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Focussed microarray analysis of apoptosis in periodontitis and its potential pharmacological targeting by carvacrol

Fares Zeidán-Chuliá [a,*], *Mervi Gursoy* [b,d], *Ben-Hur Neves de Oliveira* [a], *Daniel Pens Gelain* [a], *Eija Könönen* [b,d], *Ulvi Kahraman Gursoy* [b,d,1], *José Cláudio Fonseca Moreira* [a,1], *Veli-Jukka Uitto* [c,1]

[a] *Centro de Estudos em Estresse Oxidativo, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil*
[b] *Department of Periodontology, Institute of Dentistry, University of Turku, Turku, Finland*
[c] *Department of Periodontology, Institute of Dentistry, University of Helsinki, Helsinki, Finland*
[d] *Bacteriology Unit, Department of Infectious Disease Surveillance and Control, National Institute for Health and Welfare (THL), Helsinki, Finland*

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ABSTRACT

Objective: The objective of this study was to perform a landscape analysis of apoptosis-related genes/proteins and to study the differential gene expression by analysing array data from periodontitis patients and, second, to evaluate the anti-apoptotic effects of carvacrol, a monoterpenoid phenol, *in vitro*.

Design: A gene/protein interaction network model 'APOP' was developed by using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) version 9.05. Differential gene expression was determined by using the limma package from R and false discovery rate (FDR). With ViaComplex software, gene expression was plotted over the network. The anti-apoptotic effect of carvacrol was tested on sorbitol-treated HaCaT cells, by using a commercial kit for caspase-3 activity.

Results: The 'APOP' model characterised the landscape of interactions between apoptosis-related genes/proteins *in silico*. Forty-nine out of 70 genes from this model, such as *CSF2RB*, *NFKBIE*, *ENDOG*, *CASP10* and *CASP3*, were differentially expressed (corrected p-value < 0.05) in periodontitis samples when compared to those of healthy controls. In addition, carvacrol (0.43%) was able to inhibit the pro-apoptotic effects induced by sorbitol (0.3 M), as seen by the reduction in caspase-3 activity on HaCaT cells.

Conclusion: Our results suggest that caspase-3 can be a target protein to inhibit periodontitis-associated apoptosis of epithelial cells and that carvacrol has therapeutic potential as an anti-apoptotic agent.

* *Corresponding author at*: Centro de Estudos em Estresse Oxidativo, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Rua Ramiro Barcelos, 2600-anexo-Bairro Santana, 90035-003 Porto Alegre, RS, Brazil. Tel.: +55 51 3308 5577; fax: +55 51 3308 5535.
E-mail address: fzchulia.biomed@gmail.com (F. Zeidán-Chuliá).

[1] These authors jointly directed this work and should be considered as equal last authors.

Abbreviations: MMP, matrix metalloproteinase; EOs, essential oils; GEO, gene expression omnibus; FDR, false discovery rate; ROS, reactive oxygen species; IL, interleukin; CSF2, granulocyte–macrophage colony-stimulating factor.

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1. Introduction

Bioinformatics has established itself as an essential tool in science and the *in silico* analysis of gene expression/function has become an integral part of it by guiding the researchers towards the selection of the most relevant targets in the context of any disease.[1] Our group has successfully applied these tools in different areas of research such as pharmacognosy, neuroscience and toxicology.[2–4]

Periodontitis is an infection-induced chronic inflammatory disease of tooth-supporting tissues. Destruction initiates from the soft tissues (epithelium and gingival connective tissue) and continues to the hard tissues (alveolar bone) of the periodontium. Different bacteria, such as *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia* and *Treponema denticola*, have been associated with both the initiation and regulation of soft and hard tissue destruction during periodontal inflammation.[5] In periodontal tissues, the balance between cell death and survival of resident cells, provided by physiological programmed cell death (*e.g.*, autophagy and apoptosis), is critical for tissue homeostasis.[6] Autophagy is considered a self-degradative process, critical for the balance of energy sources at specific times as it removes aggregated proteins and/or damaged organelles as well as eliminates intracellular pathogens.[7] For instance, mitochondria play an important role in pro-inflammatory signalling and generation of reactive oxygen species (ROS), and it is regarded as an important activator of inflammasome-mediated inflammation.[8] Thus, the turnover of non-functional mitochondria (or mitophagy) would become essential in inflammatory scenarios, such as periodontitis, in order to maintain tissue homeostasis. As a matter of fact, increased levels of autophagy gene expression and high levels of mitochondrial ROS production in peripheral blood mononuclear cells from patients with periodontitis have already been reported.[9] Pathogenic bacteria induce several changes in resident cells of the gingiva (epithelial cells and fibroblasts), including the stimulation of cytokines and production of antimicrobial peptides, modulation of matrix metalloproteinase (MMP) secretion and induction of apoptosis.[10–12] Periodontitis-associated bacteria induce apoptosis in different ways: *A. actinomycetemcomitans* induces apoptosis in a caspase-3-dependent manner,[13] *P. gingivalis* initiates anti-apoptotic mechanisms in early infection and stimulates pro-apoptotic mechanisms in continuing infection[14] and *F. nucleatum* triggers apoptosis in epithelial cells by inducing caspase-3 expression; however, such an effect seems to be strain-dependent.[15]

Inhibition of tissue destruction is one of the main goals in host modulation therapies for periodontal inflammation by suppressing bacterial challenge and regulating tissue response.[16] Essential oils (EOs) have been proposed as natural therapeutic agents due to their antibacterial and anti-gelatinolytic properties.[2] *Satureja hortensis* L. EO, with carvacrol as its main active component, has been shown to exhibit strong antibacterial effects on periodontopathogenic bacteria and to decrease their cytotoxicity and excessive MMP production by epithelial cells *in vitro*.[17,18] In fact, in our recent study, it was demonstrated that 0.05% of *S. hortensis* L. EO is able to inhibit cell death and increase the proliferation of human epithelial cells.[18] These events could be a result of anti-apoptotic properties induced by *S. hortensis* L. EO. In the present continuation study, we hypothesised that its anti-apoptotic effect depends on carvacrol, which is the main compound of *S. hortensis* L. EO. Our aims were (1) to characterise the apoptotic landscape in periodontitis with an *in silico* approach and a pathway-focussed (apoptosis) microarray analysis and (2) to analyse the effect of carvacrol as an anti-apoptotic agent *in vitro*.

2. Materials and methods

2.1. Network development

The apoptosis interaction network model 'APOP' was developed by associating a total of 70 apoptosis-related genes/proteins (Table 1), provided by the Kyoto Encyclopedia of Genes and Genomes (KEGG) PATHWAY database (http://www.genome.jp/kegg/pathway.html; Apoptosis: map04210). The network was generated by using the database resource search tool Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) version 9.05 (http://string-db.org/) for the retrieval of interacting genes[19] with 'Experiments' and 'Databases' as input options and a confidence score of 0.400. STRING is a well-known public database with information about direct and indirect functional protein–protein interactions. The genes/proteins belonging to the 'APOP' model were identified by the Human Genome Organisation (HUGO) Gene Symbol[20] and Ensemble protein ID. Once they were selected, the links between two different nodes (genes/proteins) were provided by the STRING database and saved in data files to be handled in the Medusa interface.[21]

2.2. Differential gene expression analysis

Microarray data were downloaded from the international repository (Gene Expression Omnibus, http://www.ncbi.nlm.nih.gov/geo/; GEO accession No.: GSE10334; Platform: GPL570, Affymetrix), an experiment that is composed of 247 human samples (64 healthy and 183 diseased gingival samples).[22] In order to perform the differential gene expression analysis, normalised data of periodontitis samples *versus* controls were analysed by using the limma package from R and false discovery rate (FDR)[23,24] for statistical assessment of the microarray data (corrected p-values < 0.05 were considered significant). The means of gene expression in diseased samples were plotted *versus* the expression found in healthy ones to be visualised with ViaComplex.[25] This software plots gene expression over the Medusa network (in a colour scale) by overlapping functional input data (microarray expression data) with interaction information ('APOP' model) and distributes the microarray signal according to the coordinates of the nodes and its interactions in the network of interest.

2.3. Chemicals and concentrations

Carvacrol (282197) was purchased from Sigma. For every test performed on epithelial cells, a concentration of carvacrol of

Table 1 – Differentially expressed genes (down or up-regulated) from the interaction network model "APOP" (periodontitis *vs.* healthy samples).

Gene symbol	Ensemble protein ID	Gene name/description	Differential gene expression	Corrected p-value < 0.05
CSF2RB	ENSP00000384053	Colony stimulating factor 2 receptor, beta, low-affinity (granulocyte–macrophage)	UP	4.99976E−16
NFKBIE	ENSP00000275015	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon	UP	3.52219E−12
ENDOG	ENSP00000361725	Endonuclease G	DOWN	1.11789E−11
CASP10	ENSP00000286186	Caspase 10	UP	5.29366E−11
CASP3	ENSP00000311032	Caspase 3	UP	2.84703E−10
BID	ENSP00000318822	BH3 interacting domain death agonist	UP	3.88191E−10
CFLAR	ENSP00000312455	CASP8 and FADD-like apoptosis regulator	UP	1.94246E−09
IL1B	ENSP00000263341	Interleukin 1, beta	UP	5.23217E−09
ATM	ENSP00000278616	Ataxia telangiectasia mutated	UP	3.03187E−08
BCL2	ENSP00000329623	B-cell CLL/lymphoma 2	UP	6.78125E−08
PAWR	ENSP00000328088	PRKC, apoptosis, WT1, regulator	DOWN	9.79553E−08
PPP3CC	ENSP00000240139	Protein phosphatase 3, catalytic subunit, gamma isozyme	UP	1.80816E−07
IRAK3	ENSP00000261233	Interleukin-1 receptor-associated kinase 3	UP	3.67862E−07
BIRC3	ENSP00000263464	Baculoviral IAP repeat containing 3	UP	5.88785E−07
CASP8	ENSP00000351273	Caspase 8	UP	7.1311E−07
BCL2L1	ENSP00000302564	BCL2-like 1	UP	9.79044E−07
CASP9	ENSP00000330237	Caspase 9	UP	2.3836E−06
BAX	ENSP00000293288	BCL2-associated X protein	UP	3.658E−06
IL1A	ENSP00000263339	Interleukin 1, alpha	UP	4.48708E−06
MYD88	ENSP00000379625	Myeloid differentiation primary response gene (88)	DOWN	6.81905E−06
TNFSF10	ENSP00000241261	Tumour necrosis factor (ligand) superfamily, member 10	UP	7.55029E−06
DFFA	ENSP00000366237	DNA fragmentation factor, 45 kDa, alpha polypeptide	UP	1.11559E−05
MAP3K14	ENSP00000342059	Mitogen-activated protein kinase kinase kinase 14	UP	0.000101503
NFKB2	ENSP00000189444	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	UP	0.000103961
CASP6	ENSP00000265164	Caspase 6	DOWN	0.00010583
IRAK1	ENSP00000358997	Interleukin-1 receptor-associated kinase 1	UP	0.000595167
FASLG	ENSP00000356694	Fas ligand (TNF superfamily, member 6)	UP	0.000847119
APAF1	ENSP00000353059	Apoptotic peptidase activating factor 1	UP	0.001268061
IKBKG	ENSP00000358622	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma	UP	0.001300281
BAD	ENSP00000309103	BCL2-associated agonist of cell death	UP	0.002391555
TNFRSF10A	ENSP00000221132	Tumour necrosis factor receptor superfamily, member 10a	DOWN	0.002593051
NFKBIZ	ENSP00000325663	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	UP	0.002864101
PPP3CB	ENSP00000378306	Protein phosphatase 3, catalytic subunit, beta isozyme	UP	0.003828891
CIDEA	ENSP00000339344	Cell death-inducing DFFA-like effector a	DOWN	0.005352422
FAS	ENSP00000347979	Fas (TNF receptor superfamily, member 6)	DOWN	0.005897434
TRADD	ENSP00000341268	TNFRSF1A-associated *via* death domain	UP	0.006002501
TNFRSF10B	ENSP00000276431	Tumour necrosis factor receptor superfamily, member 10b	UP	0.007023252
TNF	ENSP00000392858	Tumour necrosis factor	UP	0.007409663
CASP7	ENSP00000298700	Caspase 7	UP	0.014457117
IL1R1	ENSP00000233946	Interleukin 1 receptor, type I	UP	0.015435312
IRAK2	ENSP00000256458	Interleukin-1 receptor-associated kinase 2	UP	0.016514545
CHUK	ENSP00000359424	Conserved helix-loop-helix ubiquitous kinase	DOWN	0.018964518
PRKCA	ENSP00000284384	Protein kinase C, alpha	UP	0.019393692
RELA	ENSP00000384273	v-rel reticuloendotheliosis viral oncogene homolog A (avian)	UP	0.025020711
DFFB	ENSP00000367454	DNA fragmentation factor, 40 kDa, beta polypeptide (caspase-activated DNase)	UP	0.025975985
NFKB1	ENSP00000226574	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	UP	0.027024066
IRAK4	ENSP00000349096	Interleukin-1 receptor-associated kinase 4	DOWN	0.027895001
IL3RA	ENSP00000327890	Interleukin 3 receptor, alpha (low affinity)	UP	0.028674832
TRAF2	ENSP00000247668	TNF receptor-associated factor 2	UP	0.030407183
NFKBIB	ENSP00000312988	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta	No	0.053288736
PPP3R1	ENSP00000234310	Protein phosphatase 3, regulatory subunit B, alpha	No	0.055729615
AKT1	ENSP00000270202	v-akt murine thymoma viral oncogene homolog 1	No	0.075569289
AIFM1	ENSP00000287295	Apoptosis-inducing factor, mitochondrion-associated, 1	No	0.090967934

Table 1 (*Continued*)

Gene symbol	Ensemble protein ID	Gene name/description	Differential gene expression	Corrected p-value < 0.05
BIRC2	ENSP00000227758	Baculoviral IAP repeat containing 2	No	0.099538022
PPP3CA	ENSP00000378323	Protein phosphatase 3, catalytic subunit, alpha isozyme	No	0.105342754
NTRK1	ENSP00000351486	Neurotrophic tyrosine kinase, receptor, type 1	No	0.108543576
NFKBIL2	ENSP00000386239	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-like 2	No	0.109150906
CAPN2	ENSP00000295006	Calpain 2 (m/II) large subunit	No	0.131183193
IL3	ENSP00000296870	Interleukin 3 (colony-stimulating factor, multiple)	No	0.138840006
IKBKB	ENSP00000339151	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	No	0.14825754
CAPN1	ENSP00000279247	Calpain 1 (mu/I) large subunit	No	0.171100789
NGF	ENSP00000358525	Nerve growth factor (beta polypeptide)	No	0.171317423
CIDEB	ENSP00000258807	Cell death-inducing DFFA-like effector b	No	0.191423981
RIPK1	ENSP00000259808	Receptor (TNFRSF)-interacting serine-threonine kinase 1	No	0.302440791
TP53	ENSP00000269305	Tumour protein p53	No	0.539824907
IL1RAP	ENSP00000072516	Interleukin 1 receptor accessory protein	No	0.613518474
FADD	ENSP00000301838	Fas (TNFRSF6)-associated *via* death domain	No	0.709388393
PPP3R2	ENSP00000363939	Protein phosphatase 3, regulatory subunit B, beta	No	0.822298246
TNFRSF1A	ENSP00000162749	Tumour necrosis factor receptor superfamily, member 1A	No	0.872878353
NFKBIA	ENSP00000216797	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	No	0.996703605

0.43% was used. This concentration was chosen based on the composition of *S. hortensis* L. EO and the concentration in which *S. hortensis* L. EO (5 μl ml^{-1} or 0.5%, v/v) prevented cell death in the presence of a pro-oxidant insult and stimulated the proliferation of epithelial cells, as shown in our previous study.[18] As the tested EO contained 86% of carvacrol in its composition, only 0.43% of carvacrol was then used in this continuation study. In addition, a higher concentration of carvacrol (4.3%) was used for the antibacterial experiments, as only 5% of *S. hortensis* L. EO was shown to display an antibacterial effect against periodontopathogens.[18]

2.4. Cell culture

Human epithelial keratinocytes (HaCaT cells) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing foetal calf serum (10%), l-glutamine (1%) and penicillin G (1%) in an atmosphere of 5% CO_2 at 37 °C.

2.5. Bacterial culture

The utilised bacterial strains (Table 2) came from the culture collection of anaerobic bacteria of the National Institute for Health and Welfare (THL). All bacteria were grown on brucella blood agar enriched with hemin and incubated at 37 °C in an anaerobic chamber for 5 days.

2.6. Caspase-3 activity

HaCaT cells were incubated overnight in 96-well plates to 80% confluency. Cells were pretreated with either 4.3 μl ml^{-1} (0.43%) of carvacrol or just DMEM for control cells for 10 min at 37 °C in a CO_2 incubator. Afterwards, the tests groups were incubated with sorbitol (0.3 M)[26] for 1 h. Then, caspase-3 activity was analysed according to the manufacturer's instructions provided in the kit (CASP3F; Sigma).

2.7. Antibacterial activity

Antibacterial activity of carvacrol against the selected bacterial strains was determined by a disc diffusion assay with slight modifications.[18] Briefly, one 5-day-old colony from the first passage of each bacterial strain was inoculated on brucella blood agar. Discs of 6 mm diameter were impregnated in 15 μl of carvacrol at the tested concentration (4.3%) and then placed onto the inoculated agar plates, which were incubated at 37 °C in an anaerobic chamber for 5 days. Five micrograms of metronidazole (nitroimidazole antibiotic) was used as a positive control for antibacterial effect. The solvent, dimethyl sulphoxide (DMSO), was used as a negative control. Thereafter, the inhibition zone was measured and the effect calculated. All the tests were done in triplicates (independent experiments).

2.8. Statistical analysis

Differences between the test and control groups (*in vitro* experiments) were analysed by using the Student's *t*-test. Results were considered significant (*) with a p-value < 0.05.

3. Results

3.1. The in silico network model 'APOP' and its differentially expressed genes characterise apoptosis in periodontitis

In order to perform a focussed DNA microarray analysis of apoptosis in periodontitis samples, the network model 'APOP', composed of 70 genes (Table 1) involved in apoptotic process, was developed (Fig. 1A; for further details, see Section 2). These apoptosis-related genes were submitted to further analysis with the ViaComplex software, which plots the expression

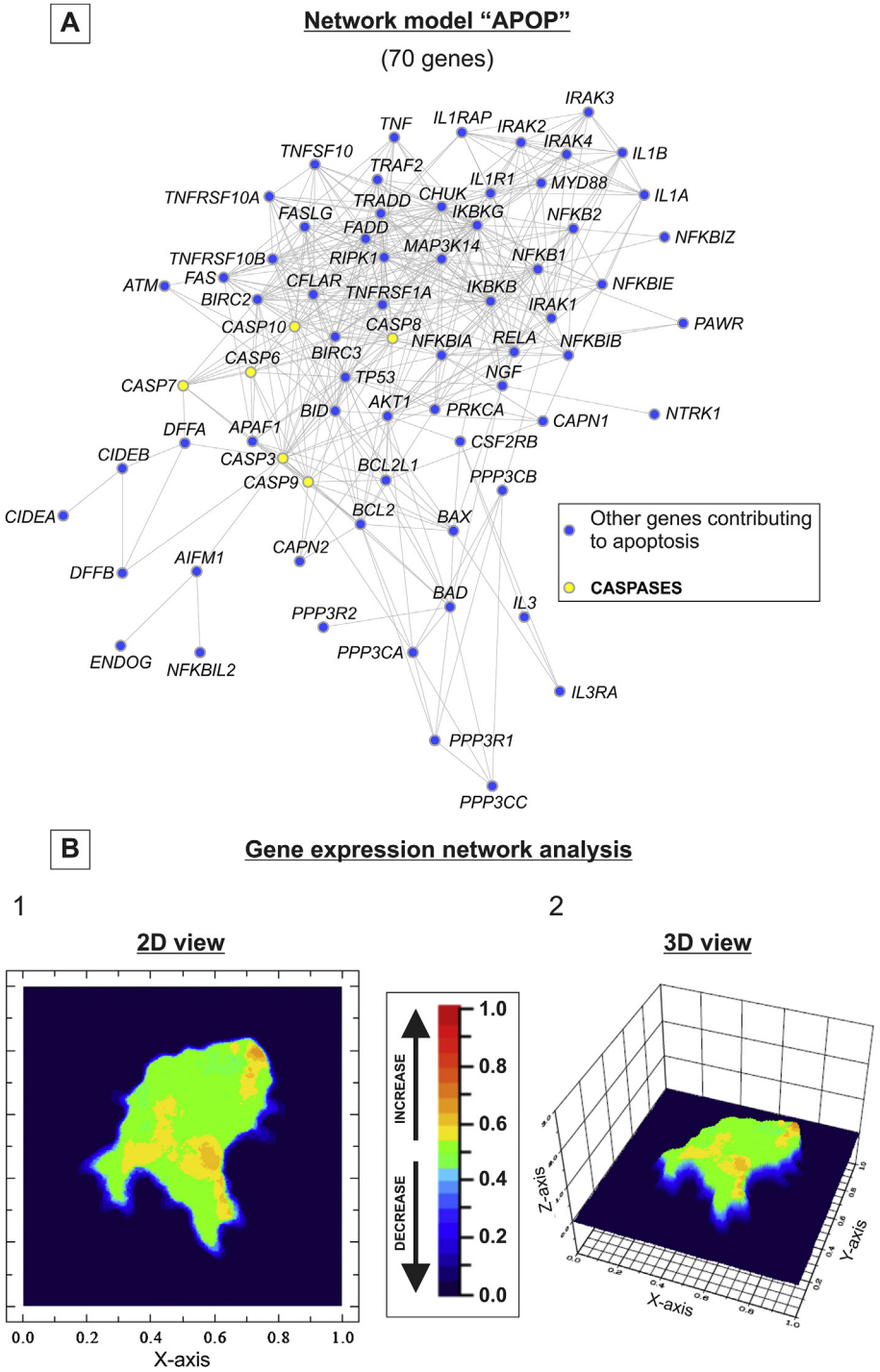

Fig. 1 – Interaction network model of apoptosis-related genes developed by using the STRING 9.05 database resource search tool, under a confidence score of 0.400 (A). Gene expression of diseased gingival samples *vs.* healthy controls plotted over the network model "APOP" (B). The Z-axis was constructed with ViaComplex software for 2D (B.1) and 3D (B.2) view representation of gene expression.

mean values derived from each diseased gingival sample over the expression of healthy ones (Z-axis) in the network model 'APOP', presenting them as two- and three-dimensional (2D and 3D) ViaComplex-generated landscapes (Fig. 1B and C). Analysis of differential gene expression showed that a total of 49 out of 70 genes belonging to the network model were differentially expressed (corrected p-value < 0.05) in periodontitis samples in comparison to healthy controls. *CSF2RB*, *NFKBIE*, *ENDOG*, *CASP10*, *CASP3*, *BID*, *CFLAR*, *IL1B*, *ATM* and *BCL2* were the most differentially expressed genes (Table 1).

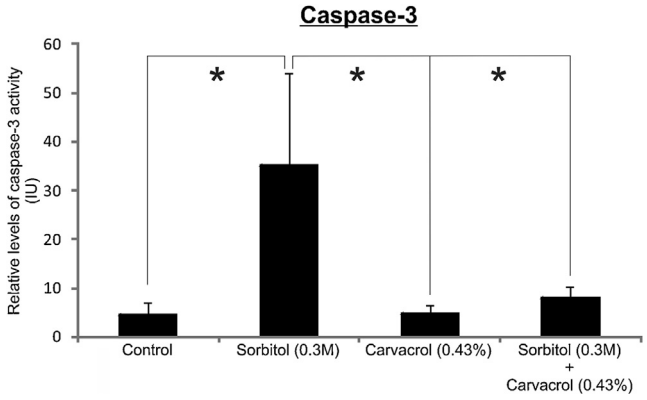

Fig. 2 – Effect of carvacrol against sorbitol-induced caspase-3 activity on HaCaT cells. Differences between treated and control cells were considered significant (*) when *p*-value < 0.05.

3.2. *Carvacrol inhibits sorbitol-induced caspase-3 activity in epithelial cells*

After treating epithelial cells with 0.3 M of sorbitol for 1 h, there was a significant increase in caspase-3 activity (p-value < 0.05) when compared to control cells (Fig. 2). Sorbitol-induced caspase-3 activation was prevented when cells were pretreated with carvacrol (0.43%) for 10 min (p-value < 0.05; Fig. 2).

3.3. *Modest antibacterial activity of carvacrol against periodontopathogenic strains when compared to metronidazole*

Carvacrol (4.3%) displayed a modest antibacterial activity against the tested periodontopathogens (Table 2) except for a strain of *P. gingivalis* (AHN 24135), when compared to our positive control (metronidazole). Metronidazole was strongly effective against the examined bacterial strains except for *A. actinomycetemcomitans* (AHN 24195 and NTCC 9710), *F. nucleatum* (AHN 9508) and *P. gingivalis* (AHN 24135). DMSO (a negative control) did not show any antibacterial effect.

4. Discussion

In the present study, we characterised the landscape of interactions of apoptosis-related genes/proteins by constructing an *in silico* network model where 70% of these genes were differentially expressed in periodontitis patients. In addition, we showed *in vitro* evidence for the anti-apoptotic properties of carvacrol on epithelial cells and its antibacterial effect against a number of periodontopathogens. To the best of our knowledge, this is the first study reporting that carvacrol is able to inhibit caspase-3 activity in human epithelial cells when challenged with sorbitol. The use of one single concentration of carvacrol for the *in vitro* experiments could be seen as a weakness in the present report. However, it has to be clearly remarked that this set of experiments represents a continuation study from our recently published work, where we showed that 0.5% of *S. hortensis* L. EO did not trigger cytotoxicity and prevented oxidative stress-induced epithelial cell death.[18] Therefore, in the present report, we tested whether the amount of carvacrol found in such a concentration of EO (0.43% carvacrol) could additionally exert anti-apoptotic properties *in vitro*. To achieve this goal, we challenged human epithelial cells with sorbitol (0.3 M)[26] which is a well-known inducer of apoptosis, mediated by oxidative/nitrosative stress rather than a canonical hyperosmotic shock,[27] and checked the potential anti-apoptotic capabilities of carvacrol *in vitro* by measuring caspase-3 activity. According to our results, 0.43% carvacrol was able to prevent the sorbitol-induced increase of caspase-3 activity in human epithelial cells (Fig. 2). Both caspase expression and activation are connected by various apoptotic signalling systems. Caspases are synthesised as inactive precursors (pro-caspases) and are

Table 2 – Antibacterial effects of carvacrol against periodontitis-associated bacteria. Symbols: −, no antimicrobial activity, inhibition zone <1 mm; + modest antimicrobial activity, inhibition zone: 2–4 mm; ++, clear antimicrobial activity, inhibition zone: 5–10 mm; +++, strong antimicrobial activity, inhibition zone >10 mm.

Bacterial strain		Metronidazole (5 μg) (positive control)	Carvacrol (43 μl ml^{-1})
Aggregatibacter actinomycetemcomitans	AHN 24195	−	+
	NTCC 9710	−	+
Fusobacterium nucleatum	AHN 9508	−	+++
	ATCC 25586	+++	++
Parvimonas micra	AHC 15107	+++	+
	AHC 15154	+++	+
	ATCC 33270	+++	+
Porphyromonas gingivalis	AHN 24135	−	−
	AHN 24155	+++	+
	ATCC 33277	+++	+
Prevotella intermedia	AHN 8290	+++	++
	ATCC 25611	+++	+
Prevotella nigrescens	AHN 8293	+++	++
	ATCC 33563	+++	+

activated upon cleavage by other caspases.[28] Cleavage and activation of pro-caspases result in an amplified proteolytic cascade reaction. Therefore, it should be remembered that the activity of caspases does not necessarily relate with its expression. Carvacrol-induced inhibition of apoptosis is likely due to its reported antioxidant properties *in vitro*.[29] This is consistent with another report where carvacrol attenuated the impairment caused by acute myocardial infarction of rats, and these beneficial effects were also attributed to its antioxidative potential.[30]

In periodontitis, epithelial cells form the first barrier against invasion and periodontitis-associated bacteria, such as *A. actinomycetemcomitans*, are able to invade epithelial cells by stealth without triggering inflammation through the induction of apoptosis.[31] These bacteria possess the ability of promoting apoptosis in macrophages which could be important also for the initiation of infection and the development of periodontitis.[32] Moreover, *A. actinomycetemcomitans*, after 24 h of exposure, is able to induce the overexpression of genes involved in tissue remodelling and bone resorption (*e.g.*, *CSF2*), genes encoding components of the low-density lipoprotein (LDL) pathway, as well as nuclear factor-κB-dependent genes and cytokines in gingival epithelial cells.[33] In addition, *A. actinomycetemcomitans* has been reported to produce leucotoxin that selectively lyses primate neutrophils and monocytes, that is, major populations of defence cells in the periodontium, and their lysis by such a toxin seems to depend on caspase-1 activation.[34] Despite these reports, the question is whether apoptosis could be considered a generalised pathological process present in periodontal inflammation. Our pathway-focussed microarray analysis revealed that the majority of genes (49 out of the 70 genes) belonging to our network model were differentially expressed (up- or down-regulated) in patients with periodontitis (Fig. 1 and Table 1), suggesting that apoptosis could be, in general terms, an important biological event in this pathogenic context. These results are consistent with other studies performed at the clinical level. For instance, Makhoul et al., who examined the putative link between apoptotic biomarkers in gingival crevicular fluid and periodontal destruction in four cases of localised aggressive periodontitis, found a significant increase in the apoptotic markers Fas/FasL together with DNA fragmentation when comparing gingival crevicular fluid from diseased *versus* non-diseased sites in patients.[35] Furthermore, periodontitis is well recognised as one of the main complications of diabetes mellitus.[36] Recently, it was demonstrated that high glucose levels induces concentration- and caspase-3-dependent increase of apoptosis in cultured human periodontal ligament fibroblasts, whereas inhibitors of caspase-3 can prevent the high-glucose-induced apoptosis in these cells.[37]

Apoptosis is also a critical process for the resolution of inflammation as it is the mechanism by which neutrophil lifespan is regulated.[38] These cells are polymorphonuclear leucocytes and represent the host's first line of defence against bacterial and fungal infections, but their overactivity can cause tissue damage and prolong the extent and severity of inflammatory periodontal diseases. Neutrophil death by apoptosis together with a safe removal (of intact apoptotic neutrophils) by phagocytic cells (macrophages) contribute to limit tissue damage during the resolution of an inflammatory response.[38,39] The mechanisms by which neutrophil apoptosis is activated/inactivated are still under intense investigation but it seems to be clear, for instance, that a number of growth factors and cytokines, such as interleukin (IL)-1 beta, IL-2, IL-15, tumour necrosis factor-α, interferon-γ, BCL2, granulocyte colony-stimulating factor, granulocyte–macrophage colony-stimulating factor (CSF2) and lipopolysaccharides, are known to prolong the survival of these cells and impede neutrophil apoptosis, impairing the resolution of inflammation.[38,40,41] In our array analysis, *CSF2RB*, *IL1B* and *BCL2* are among the top ten up-regulated genes in periodontitis samples (Table 1), suggesting that neutrophil numbers within the present diseased tissues could be extremely high. By contrast, the caspase-cascade system plays critical roles in the induction, transduction and amplification of intracellular apoptotic signals.[42] Considering that both *CASP3* and *CASP10* were also highly up-regulated in periodontitis patients (Table 1), we speculate that both increases may be indicative of apoptosis of other cellular compartments than neutrophils, such as epithelial cells. Besides, our array analysis defines the apoptotic changes at the tissue level, without taking the cellular type into account, as the analysed samples come from healthy and diseased gingival tissues.[22] In the best-case scenario, any potential drug aimed to be used for the treatment of periodontal inflammation should protect epithelial cells against apoptosis in a pro-oxidant environment without inhibiting the constitutive neutrophil death (essential for modulating neutrophil homeostasis), display an analgesic effect and show antibacterial properties against common periodontopathogens. Interestingly enough, it has been already demonstrated that pre-treatment with carvacrol is able to significantly attenuate the number of neutrophils and exert anti-nociceptive activity *in vivo*.[43,44] Our data now show evidence of anti-apoptotic properties of carvacrol in human epithelial cells when challenged with sorbitol (Fig. 2). Thus, the last question to be addressed was whether carvacrol exerts any antibacterial activity against periodontitis-associated pathogens. Our group described the antibacterial effect of *S. hortensis* L. EO against periodontitis-associated bacteria and its anti-gelatinolytic properties against PMA-induced MMP activity of human epithelial cells.[18] As this EO from *S. hortensis* L. contained 86% of carvacrol, its potential antibacterial properties were further tested against the same periodontal bacterial strains. In general, a modest antibacterial effect was seen against all tested bacteria, except for one strain of *P. gingivalis* (AHN 24135), by the disc diffusion assay. This strain was also resistant to 5 μg of metronidazole, which was used as our positive control (Table 2). Moreover, neither *A. actinomycetemcomitans* (AHN 24195 and NTCC 9710) nor *F. nucleatum* (AHN 9502) was sensitive to metronidazole. Such a discrepancy can be explained by the fact that the resistance or susceptibility of periodontal bacteria against metronidazole is strain dependent.[45] One final point to address is that, in the present study, anti-apoptotic effects of carvacrol were tested on a non-tumorigenic HaCaT keratinocyte cell line. HaCaT cells have been widely used in studies on host–bacteria interactions and our group has demonstrated infection-related responses of this same cell line (*i.e.*, proliferation, cell death, IL-8 and beta-defensin secretion) against periodontal pathogens.[46–49]

To the best of our knowledge, this is the first study showing a focussed microarray analysis of apoptosis-related genes in the context of periodontal inflammation. Several genes involved in this biological process are deregulated in periodontitis. Anti-apoptotic properties of carvacrol, displayed in the present study, refer to the need for further therapeutic exploration of this compound.

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Competing interests

None declared.

Ethical approval

Not required.

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