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Keratin-based peptide: biological evaluation and strengthening properties on relaxed hair

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Synopsis

A peptide based on a fragment of hair keratin type II cuticular protein, keratin peptide (KP), was studied as a possible strengthening agent for weakened relaxed hair. The peptide was prepared both in aqueous water formulation (WF) and organic solvent formulations (OF), to determine the effect of organic solvents on peptide interaction with hair and the differences in hair recovery. Both peptide formulations were shown to improve mechanical and thermal properties of weakened hair with peptide in OF showing the stronger effect. As a potential new hair care product, and so would necessitate contact with skin, the cytotoxicity and genotoxicity of the peptide were also evaluated through different methodologies (Alamar Blue assay, 2′-7′-dichlorofluorescein probe, cell morphology and growth and evaluation of DNA damage by an alkaline version of the comet assay) in skin fibroblasts. These tests are indicators of the potential of peptide to cause irritation on skin or to be carcinogenic, respectively. The peptide in WF did not cause cytotoxicity or genotoxicity in any of the concentrations tested. The presence of OF, however, induced a 20% decrease in cell viability in all of the range of concentrations used after 72-h incubation. Moreover, OF inhibited cell growth and was considered genotoxic at first contact with cells. The peptide was therefore considered a promising strengthening agent for hair and was shown to be innocuous when applied in WF.

Résumé

Un peptide, fragment de de la protéine cuticulaire keratine type II, le ''keratine peptide'' (KP), a été étudié comme possible agent de renforcement pour des cheveux défrisés affaiblis. Le peptide a été préparé en solution aqueuse (WF) et dans des formulations aux solvants organiques (OF), afin de déterminer l'effet des solvants organiques sur l'interaction du peptide avec les cheveux et les différences sur la restitution du cheveu. Les deux formulations du peptide ont montré une amélioration des propriétés mécaniques et thermiques des cheveux affaiblis; les formulations aux solvants organiques se montrant plus efficaces. La cytotoxicité et la génotoxicité du peptide ont également été évaluées dans les fibroblastes de la peau, par des méthodes différentes (dosage au bleu d'Alamar, sonde au 2′-7′- dichlorofluorescéine, morphologie et prolifération des cellules, évaluation des dommages à l'ADN par une version alcaline du test COMET). Ces tests sont des indicateurs du potentiel irritant du peptide ou d'être cancérigène, respectivement. Le peptide dans WF n'a pas provoqué de cytotoxicité ou de génotoxicité, à aucune des concentrations testées. La présence de OF, cependant, a induit une diminution de 20% de la viabilité cellulaire dans l'ensemble de la gamme des concentrations utilisées après incubation 72h. En outre, une inhibition de la croissance cellulaire a été observée et la formulation a été considérée comme génotoxique lors du premier contact avec des cellules. Le peptide a donc été considéré comme un agent prometteur de renforcement pour les cheveux et s'est avéré inoffensif lorsqu'il est appliqué dans WF.

Introduction

Black African hair is characterized by its extremely curly shape [1], being very difficult to comb both in its wet and dry states, hard to style and highly unmanageable [2]. As a consequence, it requires more drastic approaches to styling than any other type of hair. Whether it is alkaline relaxing or repeated hot combing, common styling techniques for this type of hair leave the hair very fragile, and it requires special care to maintain its healthy appearance. Even when intact, African hair is very brittle [3]. It, therefore, requires special handling and treatment with specifically formulated products that are different from Caucasian hair care formulas [2].

Our group has developed a 13 amino acid keratin-based peptide (KP) for application on relaxed damaged hair with proven characteristics of recovering mechanical properties of over-bleached damaged hair [4]. Because of its properties, this peptide may constitute a potential candidate for a new product development. For this reason, the peptide was evaluated for potential risks from contact with skin via the study of cell viability, cell morphology and genotoxicity. The first three tests evaluated the potential for skin damage, while the third test evaluated the induction of cell death and/or the increase in DNA mutation rate.

The safety assessment of cosmetic products is one of the most important steps when a new product is created. Cosmetics and their ingredients must not be harmful to human health under normal or foreseeable conditions of use [5]. Prior to human testing,

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[1]Calculated with ''Peptide Property Calculater'' available at www.innovagen.se.

an initial product safety assessment is usually performed *in vivo* on animals. However, ethical and financial objections to these methods have been raised as well as scientific drawbacks such as intra- and interlaboratory variability, good predictability only for strong irritants and the fundamental difference between animal and human in morphological aspects of skin [6]. As a consequence, several *in vitro* alternatives to animal testing have recently been proposed to predict the *in vivo* skin irritancy potential of a variety of test agents [7, 8]. The Alamar Blue (AB) assay has been introduced as an alternative cell viability indicator and used to estimate the cytotoxicity of chemicals on cultured cells [9, 10].

The genetic toxicity tests are also an important analysis because they are predictive not only the potential carcinogenic, but also the mutagenic hazards. They are principally regarded as short-term screening studies for the prediction of the carcinogenic potential of substances [5, 11].

In this study, KP was studied as a new strengthening agent for damaged hair. Several concentrations of peptide in the presence of both aqueous and organic solvent formulations were tested, and the cytotoxicity and carcinogenic potential was studied, as well as the reinforcement of hair physical and chemical properties.

Materials and methods

Materials

Hair

Natural African black curly human hair samples were provided by International Hair Importers & Products Inc. (Glendale, New York, U.S.A.), and previous to use, a washing procedure with a commercial shampoo was performed.

Peptide

Keratin peptide (KP) used in this work was synthesized by JPT Peptide Technologies GmbH (Germany). The peptide was developed based on the amino acid sequence of cuticular keratin type II from human hair [12] and possess a sequence with thirteen amino acids comprising two cysteine residues (TAMRA-$X_3CX_5CX_3$-$CONH_2$) with a molecular weight of 1599.8 g mol^{-1}. The characteristics of this peptide were presented in previous work [13].

Table I Peptide concentration keratin peptide (KP) and conditions used in the citotoxicity (Alamar Blue) and genotoxicity assay

	KP concentration
WF 100% Phosphate buffer	0 (Negative control)
	0.025 g L^{-1}
	0.05 g L^{-1}
	0.1 g L^{-1}
	0.2 g L^{-1}
	0.5 g L^{-1}
OF	0 (Negative control)
10% Ethanol	0.025 g L^{-1}
1.5% Propylene glycol	0.05 g L^{-1}
0.5% Benzyl alcohol	0.1 g L^{-1}
88% Phosphate buffer	0.2 g L^{-1}
	0.5 g L^{-1}

OF, organic solvent formulations.

The peptide was either dissolved in an aqueous solution (0.05 M phosphate buffer solution, pH 7.5) (WF) or in an organic solvent formulation (OF). In the latter case, the composition comprised the following: peptide, ethanol (10%), propylene glycol (1.5%), benzyl alcohol (0.5%) and phosphate buffer solution at pH 7.5 (88%). Concentrations from 0.025 to 0.5 g L^{-1} of peptide were prepared (Table I). The sequence of the peptide is described by one-letter code, in which X represents one of known amino acid residues, with the exception of cysteine residue that is represented by the letter C.

Methods

Hair relaxing treatment

The black African hair was relaxed by the conventional treatment with strong alkali, in this case, sodium hydroxide. This breaks the disulphide bonds and from these broken bonds are formed lanthionine linkages that hold the hair in its new straightened configuration [2]. This process, thus, results in the permanent rearrangement of the internal protein structure of the hair. Hair tresses of 0.2 g were placed in contact with a 0.75 M sodium hydroxide (NaOH) solution for 30 min. The high alkalinity (pH = 12–14) swells the hair and opens the cuticle, allowing the alkaline agent to penetrate the hair fibre and diffuse into the cortex. The NaOH reacts with the keratin protein, breaking the structural disulphide bonds in the hair. The hair was then extended to a straight configuration by the use of a comb. The alkaline agent was then washed out from hair, and a solution of 0.1 M acetic acid was used as a neutralizing agent to initiate the reformation of new disulphide crosslinks and to close down the cuticle. These new bonds lock and the new hair shape is created. The neutralizing agent was then washed out using a commercial shampoo.

Application of peptide on relaxed hair

Hair relaxed samples (0.2 g) were treated or with 3 mL of water formulation (WF) 0.05 M phosphate buffer solution pH 7.5; or with 3 mL of organic solvent formulation (OF) in the presence of KP at final concentration of 0.2 mg mL^{-1}. The treatment was carried out at 37°C in a bath with orbital agitation, 100 rpm for 1 h. After treatment, the hair samples were washed with commercial shampoo and under running water. As control, the hair was treated in the same way, omitting the peptide in the solution.

Mechanical properties

The effect of peptide application on both WF and OF buffers was assessed by the differences in the mechanical properties. For this test, a set of hair fibres with low variability in diameter was selected using a light microscope. The method used broadly follows the guidelines laid down in ASTM D1445-95 for the tensile testing of fibres. The measurements were performed with an Instron 4505 tensile tester with a maximum load cell capacity of 2.5 N. For each measurement, 20 single hair fibres were taken randomly from the tress. Each hair was individually mounted in the tensile jig by means of a paper template with a fixed gauge length of 20 mm. Before the tensile tests were started, the paper template was cut across, so that just the hair fibre was fixed as a continuous length within the jig. The measurements were performed under controlled conditions (20 ± 0.5°C; 55 ± 4% humidity), at a rate of 1.5 mm min^{-1}, until breakage occurred. For each hair, records of applied load against extension were taken, and using an average mean diameter of 70 μm, calculated through the analysis of microscopy images of hair transversal cuts (Fig. 1), the data were converted to stress (load/unit area) against strain (% exten-

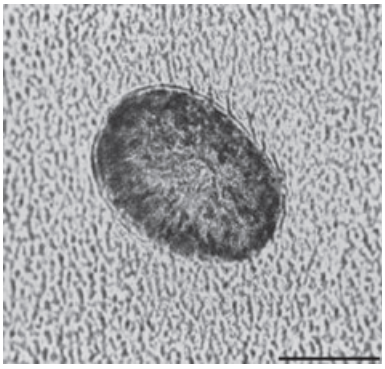

Figure 1 Light microscopy of an African hair transversal cut. Bar indicates 50 μm.

sion). Statistic Dixon's Q test was applied to identify and reject outliers.

Differential Scanning Calorimetry (DSC)
To study the thermal and chemical characteristics of hair fibres, a DSC study was performed. All investigations were conducted on a power-compensated DSC instrument (DSC-7; Perkin Elmer, Waltham, MA, U.S.A.) using pressure-resistant (25 bar), stainless steel, large-volume capsules in the temperature range of 50–250°C (heating rate: 5°C min^{-1}, sample weight: 7–8 mg). The DSC device was calibrated using indium and palmitic acid, both of high purity. Prior to analysis, the hair samples were extensively rinsed and allowed to dry at selected levels of humidity (RH of 45%) and temperature (20–22°C) for 24 h. The hair was then finely sliced into small pieces until a powder-like state, and around 7 mg of hair 'powder' was measured at least three times, to validate the results.

Peptide safety assessment
Different peptide concentrations in WF or OF buffers (Table I) were filtered in a sterile environment and, subsequently, diluted (100%, 10% (v/v)) in complete culture medium. The culture medium itself was used as a negative control, whereas a 200 μM solution of the oxidant *tert*-butyl hydroperoxide (*t*-BOOH) (Sigma, Sintra, Portugal) prepared in fresh culture medium was used as a toxic positive control.

Culture of human skin fibroblasts cell line (BJ-5ta)
The BJ-5ta cell line (ATCC, CRL-4001) was maintained according to ATCC recommendations (four parts Dulbecco's BJ5TA Modified Eagle's Medium (DMEM) containing 4 mM L-glutamine, 4.5 g L^{-1} glucose, 1.5 g L^{-1} sodium bicarbonate and 1 part of Medium 199, supplemented with 10% (v/v) of foetal bovine serum (FBS), 1% (v/v) of penicillin/streptomycin solution and 10 μg mL^{-1} hygromycin B). The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. Culture medium was replaced every 2 days.

Cytotoxicity evaluation

Alamar Blue assay
Cells were seeded at a density of 20×10^3 cells per 100 μL per well on 96-well tissue culture polystyrene (TCPS) plates (TPP, Switzerland) the day before experiments and then exposed to the peptide and incubated at 37°C in a humidified atmosphere with 5% CO_2. Cells were examined at 24, 48 and 72 h for signs of toxicity, using Alamar Blue assay (alamarBlue® Cell Viability Reagent; Invitrogen, Alcobendas, Madrid, Spain). Resazurin, the active ingredient of alamarBlue® reagent, is a non-toxic, cell-permeable compound that is blue in colour and reduced to resorufin, red colour compound, by viable cells. Of 10 μL of alamarBlue® reagent was added to each well containing 100 μL of culture medium. After 4 h of incubation at 37°C, the absorbance at 570 nm was measured, using 600 nm as a reference wavelength, in a microplate reader (Spectramax 340PC, Molecular Devices, Sunnyvale, CA, U.S.A.). The quantity of resorufin formed is directly proportional to the number of viable cells. The procedure was performed at least three times with duplicates, and the average values are presented.

2′-7′-Dichlorofluorescein assay
The 2′-7′-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA) (molecular probes) has been used as a substrate for quantitatively measuring intracellular oxidant production. $DCFH_2$-DA is deacetylated by esterases to 2′-7′-dichlorodihydrofluorescein (DCFH2), which is not cell permeable, so it remains within the cell. This non-fluorescent molecule is then oxidized to fluorescent 2′-7′-dichlorofluorescein (DCF) by the action of cellular oxidants. DCF can be detected by its fluorescence at 525 nm. Cell culture medium was removed, and cells were washed twice with PBS to remove endogenous esterase activity. Cells were loaded with 100 uM $DCFH_2$-DA for 30 min in the dark at 37°C. Extracellular $DCFH_2$-DA was removed by washing twice with PBS. Hydrogen peroxide was added to the positive control in this phase (1 : 5 v/v). The cells were lysed by adding 500 μL of 90% DMSO solution and 10% PBS (v/v) for 10 min in the dark at room temperature with shaking. Cell lysates were transferred to a 96-well black assay plate, and fluorescence intensity in each well was read at 25°C with a Fluoroskan Ascent FL plate reader (Thermo Scientific, Alcobendas, Madrid, Spain), using an excitation wavelength of 485 nm excitation and an emission wavelength of 520 nm.

Cell morphology
Morphological changes in cells were also followed by phase contrast microscopy (IX71; Olympus), after 1 h and 72 h of contact with peptide.

Genotoxicity evaluation (Comet assay)
One day before use, fibroblasts were seeded at 40 000 cells mL^{-1} for the 72-h genotoxicity assays and at 100 000 cells mL^{-1} for the 1-h genotoxicity assays. After treatment, cells were harvested by trypsinization and used for the comet assay. The alkaline version of the comet assay was performed as previously described [14]. In brief, 40 000 cells were embbebed in 1% w/v low-melting-point agarose and spread onto agarose-coated slides. Slides were immersed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10 with NaOH, Triton X-100 1% v/v added fresh) at 4°C for 2 h. Then slides were immersed in electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13) at 4°C and incubated for 40 min for alkaline unwinding of DNA. Afterwards, electrophoresis was performed for 20 min, 300 mA, at 0.8 V cm^{-1} in a cold room (4°C). Finally, the slides were neutralized by washing them three times for 5 min each with 0.4 M Tris, pH 7.5, at 4°C. For the analysis of the comet images, slides were stained with SYBR Gold (Invitrogen), and images were taken under a fluorescent microscope (IX71; Olympus, Hamburg, Germany). Images were then analysed using a free comet scoring software Comet Score (TriTek Corp., Sumerduck, VA, U.S.A.).

Data analysis

Replicate samples were used for each measurement, and all the values are presented as averages ± the respective standard deviations (SD) or standard error of mean (SEM). Results were analysed using GRAPH PAD PRISM version 5.04 for WINDOWS (Graph Pad Software, San Diego, CA, U.S.A.). Statistical significances were determined using a one-way ANOVA followed by the Dunnett *post-hoc* test or by the unpaired two-tailed Student's *t*-test method. P values ≤ 0.05 were considered statistically significant.

Results and discussion

KP is able to recover the mechanical and thermal properties of damaged hair

Keratin peptide, a peptide derived from keratin intermediate filaments, was previously shown to partially recover the mechanical and thermal properties of over-bleached blond hair [4]. For that reason, we have used the same peptide to study its ability to strengthen weakened relaxed African hair.

To develop KP, a fraction of the amino acid sequence of keratin type II cuticular Hb5, a protein that in humans is encoded by the KRT85 gene [15, 16], was mimicked, and a fragment of 13 amino acids comprising two cysteines separated by four other amino acids was then selected and chemically synthesized. Because of its origin, from keratin intermediate filaments (KIF), this peptide is thought to comprise affinity towards hair surface and ability to restore damaged cuticle parts. Also, as type II alpha-keratin, it might possess a resilient and pliable properties [17], which can be translated to the hair fibre. This peptide constitutes a novel approach for the development of a cosmetic formulation, because previously reported keratin-based peptides for hair reinforcement are based on the cysteine-rich keratin-associated proteins (KAP) from hair [18–20].

Characterized by its extremely curly shape, African hair requires drastic approaches such as relaxing treatments to style it. These techniques, used to permanently straighten curly hair, use strong bases, such as sodium hydroxide or guanidine hydroxide, etc., to disrupt the disulphide bonds responsible for the hair shape and rearrange them to make the hair straight.

It is an effective treatment, but very aggressive to the hair fibre inducing cuticular damage at the surface of the hair and weakening of the fibre [2]. As KP was found in a previous study to increase the mechanical resistance and thermal properties of over-bleached hair to similar values of untreated virgin hair [4], the ability of this peptide to improve the relaxed weakened hair properties was evaluated.

The fragility of the hair fibre after this treatment is because of the fact that one-third of the disulphide bonds are transformed into lanthionine bonds [2]. Young's modulus and tensile strength are therefore important parameters to determine the hair's resistance. The relaxing treatment induced 35% of resistance loss when compared with virgin, unaltered hair, measured in terms of Young's modulus. The maximum strength of the hair fibres also decreased by *c.* 36% (Fig. 2).

The application of KP on hair was tested in two different environments, WF and OF. The reason for using this organic solvent formulation is that, despite this peptide being soluble in aqueous solutions, it possess a certain hydrophobicity (ratio of hydrophilicity = −0.4).[1] Hydrophobic peptides are usually applied together with a formulation comprising a surfactant such as dipalmitoylphosphatidylcholine or an organic solvent such as ethanol.

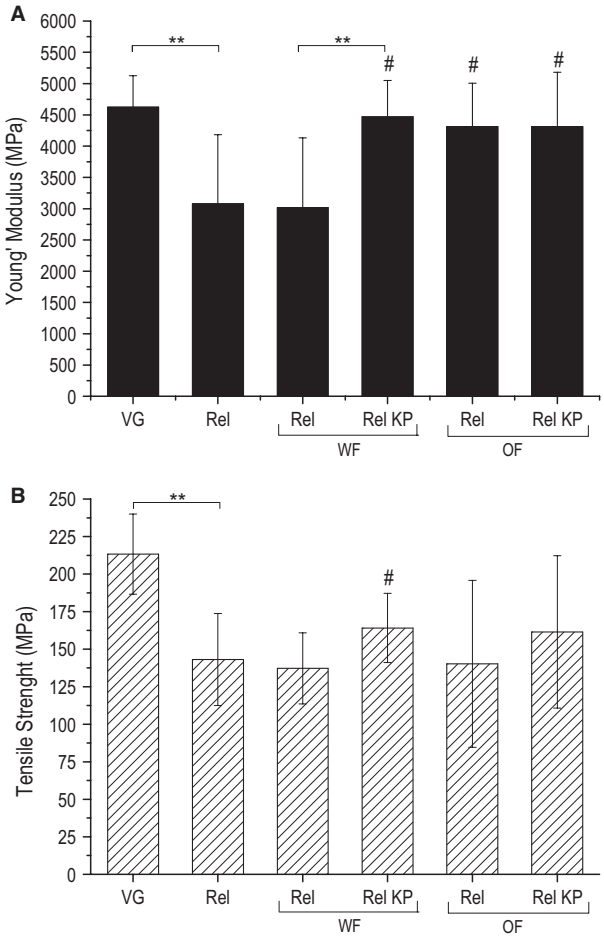

Figure 2 Mechanical resistance parameters: (A) Young's modulus and (B) tensile strength of virgin hair (VG) of relaxed black hair (Rel) and relaxed black hair treated with 0.2 mg mL^{-1} keratin peptide keratin peptide in the presence of aqueous formulation (WF) and organic solvent formulation. Values are mean ± SD (standard deviation) of eight independent experiments. $^{**}P \leq 0.01$ when compared with each other, $^{\#}P \leq 0.05$ when compared with relaxed hair. No significant differences were observed between the other conditions and the relaxed hair.

These formulations are used to attain a peptide formulation compatible with a water environment [21], thus improving the interaction with biological membranes. Using the same approach, we have used a mixture of 10% ethanol, 1.5% propylene glycol and 0.5% benzyl alcohol in 0.05 M phosphate buffer solution, pH 7.5 (OF formulation). Here, it is thought that the peptide might possess a different conformation to that in phosphate buffer solution alone, thus perhaps facilitating its penetration into the hair fibre.

Evidences that KP treatment improved hair mechanical properties when it was applied either in WF or OF environment were observed. Both Young's modulus and tensile strength results suggest that the hair resistance loss caused by the relaxing treatment was partially regained after KP treatment (Fig. 2). The hair regained 28–31% of its Young's modulus and 13% of maximum strength, in both peptide formulations. An increase in the elongation values was also observed (data not shown); however, these results were not statistically different.

An interesting result was that application of OF alone, that is, in the absence of peptide, was able to increase the Young's modulus of hair by ~28%, despite the maximum strength parameter still low. It is widely accepted that mechanical and thermal properties of hair are strongly influenced by their immediate chemical environment, for example, treatment formulations [22–24]. Therefore, a DSC study was also performed.

Although human hair exhibits complex morphology as an α-keratin fibre, for the context of thermal analysis, it is considered as a filament/matrix composite, as originally proposed by Feughelman [25, 26]. In this model, the α-helical fraction of the intermediate filaments (IF) comprises the crystalline filament phase that is embedded in an amorphous matrix, which is represented by the intermediate filament-associated proteins (IFAPs) and the rest of the amorphous morphological components.

Four main parameters were analysed by DSC: the temperature and enthalpy of water removal (Td_{H2O} and ΔHd_{H2O}), which indicate the extent of water absorption on amorphous matrix phase; and the temperature and enthalpy of α-helix keratin denaturation ($Td_{\alpha\text{-helix}}$ and $\Delta Hd_{\alpha\text{-helix}}$), which indicate the chemical changes occurring in the crystalline phase (α-helix chain) of the fibre.

Tensile strength parameter may be correlated and compared with Td_{H2O} and ΔHd_{H2O}. When the hair fibre is damaged, both parameters are expected to be decreased. The former parameter is decreased because of cuticle layer depletion after relaxing treatment, while the latter is decreased because of higher water evaporation escape pathways in cuticle. Therefore, if KP is able to penetrate the hair shaft or simply cover the hair's surface by filling in voids, the robustness of the fibres will increase along with the increase in temperature at which the water is evaporated.

Similarly, the Young's modulus of the hair fibre may be correlated with $Td_{\alpha\text{-helix}}$ and $\Delta Hd_{\alpha\text{-helix}}$ parameters. The latter parameter is a measure of the chemical environment on the crystalline phase of hair, while the former parameter is a measure of the resistance of alpha-helix chain to be unfolded (alpha-helix transformation in beta-sheet).

In fact, there are evidences that these parameters were decreased when hair was subjected to relaxing treatments (Table II), which confirmed a degree of chemical damage to the fibre. The temperature of water removal dropped by around 6°C, because of the plasticizing effect of water on the fibre, which more readily penetrates the damaged hair shaft, thereby decreasing the energy for it to occur (Table II). The α-helix denaturation temperature decreased by 2°C, and the α-helix denaturation enthalpy dropped from 6.76 J g^{-1} to 3.33 J g^{-1}. The relaxing treatment breaks the disulphide bonds and chemically reforms them to change the shape of the hair leading to the weakening of the hair structure, explaining the decrease of thermal properties.

Table II Thermal characteristics of relaxed black hair treated with 0.2 mg mL^{-1} keratin peptide (KP) in the presence of aqueous formulation (WF) and organic solvent formulation (OF)

	Td_{H2O}(°C)*	ΔHd_{H2O} (J g^{-1})*	$Td_{\alpha\text{-helix}}$(°C)*	$\Delta Hd_{\alpha\text{-helix}}$ (J g^{-1})*
Virgin hair	92.8 ± 0.8	244 ± 4	229.7 ± 0.1	6.8 ± 0.2
Relaxed hair	87 ± 9	206 ± 49	227.9 ± 0.5	3.33 ± 0.05
Relaxed hair				
WF	88 ± 3	207 ± 23	227.2 ± 0.9	3.41 ± 0.8
KP in WF	91 ± 1	262 ± 2	228.9 ± 0.1	4.66 ± 0.03
OF	97 ± 1	267 ± 1	227.9 ± 0.2	4.9 ± 0.1
KP in OF	93 ± 1	270 ± 16	228.2 ± 0.4	4.8 ± 0.3

*Data present mean ± SD (standard deviation) from a minimum of three experiments

The application of KP in WF significantly increased the temperature and enthalpy of water removal, indicating that less absorption of water by the amorphous matrix phase occurred, probably due to the fact that KP is able to replenish the holes created after relaxing treatment on hair's cuticle [4]. An increase in the temperature and enthalpy of α-helix keratin denaturation of damaged hair was also observed, indicating a higher energy needed to disrupt the keratin α-helix structure because of the chemical interaction of peptide with α-helix filament of the fibre.

Interestingly, when KP is applied in OF, the same behaviour observed for Young's modulus parameter is detected when analysing the thermal properties, which showed an increase when OF was applied alone without peptide. The OF may be considered as a hair-swelling formulation, in which organic solvents are able to absorb and diffuse easily into hair via the defects on the surface because of the depletion of the hydrophobic lipid protection layer after the relaxing treatment. Less water is expected to be absorbed with increased content of organic solvents, which could explain the increase in the temperature and enthalpy of water removal. The increase in both Young's modulus parameter and enthalpy of α-helix denaturation could be explained by the ability of OF to interact with the hydrophilic molecules of the inner cellular structure of hair, changing the environment around α-helix chains.

Apparently, OF makes a positive influence on the recovery of hair mechanical and chemical properties. However, OF does not give rise to recovery of tensile strength (Fig. 2). On the other hand, when KP is applied with WF, significant increases in both hair resistance and thermal properties are also observed as a consequence of peptide interaction with hair fibre, showing the potential of peptide itself to truly repair damaged hair.

It is therefore proposed that a synergistic effect between OF and KP is observed, in which OF opens the structure of the hair fibre,

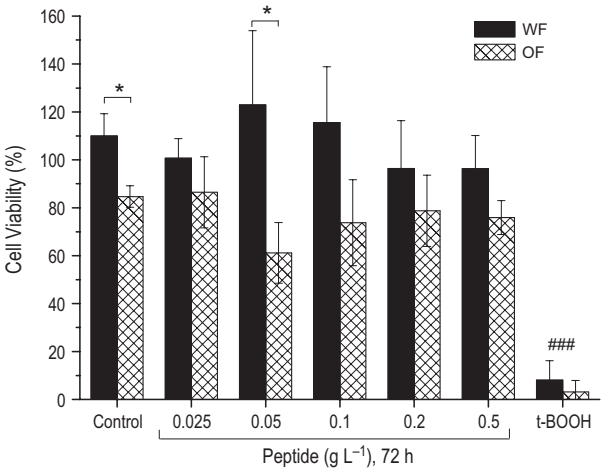

Figure 3 Viability of human normal skin fibroblasts cells after 72 h of contact with conditioned peptide keratin peptide. Values are mean ± SD (standard deviation) of four independent experiments. $^{*}P \leq 0.05$, when compared with each other, $^{\#\#\#}P \leq 0.001$ when compared with respective control. No significant differences were observed between each concentration of peptide and the respective control.

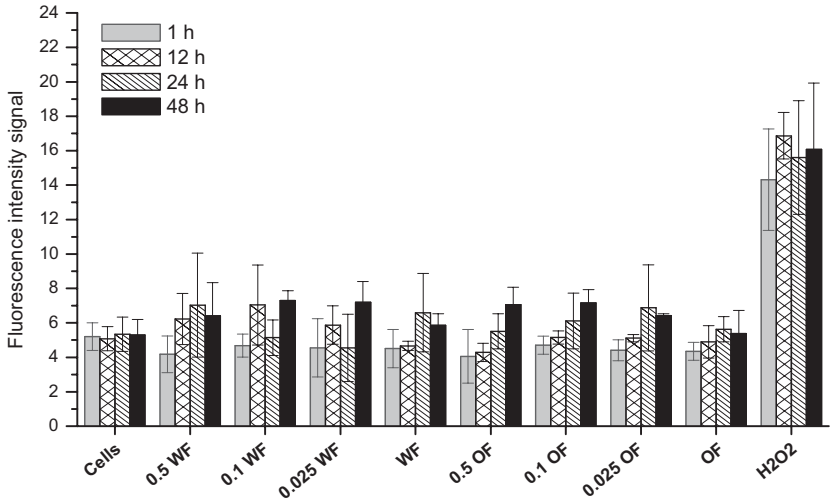

Figure 4 Fluorescence signal indicating the oxidative stress in human fibroblasts cells lysate, which were previously exposed to different concentration of peptide with aqueous (WF) and organic solvent formulations. Values are mean ± SD (standard deviation) of three independent experiments. $P \leq 0.001$ for all conditions tested when compared with H_2O_2 control. No significant differences were observed between each concentration of peptide and control cells.

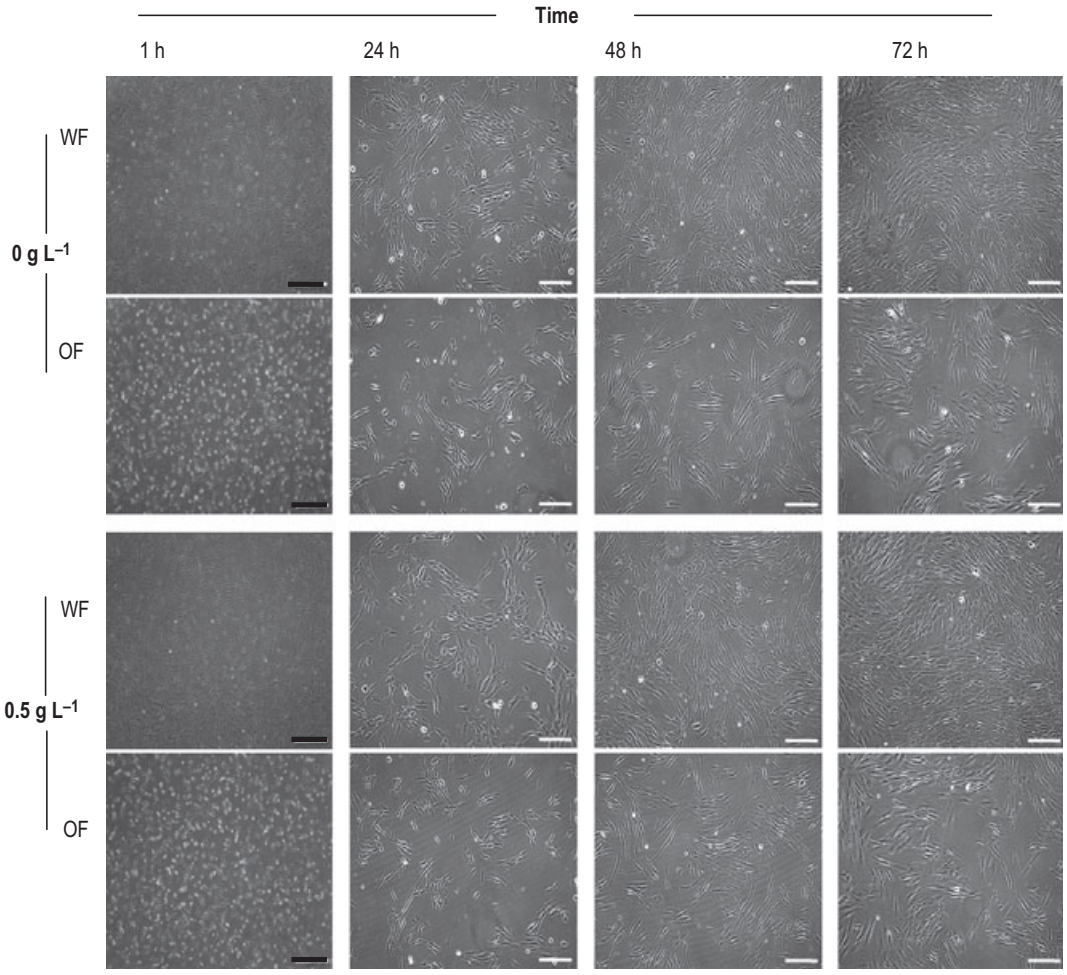

Figure 5 Effect of peptide on aqueous (WF) and organic solvent formulations on human fibroblasts after 1 h and during 72 h of incubation. Photographs are of a representative experiment from a total of four independent experiments. Black bar indicates 500 μm, while white bar indicates 200 μm.

allowing KP to penetrate inside the fibre cortex, and therefore increasing its mechanical (Fig. 2) and thermal properties (Table II). It would thus seem that combinatorial approaches based on KP and OF interaction comprise a good approach to developing new hair care formulations for treating damaged hair.

Peptide cytotoxicity evaluation

As these peptide formulations are to be applied on human hair and would therefore come into contact with the human scalp, the cytotoxicity was evaluated using an immortalized cell line of normal human foreskin fibroblasts (BJ5TA). A wide range of peptide concentrations were tested for the cytotoxic evaluation (Table I). The concentrations were chosen according to the conditions tested for treatment of hair samples.

Using the same approach of Lee *et al.* [6] to identify a possible skin irritation potential of peptide in both WF and OF environments, Alamar Blue (AB) assay was applied to cultured cells exposed to peptide. AB is reduced by cellular reductases to a pink-coloured chromophore, which can be quantified by either fluorimetric or spectrophotometric methods. It is a simple and rapid method used to estimate the cytotoxicity of chemicals on cultured cells [9]. Figure 3 shows the cell viability of human skin fibroblasts after 72 h of incubation with KP in WF and OF. The cell viability at 24-h and 48-h time points was also evaluated (data not shown), and the same behaviour represented for 72 h was observed. When the peptide was applied in the presence of WF, the cell viability is close to 100%, indicating that this formulation is not toxic and can be used in direct contact with hair scalp (Fig. 3). No significant differences in cell viability were observed between the different concentrations of peptide tested, which means that at these concentrations, the peptide should not cause skin irritation.

However, when the peptide was in the presence of OF, less cell viability was observed. We believe that this toxicity is because of the OF environment and not because of the peptide, as there are no significant differences between the peptide concentrations in OF and the negative control (OF alone) (Fig. 3).

To corroborate these results, the oxidative stress caused by the peptide formulations was tested using the 2′7′-dichlorofluorescein (DCF) assay [27–29]. This assay quantifies endogenously produced reactive oxygen species (ROS). ROS are essential to life, being involved in several biological functions [30, 31], but when overproduced (e.g. because of exogenous stimulation), or when the levels of antioxidants become severely depleted, these reactive species cause oxidation of biomolecules, leading to cellular damage that may become irreversible and cause cell death [32, 33]. It is therefore important to properly assay the propensity of new product to induce the overproduction of ROS in order to have a guideline for their proper use. The peptide in the presence of WF or OF induced low ROS on cells after 1 h, which started to increase with time to 72 h (Fig. 4). However, the ROS levels produced were not considered cytotoxic. The OF, in this case, did not cause oxidative stress on cells.

To corroborate the previous results, the morphology of cells in contact with peptide formulations was followed for 72 h by phase contrast microscopy. When fibroblasts were incubated with peptide dissolved in WF for 1 h, no cytotoxicity was observed (Fig. 5), with or without peptide. Cells presented normal morphology and grew continuously within the time line, as observed by the increased cell density. The peptide did not perturb cell growth. However, when cells were incubated with OF, significant cell morphological damages, that is, cells detach and round up, with or without peptide, were observed (Fig. 5), with smaller cell density. In addition, some cells were floating and others morphologically affected (stressed), indicating induction of cell damage and death by OF. The peptide did not improve or worsen the damaging effects of OF in cells until the highest concentration tested.

The decrease in cell viability observed when KP is in OF formulation (Fig. 5) can thus be explained by the OF capacity of inhibiting cell growth.

Genotoxicity evaluation of peptide

Damage to DNA has strong cellular implications and is involved in the termination of cell cycle, in the induction of cell death and/or in the increase in DNA mutation rate and carcinogenesis. The

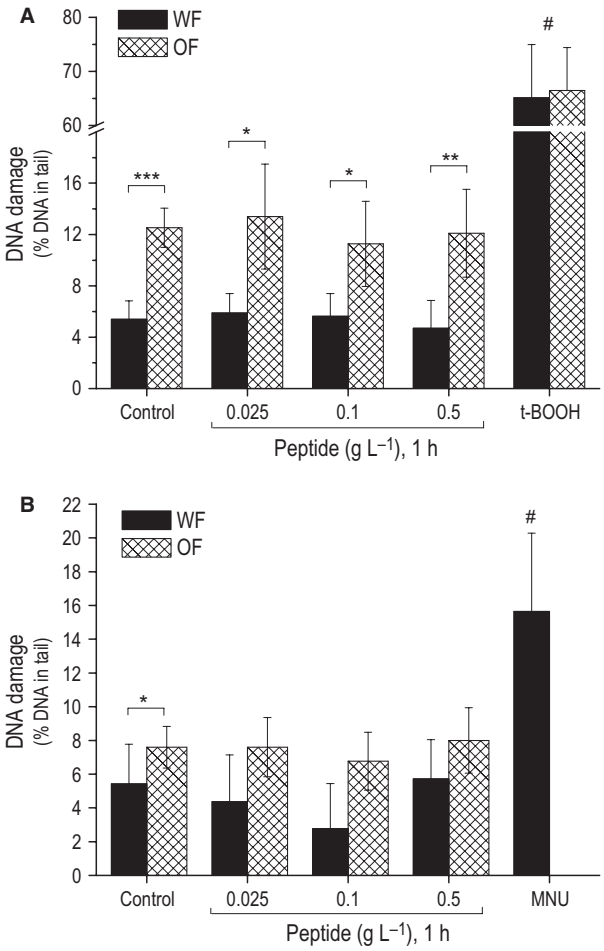

Figure 6 Effect of peptide and solvent formulations on DNA damage, expressed by the per cent of DNA in the tail of the comet (values are the average of 100 cells counted per condition), of human fibroblasts after (A) 1 h and (B) 72 h of incubation, measured by comet assay. *tert*-Butyl hydroperoxyde (*t*-BOOH) was used as positive control for 1-h evaluation and N-methyl-N-nitrosourea (MNU) for 72-h evaluation. Values are mean ± SD of four independent experiments. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ when compared with each other. $^{\#}P \leq 0.05$ when compared with the respective control. No significant differences were observed between each concentration of peptide and the respective control.

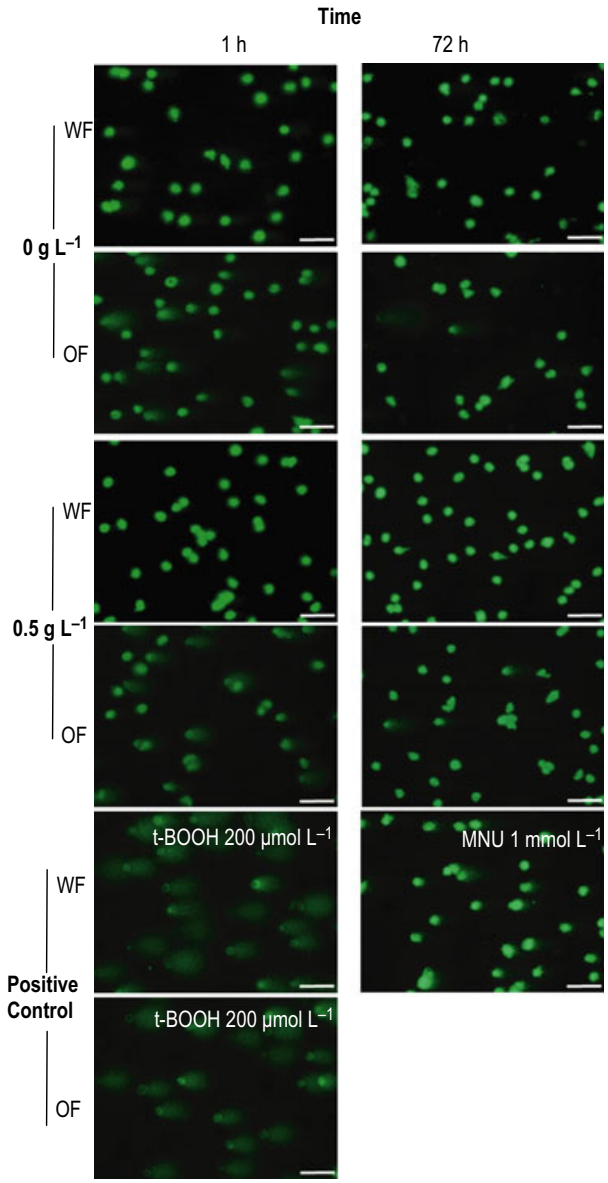

Figure 7 Effect of peptide in aqueous (WF) and organic solvent formulations on DNA damage of human fibroblasts after 1 h and 72 h of incubation. Photographs are representative of a comet assay experiment from a total of four independent experiments. Bar indicates 100 μm.

single-cell gel electrophoresis, or comet assay, is a sensitive, quick and simple method for evaluating DNA damage in single cells, and commonly used nowadays in genotoxic studies. After embedding cells in agarose and following cell lysis, and alkaline unwinding of the DNA, the negatively charged DNA is pulled towards the anode. However, only loops of DNA that contain breaks are able to migrate in the gel from the supercoiled DNA (head) forming the comet tail [34]. The percentage of DNA that is in tail, evaluated under fluorescence microscopy and using image analysis programs, is a reliable and one of the most used parameters to express DNA damage [34].

The potential genotoxicity of the peptide was evaluated by the comet assay exposing human fibroblasts to the peptide for a short (1 h) and prolonged (72 h) period of time. This methodology allows discriminating among short and direct damage to DNA that can be or not then repaired, and prolonged DNA damage including crosslinking of DNA with DNA and protein.

Cells were used for the comet assay to evaluate DNA damage. As shown in Fig. 6, the peptide did not induce DNA damage until the highest concentration tested (0.5 g L^{-1}). However, OF induced significant DNA damage and was not dependent on peptide. Damage induced by OF was not, however, significant to almost every cell, contrary to what happens to the positive control. In this case, there was heterogeneity on cell damage between cell populations. That can also be observed in a representative image taken from the comet assay (Fig. 7, 1 h). Taken together, we observed that some cells are more prone to DNA damage than others in the presence of OF for 1 h.

After 72 h of incubation, cells were used for the comet assay to evaluate DNA damage. As shown in Fig. 6, the peptide did not induce DNA damage until the highest concentration tested (0.5 g L^{-1}). OF still induced significant DNA damage and does not seem dependent on peptide. However, the extent of damage was remarkably smaller than the one observed after 1 h of incubation (Fig. 6), indicating that most of the cells after 72 h of incubation repaired DNA damage or are resistant cells. The carcinogenic compound N-methyl-N-nitrosourea (MNU) used as positive control induced significant DNA damage (Fig. 6) after 72 h of incubation. The overall DNA damage after 72 h of incubation can also be observed in a representative image taken from the comet assay (Fig. 7).

Comet images after 72 h of incubation with peptide and/or alcohol formulation did not show DNA condensation when compared with controls (Fig. 7), which indicates no significant DNA–DNA and/or DNA–protein crosslinking induced both by peptide and OF. In addition, after 72 h of incubation of cells with peptide and/or OF did not prevent extensive DNA damage induced by the oxidant t-BOOH when compared with controls (data not shown), confirming therefore that DNA crosslinking did not take place.

Conclusions

KP was able to improve both mechanical and thermal properties of relaxed hair, corroborating the results previously obtained for this peptide. In the presence of organic solvent formulation (OF), KP demonstrated even higher recovery of these properties, supporting its application as a cosmetic product. However, the study of its cytotoxicity and genotoxicity indicated that OF induced decreased cell viability, indicating the potential of OF to induce skin irritation. The peptide in WF, on the other hand, did not show toxicity, meaning that this peptide can be safely applied with all the concentrations tested and will not induce skin irritation.

The observations by cell morphology and by the comet assay corroborate the previous results. The peptide was found to be non-cytotoxic, and it does not inhibit cell growth nor induce DNA damage or DNA crosslinking in human foreskin fibroblasts in culture up to a concentration of 0.5 g L^{-1} and 72 h of exposure. The OF formulation, used to dissolve peptide, at a concentration of 10% (v/v) in culture medium inhibited cell growth, is cytotoxic and genotoxic, but did not induce DNA crosslinking in human foreskin fibroblasts.

Despite the fact that KP together with OF seems to promote the highest recovery of relaxed hair properties, it seems to induce

certain toxicity on cells in this medium. Conversely, the KP in WF does not induce toxicity or genotoxicity and is also able to recover the properties of damaged hair. This formulation is therefore an excellent candidate for a new hair care product development.

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