QUANTITATIVEN BESTIMMUNG VON HUMAN ENDOTHELIN (1-21) IN SERUM, EDTA PLASMA, URIN, SPEICHEL UND ZELLKULTURÜBERSTAND.
KAT. NR. BI-20052. 12 X 8 TESTE

ENZIMOINMUNOENSAYO PARA LA DETERMINACION CUANTITATIVA DE ENDOTELINA HUMANA (1-21) EN SUERO, PLASMA EDTA, ORINA , SALIVA Y EN SOBRENADANTE DE CULTIVOS CELULARES
CAT. NO. BI-20052, 12 X 8 TESTS

rev.no. 080310 (replacing 071220)

CE

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1) INTRODUCTION

Cleavage of Big Endothelin by a membrane-bound metalloproteinase, the Endothelin Converting Enzyme (ECE) leads to the active ET (1-21), a potent vasoconstrictor and to the biological inactive C-terminal fragment (22-38). The half-life of ET in the plasma is less than one minute, whereas clearance of Big ET is much slower. Endothelin has been identified in a variety of tissues, including lung, kidney, brain, pituitary and peripheral endocrine tissues and placenta. The biological role of ET extends beyond regulating vascular tone also in its growth regulatory properties. The peptide interacts in an autocrine/paracrine manner with specific ET receptors found on numerous cells, including smooth muscle cells, myocytes, and fibroblasts.

POSSIBLE INDICATIONS

- Heart failure and acute myocardial infarction
- Oncology
- Marker for endothelial dysfunction, liver damage and renal disease
- Hypertension

2) CONTENTS OF THE KIT

CONT	KIT COMPONENTS	QUANTITY
PLATE	Polyclonal anti Endothelin antibody, microtiter plate strips in stripholder packed in alubag with desiccant	12 x 8 tests
WASHBUF	Wash buffer concentrate 20x, natural cap	1 x 50 ml
AB	Detection antibody, monoclonal mouse anti Endothelin antibody, green cap, ready to use	1 x 22 ml
STD	Standards (0-10 fmol/ml), synthetic human Endothelin-1 (1-21) in human plasma, white caps, lyophilised	6 vials lyophilised
CTRL	Controls, synthetic human Endothelin-1 (1-21) in human plasma, yellow caps, lyophilised, exact concentration after reconstitution see label	2 vials lyophilised
CONJ	Conjugate (anti mouse IgG antibody-HRPO), amber cap, ready to use	1 x 22 ml
SUB	Substrate (TMB solution), blue cap, ready to use	1 x 22 ml
STOP	Stop Solution, white cap, ready to use	1 x 7 ml
ET-STOCK	Endothelin Stock, synthetic human Endothelin-1 (1-21), lyophilised, red cap, exact concentration after reconstitution see label	1 vial lyophilised

3) ADDITIONAL MATERIAL ADDED TO THE KIT

- 2 self-adhesive plastic films
- QC protocol
- Protocol sheet
- Instruction manual for use

4) MATERIAL AND EQUIPMENT REQUIRED BUT NOT SUPPLIED

- Precision pipettes calibrated to deliver 50-1000 µl and disposable tips
- ELISA reader for absorbance at 450 nm (or from 450 nm to 620 nm)
- Graph paper or software for calculation of results
- Distilled or deionised water

5) REAGENTS AND SAMPLE PREPARATION

Reconstitution/Handling:

- STD (Standards, white caps) in 0.5ml distilled water, at room temperature (18-26°C) for 30 min, shake well. Reconstituted standards are stable at -20°C or -70°C until expiry date stated on label. Avoid repeated freeze-thaw cycles!
- CTRL (Controls, yellow caps) in 0.5 ml distilled water at room temperature (18-26°C) for 30 min, stable at -20°C or -70°C until expiry date stated on label, avoid freeze-thaw cycles.
- WASHBUF (Wash buffer) dilute the concentrate 1:20 with distilled water (50 ml concentrate + 950 ml distilled water). Crystals in the buffer concentrate will dissolve at room temperature. Buffer is stable at 2-8°C until expiry date stated on label.
- ET-STOCK (Endothelin stock, red cap): Direct measurement of Endothelin in cell culture supernatants: Reconstitute in 2 ml cell culture medium at room temperature (18-26°C) for 30 min, shake well.
  Exact concentration after reconstitution see label, eg. 10 fmol/ml. Reconstituted ET stock is stable at -20°C or -70°C until expiry date stated on the label. Avoid repeated freeze-thaw cycles.

Sample type:
Serum, EDTA plasma, urine, saliva and cell culture supernatants are suitable for use in this assay. Don't change sample type during studies.

Sample collection:
Freshly collected EDTA plasma or serum is put on ice immediately and centrifuged within one day. Samples should be stored at -20°C, for long-term storage store at -70°C.
Urine samples can be used without any pre-treatment.
Avoid freeze-thaw cycles. Lipemic or hemolyzed samples may give erroneous results. Samples should be mixed well before assaying. We recommend duplicates for all values. If it is necessary to dilute samples with a high concentration please use 0.9% sodium chloride solution.

For cell culture:

Do not use the plasma-standards (white caps) and controls (yellow cap)!

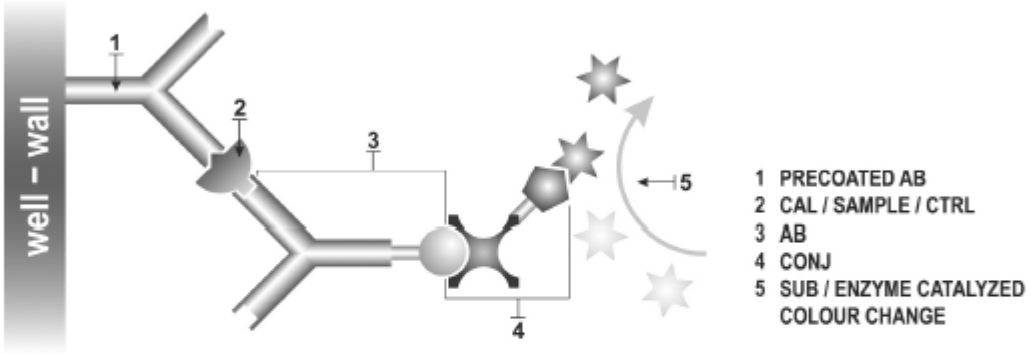
- Prepare a serial dilution of the cell culture ET-STOCK (Endothelin- stock, red cap) with cell culture medium down to appr. 0.625 fmol/ml (e.g. 10 / 5 / 2.5 / 1.25 / 0.625 fmol/ml). Cell culture medium is used as a zero standard.
- Dilute cell culture supernatant according to the expected concentration with the culture medium. Dilution of supernatant is dependent on amount of Endothelin secreted by the respective cell type.

For saliva:

For sample collection we recommend Sarstedt Salivettes.
Sample collection:
Last meal or cigarette should have been consumed at least one hour before sample collection.

- Rinse mouth with water, wait 10 minutes.
- Put cotton roll of the Sarstedt Salivette tube into the mouth, chew for 30 sec., keep in mouth for two additional minutes.
- Place the cotton roll into the flat bottom upper tube of the Salivette, seal with the stopper and centrifuge for 3 min. at 1000 rcf (rotational centrifugal force = g).
- Remove the flat bottom tube from the Salivette and pipette the clear saliva from the bottom of the V-tube, aliquot and store at -20°C or -70°C.
- Use the clear saliva according to the assay protocol.

6) PRINCIPLE OF THE ASSAY



7) ASSAY PROTOCOL

All reagents and samples must be at room temperature (18-26°C) before use in the assay.
Mark position for BLANK/STD (Standards)/SAMPLE/CTRL (Control) on the supplied protocol sheet.
Take microtiter strips out of the alu bag, take a minimum of one well as Blank. Store unused strips with desiccant at 2-8°C in the alu bag. Strips are stable until expiry date stated on the label.

Add 50 µl STD/SAMPLE/CTRL (Standard/Sample/Control) in duplicate into respective well, except blank.
Add 200 µl AB (Detection antibody) into each well, except blank, swirl gently.
Cover tightly and incubate overnight (16-24 hours) at room temperature (18-26°C).
Aspirate and wash wells 5x with 300 µl diluted WASHBUF (Wash buffer), remove remaining WASHBUF by hitting plate against paper towel after the latest wash.
Add 200 µl CONJ (Conjugate) into each well.
Cover tightly and incubate 1 hour at room temperature (18-26°C).
Aspirate and wash wells 5x with 300 µl diluted WASHBUF (Wash buffer), remove remaining WASHBUF by hitting plate against paper towel after the last wash.
Add 200 µl SUB (Substrate) into each well.
Incubate for 30 minutes at room temperature (18-26°C) in the dark.
Add 50 µl STOP (Stop solution) into each well, shake well.
Measure absorbance immediately at 450 nm with reference 620 nm, if available.

8) CALCULATION OF RESULTS

Subtract the blank extinction from all other values. Construct the standard curve from the standard values. Use commercially available software or graph paper. Obtain sample concentration from this calibration curve. The assay has been evaluated using a 4PL algorithm. Different curve fitting method needs to be evaluated by the user. Respective dilution factors have to be considered.

The quality control protocol supplied with the kit shows the results of the final release QC for each kit. Data for optical density obtained by customers may differ due to various influences and/or due to the normal decrease of signal intensity during shelf life. However, this does not affect validity of results as long as an optical density of 1.50 is obtained for the standard with the highest concentration.

9) ASSAY CHARACTERISTICS

Reference data	A panel of 70 blood donors had a median of 0.26 fmol/ml. Each laboratory should establish its own reference data.
Standard range:	0-10 fmol/ml
Sample volume	50 µl human EDTA plasma, serum, urine, saliva or cell culture supernatant
Detection Limit:	(0 fmol/ml + 3 SD): 0.02 fmol/ml
Incubation time:	overnight / 1 h / 30 min
Cross reactivity:	ET(1-21): 100%, ET2 (1-21): 100%, ET3 (1-21): <5%, Big Endothelin (1-38): <1%, Big Endothelin (22-38): <1% In normal human plasma samples ET-2 is estimated to be present at less than 20% of the ET-1 level. ET-3 is estimated to be present at 50% of the ET-1 level. Animal sera: Horse, cat, pig 100%; dog 66% and rat 79%, Mouse sera can not be measured in this ELISA.

Recovery

(n = 4)	Spike 1 fmol/ml	Spike 5 fmol/ml
Recovery (fmol/ml)	0.96	4.3
Recovery (%)	96%	86%

10) PRECISION

Intra-Assay (n=18)		
Mean (fmol/ml)	2.02	7.01
SD	0.08	0.21
CV%	4%	3%

Inter-Assay (n=24)		
Mean (fmol/ml)	3.8	0.8
SD	0.2	0.04
CV%	6 %	5%

11) TECHNICAL HINTS

- Do not mix or substitute reagents with those from other lots or sources.
- Do not mix stoppers and caps from different reagents or use reagents between lots.
- Do not use reagents beyond expiration date.
- Protect reagents from direct sunlight.
- Substrate solution should remain colourless until added to the plate.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.

12) PRECAUTIONS

All test components of human source were tested with 3rd generation tests against HIV-Ab and HBsAg and were found negative. Nevertheless, they should be handled and disposed as if they were infectious.
All liquid reagents contain 0.1%Proclin 300 as preservative. Proclin 300 is not toxic in concentrations used in this kit. It may cause allergic skin reactions – avoid contact with skin or eyes.

- Do not pipette by mouth.
- Do not eat, drink, smoke or apply cosmetics where reagents are used.
- Avoid all contact with the reagents by using gloves.
- Sulfuric acid is irritating to eyes and skin. Flush with water if contact occurs. Avoid contact with skin and mucous. Irritations are possible – Flush with water after contact!

13) LITERATURE

- "Plasma levels of endothelin, lipid peroxides and prostacyclin in diabetic patients with macroangiopathy" Migdalis IN et al.; Diabetes Res Clin Pract 2001 Nov;54(2):129-36
- "Plasma endothelin in patients with acute aortic disease" Wagner A et al.; Resuscitation 2002 Apr;53(1):71-6
- "Plasma endothelin-1 levels and clinical correlates in patients with chronic heart failure" Kinugawa T et al.; J Card Fail 2003 Aug;9(4):318-24