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Exploring the Protective Effects of *Phaeodactylum tricornutum* Extract on LPS-Treated Fibroblasts

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Abstract: Background: Microalgal extracts are an important source of bioactive compounds with antioxidant and anti-inflammatory properties that can be used in cosmetics. The microalgae *Phaeodactylum tricornutum* (PT) is known for its high content of omega-3 fatty acids, which are known to attenuate inflammation. Here, we explore the effects of aqueous microencapsulated extract of *PT* on lipopolysaccharide (LPS)-stimulated normal human dermal fibroblasts (NHDF) to underline its application as an active ingredient in cosmetics. Methods: We assessed cell viability using MTT assay, so as to target any potential cytotoxicity of the extract. Moreover, with the aid of RT-qPCR, we studied the transcript accumulation of genes involved in cell antioxidant response, cell proliferation, and inflammation. Results: Our results revealed that the hydrolyzed rice flour-encapsulated (HRF) *PT* extract promotes anti-inflammatory and antioxidant response, increasing cell proliferation in NHDF cells. Conclusions: Our data indicate a promising use of HRF-encapsulated PT extract in cosmetics by reducing skin inflammation.

Keywords: inflammation; marine extract; human dermis; microalgae

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1. Introduction

Skin is the main target of inflammation, as it is exposed to various environmental stress factors [1]. Fibroblasts, which are traditionally recognized as cells responsible for extracellular matrix production and organization, are more and more appreciated as an active key player of the immune system [2,3]. Inflammation, which is an immune-related process, is associated with the development of skin diseases [4–6], while current research is seeking to discover new natural anti-inflammatory agents

Microalgae are a rich source of essential nutrients such as carbohydrates, vitamins, minerals, trace elements, and phytochemicals (e.g., fatty acids, carotenoids). In addition, microalgae contain a significant amount of protein (up to 10%), polyunsaturated fatty acids, and carotenoids [7–9]. Moreover, microalgae could be a natural source of anti-inflammatory compounds. Specifically, fucoxanthin derived from *Phaeodactylum tricornutum* (*PT)* was shown to exhibit pro-inflammatory properties *in vitro*, and it can serve as a key candidate in the development of potential therapeutic agents for inflammatory diseases as well as neurodegenerative diseases caused by NFkB and NLRP3 inflammasome activation [10]. Furthermore, *PT* is a marine diatom with high amount, up to 5% based on its growing conditions, of the ω-3 fatty acid eicosapentaenoic acid (EPA) [11,12]. Additionally, PT is a rich source of mono-unsaturated fatty acids such as palmitoleic acid (C16:1n7), minerals, as well as carotenoid (e.g., fucoxanthin) [11]. A previous study [13] presented the protective effects of several ethanolic *PT* extracts against inflammation on the cell line RAW 264.7. Additionally, a recent study presented the anti-inflammatory effects of *PT* extract on human mononuclear cells, underlining the potential health benefits of its usage in both animal and human nutrition [14]. In addition, LPS constitutes an outer membrane structure and an

important virulence factor of the cell wall of Gram-negative bacteria, which is commonly used as an inflammatory agent [15,16].

In this study, we investigated the putative inflammatory properties of hydrolyzed rice flour (HRF)-encapsulated *PT* extract (HRF-PT) on lipopolysaccharide (LPS)-stimulated normal human dermal fibroblasts (NHDF) to underline its application as an active ingredient in cosmetics.

2. Materials and Methods

2.1. NHDF Culture, Reagents, as Well as Lipopolysaccharides (LPS) Treatment

Primary Normal Human Dermal Fibroblasts (NHDF) (Lonza Walkersville, USA) isolated from normal human adult skin were used. NHDF were cultured following producer's recommendations and grown in a FGM-2 BulletKit media containing 2% serum and subcultured until they reached 70–80% confluence. The NHDF cells were exposed to lipopolysaccharides (LPS) from *Escherichia coli* O111:B4 (10 μg/mL) for 24 h, as described before [14–16]. After the 24 h treatment with LPS and HRF-PT, cytotoxicity was assessed following by RNA isolation and transcriptomic analysis. Three independent experiments were carried out.

2.2. Cytotoxicity Assessments

Cell viability was determined using an MTT colorimetric assay kit (Vybrant® MTT Cell Proliferation Assay Kit, Thermo Fisher Scientific, USA) following the manufacturer's protocol. Briefly, after cell treatment (extract or LPS), a mix of 100 μL FBM and 10 μL MTT labeling reagent (5 mg/mL) was added in each well, and the plate was incubated for 3 h at 37 °C. In the following, 50 μL DMSO was added, and the plate was incubated for 10 min at 37 °C. Finally, the absorbance of the reaction solution was measured at 570 nm using microplate reader (infinite 200M Pro, Tecan, Switzerland). Measurements were performed and percentage of cell viability was determined as follows: Cell viability (%) = Mean OD/Control OD × 100%.

2.3. RT-qPCR Gene Expression Analysis

RNA isolation, cDNA synthesis, as well as RT-qPCR method analysis were carried out as previously outlined [17–19]. Briefly, for RNA isolation (500 ng), the Nucleospin RNA kit (Macherey-Nagel, Germany) was used, and for cDNA (reaction volume of 20 μL), the SuperScript™ First-Strand Synthesis kit (Invitrogen, UK) was used. Moreover, quantitative RT-PCR reactions were performed using a KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems, Inc., Wilmington, MA, USA), using 0.5 μM for each specific gene primer pair and 1 μL of the cDNA. Supplementary Tables S1 and S2 provide a list of the genes of the study along with their specific primer pairs. Primer Express 1.5 software (Applied Biosystems, Darmstadt, Germany) was used for the designing of the primer pairs. The relative transcript levels of the target genes (X) were normalized with the geometric average (geomean) of the endogenous reference genes (Actin beta (ACTB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH)), and calculated using the comparative threshold cycle (CT) method [(1+E)−ΔΔCT] [20,21]. For PCR efficiency, LinRegPCR software was used [22]. The RT-qPCR reactions were carried out in three independent biological replicates. Six technical repetitions were used for each biological replicate during the experimental procedure.

2.4. Statistical Analysis

The results are presented as mean ± SEM. Analysis of variance (ANOVA) following by Bonferroni's multiple corrections was used for the assessments among the different experimental states. The level of significance was * $p < 0.05$. Statistical analysis was carried out using Graphpad Prism (Graphpad Software, La Jolla, CA, USA).

3. Results

3.1. Cell Viability Evaluation

MTT assay was used to assess cell viability of the NHDF cells under the treatment of PT extract and LPS. Figure 1 presents the main outcomes of this analysis. Specifically, we observed a statistically significant increase in cell viability of NHDF cells treated with different concentrations of PT extract (0.05 µg/mL) (Figure 1a), underlining its non-cytotoxic effect. Moreover, we observed that the cell viability was increased in NHDF cells with the addition of PT extract even under the LPS treatment, which means that PT extract not only promotes cell proliferation but also protects the cells against inflammation (Figure 1b). For the LPS treatment evaluation, we opted for the lowest concentration of PT extract, as it represents the optimal concentration of extracts in cosmetic formulations.

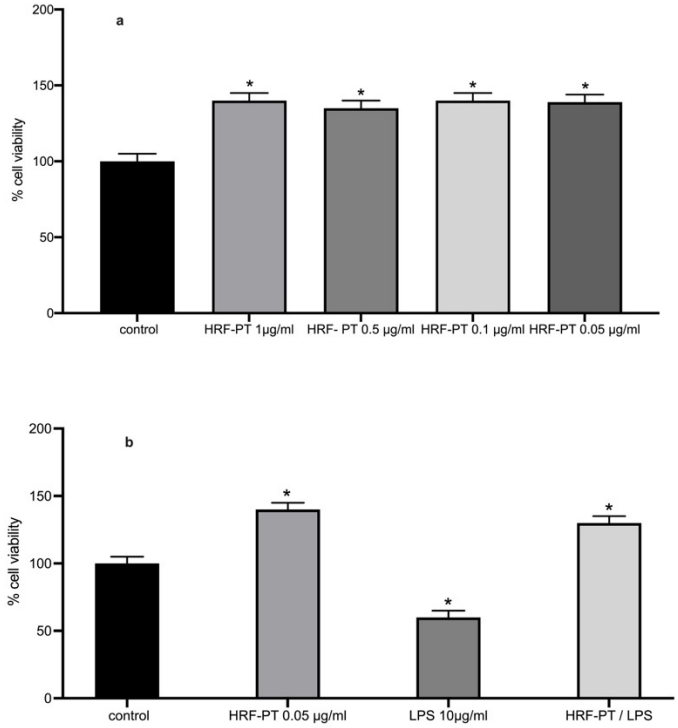


Figure 1. Cell viability (%) assessment based on MTT assay expressed as mean ± SEM for (**a**) control (untreated NHDF cells), NHDF cells treated with different concentrations of HRF-PT (0.05–1 µg/mL). Cell viability is increased with the addition of HRF-PT in different concentrations (0.05–1 µg/mL) compared to control ($p < 0.05$); (**b**) control (untreated NHDF cells), NHDF cells treated with different concentrations of HRF-PT (0.05 µg/mL), NHDF cells under LPS (10 µg/mL) treatment (LPS 10 µg/mL), NHDF cells under LPS treatment with HRF-PT (0.05 µg/mL) (HRF-PT/LPS). Cell viability is increased with the addition of HRF-PT (0.05 µg/mL) under or without LPS treatment ($p < 0.05$). * $p < 0.05$ significantly different from the control (ANOVA test).

3.2. RT-qPCR Analysis

To evaluate the bioactivity of HRF-PT in LPS-treated NHDF cells, we investigated the expression of genes related to inflammatory and antioxidant cellular response. Figure 2 demonstrates the significant modifications of genes expression with HRF-PT under or without LPS treatment. As shown in Figure 2, the expression of inflammation-related genes, inducible nitric oxide synthase (iNOS), interleukin-6 (IL6), interleukin-1 beta (IL1B), interleukin-10 (IL10), tumor necrosis factor alpha (TNFα), inhibitor protein IκBα (pikBα), and nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) was down-regulated in NHDF cells with the addition of HRF-PT under or without LPS treatment compared to control ($p < 0.05$). Furthermore, the expression of glutathione

peroxidase-1 (GPX1), was up-regulated in NHDF cells with the addition of HRF-PT under LPS treatment compared to control ($p < 0.05$).

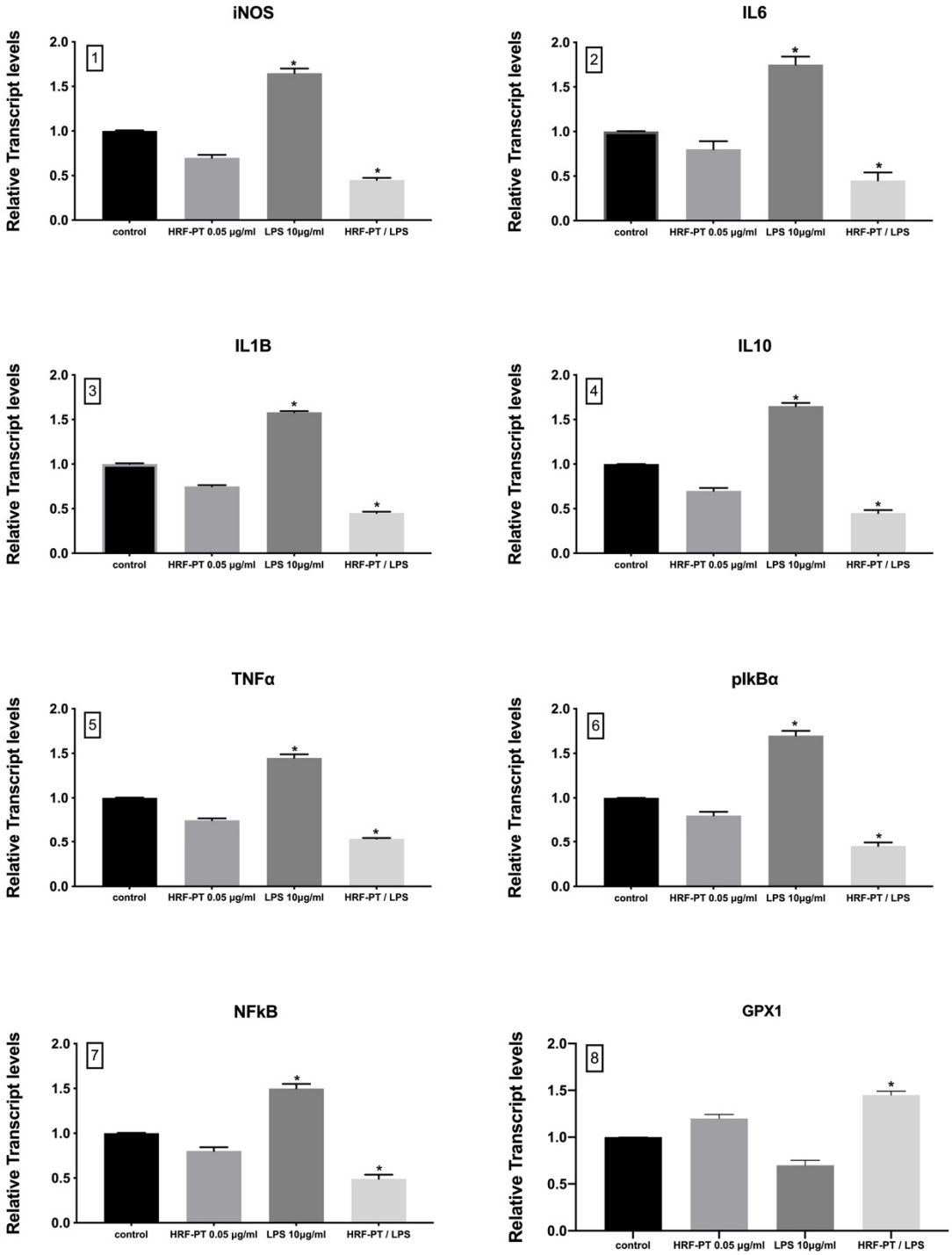


Figure 2. The relative gene expression of iNOS, IL6, IL1B, IL10, TNFα, pikBα, NFkB, GPX1 presented as a fold change ± SEM compared to untreated NHDF cells. The expressions of iNOS (**1**), IL6 (**2**), IL1b (**3**), IL10 (**4**), TNFα (**5**), pikBα (**6**), and NFkB (**7**) were down-regulated in NHDF cells with the addition of HRF-PT under or without LPS treatment compared to control ($p < 0.05$). The expression of GPX1 (**8**), was up-regulated in NHDF cells with the addition of HRF-PT under LPS treatment compared to control ($p < 0.05$). Experimental conditions: control (untreated NHDF cells), NHDF treated with HRF-PT (0.05 µg/mL), LPS-treated NHDF (10 µg/mL), LPS-treated NHDF (10 µg/mL) with HRF-PT (0.05 µg/mL). * $p < 0.05$ significantly different from the control (ANOVA test). The data correspond to the mean ± SEM of three independent experiments and six replicates each time.

4. Discussion

Skin is the main target of inflammation, as it is exposed to various environmental stress factors [1]. Fibroblasts change the quantity, quality, and duration of the inflammatory process and have a critical role during it [23,24]. Additionally, fibroblasts are a typical in vitro cell-based model for cytotoxicity determination as they recruit immune cells via soluble mediators contributing to the progression of inflammatory processes [25]. Here, we present the anti-inflammatory effects of the natural based extract, the HRF-encapsulated *Phaeodactylum tricornutum* extract in LPS-treated human fibroblasts in an attempt to be used in future cosmetic formulations as an anti-inflammatory agent.

Initially, our results demonstrated that different concentrations of HRF-PT increased the viability of human dermal fibroblasts. This increase is related to a lack of cytoxicity as well as mitochondrial activity-related cell proliferation according to previous reported works [26]. In line with this, HRF-PT confers cell viability increase under LPS treatment, which can be related to the decrease of inflammatory process and the improvement of other essential cellular processes [14–16,27].

To assess for the molecular HRF-PT activity in NHDF cells, we targeted genes related to inflammatory and antioxidant cellular response. According to our results, we observed significant alterations of different gene expressions with the addition of HRF-PT. Initially, we focused on the genes related to the inflammation cellular process. NF-kB has a significant role in the inflammatory response in cells [15,28,29]. In addition, under inflammatory stimuli, the inhibitor protein IκBα (pikBα) is phosphorylated and released from NF-kB which, once activated, migrates to the nucleus and up-regulates inflammation-related genes such as pro and anti-inflammatory cytokines and iNOS [30]. The gene expression of iNOS, pikBα, and NFkB were down-regulated with the addition of HRF-PT in LPS treated-NHDF cells compared to control in accordance with previous works [14–16,31,32]. These results underline the protective role of HRF-PT against inflammation in the cellular process. Moreover, we proceed on the assessments of pro- and anti-inflammatory cytokines. We have observed that the transcripts of TNFα, IL1B, IL6, and IL10 were increased after LPS treatment in NHDF cells; however, the addition of HRF-PT significantly reduced the expression of these genes in LPS-treated NHDF cells compared to control and in accordance with other studies even after the use of various compounds such as porins, lipotheichoic acid, and protein A [25,32,33]. The above-mentioned outcomes highlight the significant contribution of HRF-PT in the modulation of the inflammatory response. Furthermore, we assessed genes that are related to antioxidant cellular response such as GPX1, which has a fundamental role in the entire antioxidant cellular defense grid [30]. Specifically, GPX1, SOD1, and NRF2 are key antioxidant markers ([34–36] and are up-regulated under stress [37,38]. In our study, we observed that the gene expression of GPX1 was up-regulated with the addition of HRF-PT in LPS-treated NHDF cells compared to control, suggesting that HRF-PT induces a potential antioxidant response of NHDF under inflammation. This induction is in accordance with previous reports [14,15,27,39]. In line with these, we observed an increase of cell viability with the addition of HRF-PT under inflammation, confirming its protective role.

In conclusion, this study demonstrated the protective role of the HRF-encapsulated *PT* extract against in vitro inflammation. We observed that the addition of HRF-encapsulated *PT* extract reduces the expression of genes related to inflammation while increasing the expression of genes related to antioxidant response. Our results underline the potential use of HRF-encapsulated *PT* extract in cosmetic formulations as an anti-inflammatory agent. However, additional in vitro and in vivo studies are necessary to understand in depth the bioactivity of this extract as well as the molecular mechanisms that regulate the HRF-*PT*-mediated anti-inflammatory effect.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/cosmetics8030076/s1, Table S1: Gene symbol, Primer pairs, Table S2: Relative gene expression levels per experimental group.

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