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Development of an *in Vitro* Assay to Evaluate the Biological Impact of 5G Technology on Human Skin—Shield Effect of a Tannin-Rich Plant Extract

Katharina Kappler[1], Franziska Wandrey[1*], Jean-Eric Branka[2], Stéphane Poigny[1], Fred Zülli[1]

[1]Mibelle Group Biochemistry, Mibelle AG, Buchs, Switzerland
[2]Ephyscience, Nantes, France
Email: *franziska.wandrey@mibellegroup.com, jebranka@ephyscience.fr

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Abstract

Background: The new 5G telecommunication technology has stirred concerns about potential negative effects on human health by radiofrequency electromagnetic fields. As to whether skin biology can be affected by 5G waves has remained an unsolved challenge despite recent studies dealing with this issue. In particular, a strategy for rational design of an assay allowing to 1) reproducibly evaluate and decipher the 5G effects on skin as well as 2) test the potential protective effects of cosmetic active ingredients, has yet to be found. Here we describe an *in vitro* model of human normal keratinocytes irradiated by 5G waves and show their impact on two biomarkers of inflammatory stress, *i.e.* interleukin-1β (IL-1β) and reactive oxygen species (ROS) production. In addition, the capacity of a tannin-rich plant extract to protect against 5G impact is evaluated. Materials and Methods: In the first series of experiments, monolayers of human normal keratinocytes were irradiated or not (control) by 5G waves (3.5 MHz) in an anechoic chamber and were incubated at 37˚C for 24 hours. At the end of the incubation period, extracellular IL-1β and intracellular ROS were quantified using specific ELISA and colorimetric assays, respectively. In the second series of experiments, the effect of an overnight pre-incubation with increasing concentrations of a tannin-rich plant extract was evaluated. Additionally, we studied in a prospective way the expression of a set of 88 genes selected for their relevance to keratinocyte homeostasis, in relation to the 5G challenge as well as the protective effect of a tannin-rich plant extract. Results: 5G waves significantly increased IL-1β production by 48.4% ($p < 0.001$) and ROS generation by 16.7% ($p < 0.001$). Furthermore, the tannin-rich plant extract dose-dependently protected the keratinocytes against the deleterious effects of 5G as measured by IL-1β and

ROS production. Finally, the expression of 47 genes was modified by 5G waves and/or by the tannin-rich plant extract. Conclusion: This is to our knowledge the first evaluation of the impact of 5G technology on inflammatory biomarkers of human normal skin cells. Here we provide an innovative and pertinent tool to screen for natural compounds with protective effects against 5G waves to develop cosmetic products shielding against the potentially deleterious effects of electromagnetic waves on human skin.

Keywords

5G waves, Human Normal Keratinocytes, Inflammation, ROS, Interleukin-1, mRNA

1. Introduction

In the world of wireless communication, lower frequency bands cover much greater distances but offer slower data speed, while high-frequency bands cover much smaller areas but can transfer more data. In these conditions, and as low frequencies (between 800 and 3000 MHz) are heavily congested with TV and radio signals, as well as current 4G networks, development of 5G networks seems to be a good solution to ensure consistent coverage over large areas. This goal can be reached by using two types of 5G waves, *i.e.* "mm waves" and "sub-6 GHz" waves. The first one, referring to a specific part of the radio frequency spectrum between 24 and 100 GHz, will allow to simultaneously transfer the large data volumes where data congestion might be a problem, and the second one with a frequency below 6 GHz will play a more crucial role in rural towns and villages in ensuring consistent coverage over large areas (for a review, see [1]).

As of today, 5G networks emerge as new players with respect to public health issues, by focusing attention on their putative harmful effects (for reviews, see [2] [3] [4]). A range of publications has addressed the issue of possible 5G impact on the first line of defense of the human body, the skin. They include the work of Szabo *et al.* who demonstrated in 2001 that 5G millimeter waves (frequency: 61.22 GHz) significantly increased the interleukin-1β (IL-1β) production of HaCaT cells [5]. On the other hand, Mahamoud *et al.* showed in 2016 that 5G waves (frequency: 60.4 GHz) did not induce transcriptional changes in cultured human keratinocytes unless they were applied in combination with a glycolysis inhibitor [6]. Kim *et al.* showed in 2020 that 5G electromagnetic fields with a frequency of 28 GHz did not induce any modification in the melanin production of the murine B16-F10 and human MNT-1 melanoma cell lines [7].

In fact, our review of the scientific literature points to a huge heterogeneity of the model systems used to evaluate the impact of 5G waves on health. Further complexity is added by the fact that the frequency of 5G waves as well as the ways to generate them are substantially different among research groups.

Therefore, we chose to develop a standardized *in vitro* model by using 1) mo-

nolayers of human normal adult keratinocytes and 2) a 5G antenna delivering waves with a frequency of 3.5 GHz which is in the range of the frequencies used by most mobile phone operators [8]).

Electromagnetic fields generated by 5G waves are suspected to induce oxidative stress (for a review, see [9] [10]). Oxidative stress triggers inflammation in the skin (for a review, see [11] [12]) by increasing the cellular production of interleukin-1β (IL-1β) [13], a component of the so-called inflammasome (for a review see [14]). Interestingly, oxidative stress is also involved in skin aging [15] [16] [17], a major topic in the cosmetic industry that constantly strives to improve the skin appearance. In this context, we first used our model 1) to evaluate the 5G waves effect on reactive oxygen species (ROS) and IL-1β production by human normal keratinocytes, and 2) to assess the shielding effect of a powerful antioxidant compound (a tannin-rich plant extract) against the 5G-induced oxidative and inflammatory stresses. In order to investigate more deeply the 5G waves' impact on human cutaneous tissue, we decided to analyze the expression of 88 genes involved in skin homeostasis and inflammatory status before and after the treatment of keratinocytes with 5G irradiation as well as after the treatment with the tannin-rich plant extract.

2. Materials and Methods

2.1. Cell Culture and Treatments

Primary human keratinocytes were isolated from an abdominal skin residue resulting from plastic surgery. Cells were grown in Keratinocytes Growth Medium 2 (Promocell, Heidelberg, Germany). Second passage keratinocytes were seeded into 24 well plates at a cell density of 20,000 cells per well and were cultured at 37˚C in an atmosphere containing 5% of CO_2.

After reaching confluency, cells were incubated in the absence (control) or in the presence of test compound E-0825 at 0.1% and 0.5% (v/v) and immediately irradiated or not (control) with 5G waves for 1.5 and 3 hours.

At the end of the irradiation periods, cells were incubated again for 24 hours at 37˚C in an atmosphere containing 5% of CO_2.

2.2. 5G Wave Irradiation

Keratinocytes were irradiated with 5G waves at 37˚C in an anechoic chamber. For the generation of 5G waves, a vector signal generator (EXG N5172B 9 kHz - 6 GHz; Keysight Technologies, Les Ulis, France) coupled with dedicated software (Pathwave Signal Generation N7631C 5G NR Signal Creation and Playback) were used. The shape of the wave loaded on the instrument was a 5G signal with a frequency of 3.5 GHz. Transmission of signal was done with a directive antenna (Siretta, Spencers Wood, UK) with a gain of 10 dBi.

2.3. IL-1β Release

At the end of the incubation period, culture media were collected and IL-1β re-

lease was measured using a specific ELISA kit (human IL-1β/IL-1 F2 Duoset development Kit, Biotechne, Noyal, France). The colorimetric signal at 450 nm was analyzed by using an appropriate spectrophotometry plate reader (Victor V, Perkin Elmer, Villebon-Sur-Yvette, France).

2.4. Protein Quantification

Total protein content in the cell lysates was quantified by using a colorimetric method (Bradford method [18]).

2.5. ROS Generation

At the end of the incubation period, cell monolayers were rinsed prior to incubation with a HCDF-DA probe for 45 minutes. Cell monolayers were rinsed again and the fluorescence of the cells was analyzed at 485/535 nm (Ext/Em) by using an appropriate spectrophotometry plate reader (Victor V, Perkin Elmer).

2.6. Q-PCR Array

At the end of the incubation period, total RNAs were extracted using a RNeasy kit (QIAGEN, Hilden, Germany). cDNAs were generated from RNA using High capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Villebon-Sur-Yvette, France). qPCR was performed on cDNA using GeneQuery™ Human Skin Wound Healing qPCR Array Kit (Sciencell™, Carlsbad, USA) and PowerTrack SYBR Green Master Mix (Applied Biosystems™). Measurements were performed using MX3005P thermocycler (Stratagene™, La Jolia, USA).

2.7. Statistics

Data are expressed as means ± S.E. of one to two different experiments performed in triplicates (n = 3). Statistical significances of any observed differences were assessed (as indicated) by using Student t-tests or by using one-way analysis of variance (one-way ANOVA) followed by Holm-Sidak's tests.

3. Results and Discussion

Until recently the demonstration of a biological impact of 5G waves on skin was restricted to a unique report based on a spontaneously transformed human keratinocyte cell line, showing increased production of IL-1β upon exposure to 5G waves [5]. In this context, we were spurred to design an *in vitro* model system based on cultured human normal keratinocytes that would allow a multiparametric analysis of the effects of 5G waves on human skin.

In the first series of experiments, we studied the effect of 5G waves on the IL-1β and ROS production by human normal keratinocytes after an irradiation time of 1.5 or 3 hours. As only mobile phone operators are authorized to use 5G wave emitters in the public space, all these experiments were realized in an anechoic chamber able to hold waves in a confined space. As shown in **Figure 1**, 5G waves were able to significantly increase keratinocyte production of IL-1β by 19.4%

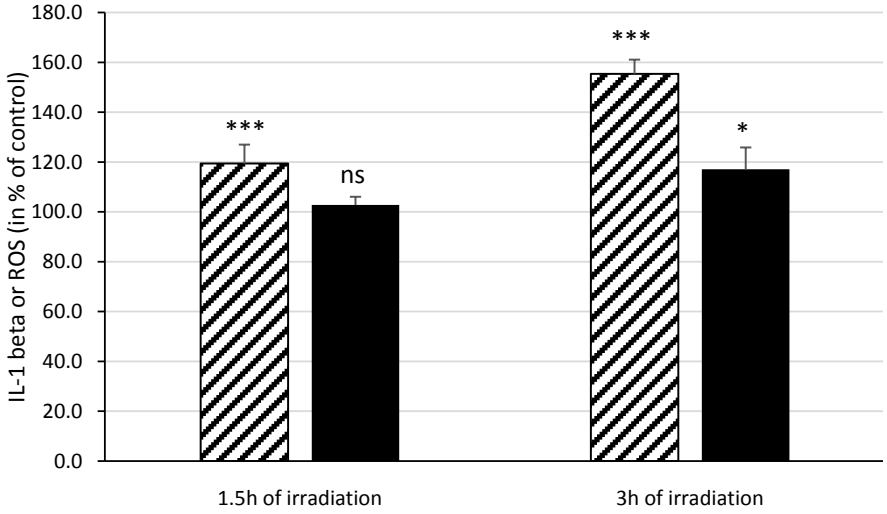

Figure 1. Effect of 5G-wave irradiation of 1.5 and 3 hours on the production of IL-1β and ROS in human normal keratinocytes (n = 6 from 2 different experiments). IL-1β: hatched bars; ROS: black bars; ns: Non-significantly different from the "control" value ($p > 0.05$; student t-test); *: Significantly different from the "control" value ($p < 0.05$; student t-test); ***: Significantly different from the "control" value ($p < 0.001$; student t-test).

($p < 0.05$) and 55.4% ($p < 0.001$) after 1.5 and 3 hours, respectively. 5G irradiations were also able to increase the keratinocyte production of ROS by 2.8% ($p > 0.05$) and 17.1% ($p < 0.05$) after 1.5 and 3 hours, respectively.

In a second step, reasoning that a higher signal could allow refining the evaluation of the underlying mechanisms and a better assessment of our tannin-rich plant extract shield effect, we chose to conduct our experiments on the production of IL-1β and ROS by keratinocytes after a 5G irradiation of 3 hours. As shown in **Figure 2** and **Figure 3**, in the selected experimental conditions, 5G waves significantly increased the production of IL-1β and ROS in keratinocytes by 48.4% ($p < 0.001$) and 16.7% ($p < 0.001$), respectively. This confirmed the results obtained in the first experiment and points to the potential of 5G irradiation to enhance oxidative stress and inflammatory processes in human keratinocytes.

Tannins are powerful antioxidant compounds. They can act as a primary antioxidant by providing a hydrogen atom or as a secondary antioxidant by chelating metal ions such as Fe and interfering with the Fenton reaction (for a review, see [19]). Reasoning that a powerful antioxidant agent could be able to protect against the 5G wave effects, we developed a tannin-rich plant extract (here called E-0825) and tested its capacity to inhibit 5G-induced IL-1β and ROS production in keratinocytes. As shown in **Figure 4** and **Figure 5**, E-0825 tested at 0.1% and 0.5% (v/v) significantly decreased the production of IL-1β by 47.1% and 76.5% ($p < 0.001$), respectively, and the production of ROS by 13.9% and 19.4% ($p < 0.001$), respectively. This is a clear demonstration of the presence of a powerful antioxidant product not only able to inhibit the 5G-induced production of IL-1β and ROS in keratinocytes but even to reduce them below basal expression levels.

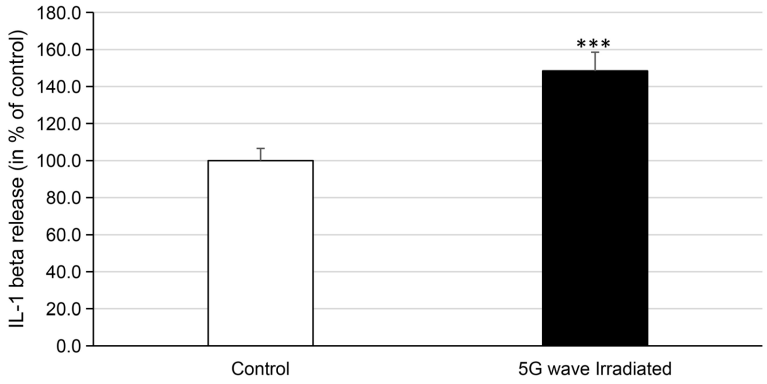

Figure 2. Effect of 5G-waves on the production of IL-1β in human normal keratinocytes (n = 6 from 2 different experiments). Control: white bar; "+5G waves": black bar; ***: Significantly different from the "control" value ($p < 0.001$; student t-test).

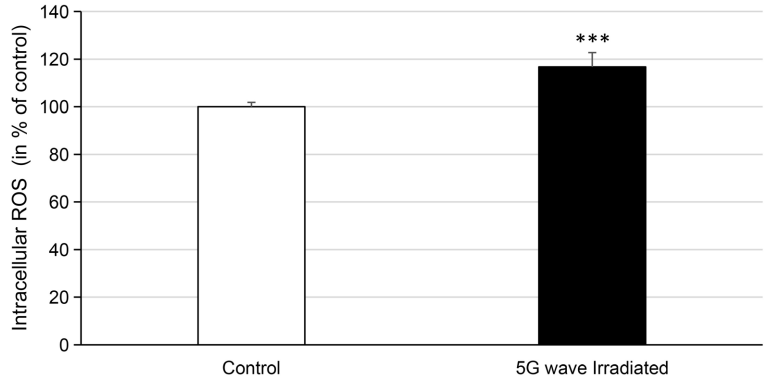

Figure 3. Effect of 5G-waves on the production of ROS in human normal keratinocytes (n = 6 from 2 different experiments). Control: white bar; "+5G waves": black bar; ***: Significantly different from the "control" value ($p < 0.001$; student t-test).

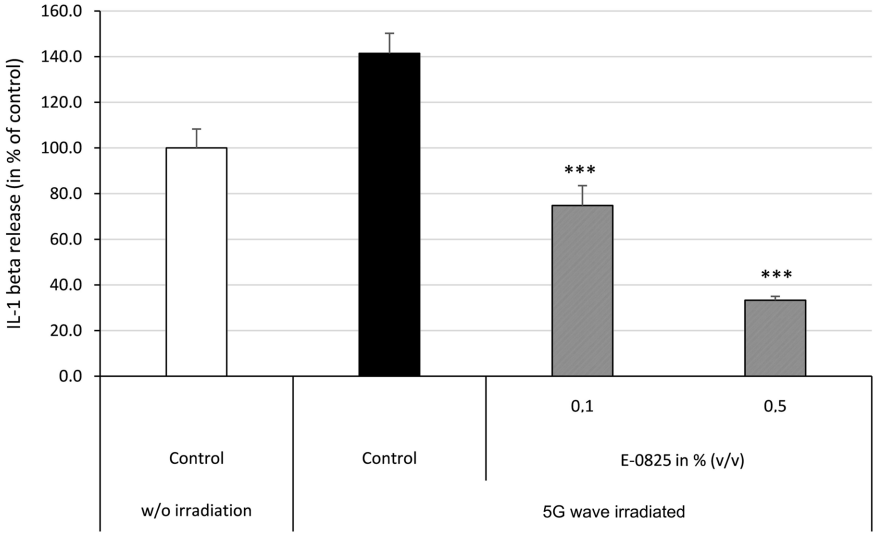

Figure 4. Effect of the compound E-0825 on the 5G-induced IL-1β production in keratinocytes (n = 3 from 1 experiment). Control w/o irradiation: white bar; "control 5G waves": black bar; "+5G waves + E-0825": grey bars; ***: Significantly different from the "control 5G waves" value ($p < 0.001$; student t-test).

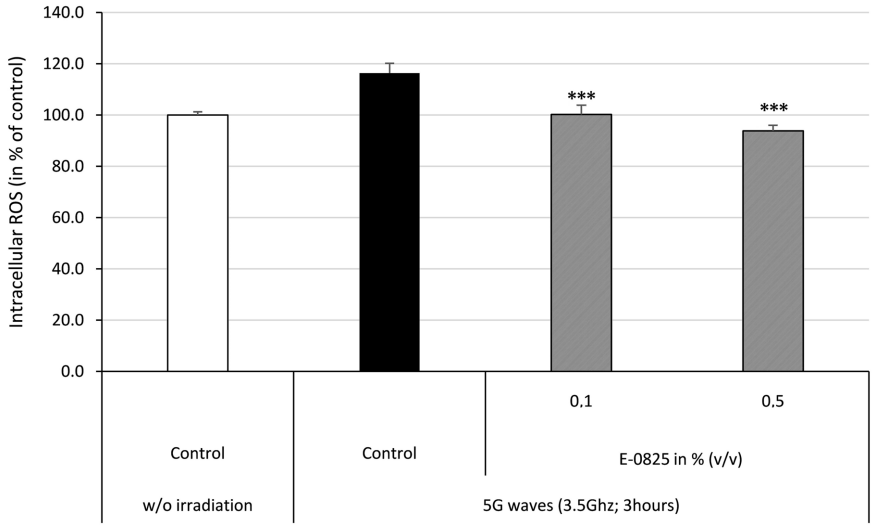

Figure 5. Effect of the compound E-0825 on the 5G-induced ROS production in keratinocytes (n = 3 from 1 experiment). Control w/o irradiation: white bar; "control 5G waves": black bar; "+5G waves + E-0825": hatched bars; ***: Significantly different from the "control 5G waves" value ($p < 0.001$; student t-test).

To extend and deepen our study, we examined the effects of 5G waves on the expression of a set of 88 genes known to be involved in the homeostasis and inflammatory status of keratinocytes. In our experimental conditions, we were able to discern a trend toward a modification of the expression of 47 out of the 88 genes upon exposure to 5G waves. For these genes, the expression was decreased or increased by more than 30% (data not shown). A set of genes, consisting of IL-1β, TNF, CXCL1, PTGS2, WNT5A and GM-CSF, displayed an increased expression, in line with an inflammatory reaction, supporting the results we obtained at the protein level of IL-1β and for the production of ROS. The expression of genes that are important for extracellular matrix structure and cell adhesion, such as COL1A2, COL3A1 (encoding type 1 and 3 collagen chains), ITGB1, ITGB3, ITGB6 (encoding integrins) and TIMP1 (encoding the matrix metalloproteinase 1 inhibitor) were decreased, whereas MMP1 (encoding matrix metalloproteinase 1), responsible for collagen degradation, showed an increased expression. Taken together our findings point to potentially deleterious effects of 5G waves on human skin cell biology.

Under the same experimental conditions, we observed a protective effect of E-0825 against irradiation with 5G waves. These beneficial effects include the dampening of the inflammatory reaction through the downregulation of IL-1β, PTGS2 and GM-CSF gene expression as well as a normalization of the expression of COL1A2, ITGB6 and MMP1, which are important genes for the preservation of the extracellular matrix structure and cell adhesion. Additionally, E-0825 is likely to exert *per se* biological actions unrelated to its 5G-shield effect. For example, the treatment of irradiated cells with E-0825 strongly decreased the expression of IL-6 and IL-8, two cytokines involved in the inflammatory response, compared to both irradiated and unirradiated control cells. In a similar

manner, treatment of irradiated cells with E-0825 also reduced the expression of STAT3, a protein notably linked to psoriasis [20], and increased the expression of COL5A1, a collagen chain implicated in the dermo-epidermal junction cohesion, whereas 5G irradiation itself did not affect the expression of these genes.

In conclusion, we have developed an accurate and reproducible model to evaluate the actual impact of electromagnetic fields generated by 5G waves on human skin biology. Moreover, our model system is relevant to the development of cosmetic active ingredients aimed to prevent the potentially harmful effects of 5G waves on human cutaneous tissue. At last, additional work regarding the expression of candidate genes involved in the skin response to 5G waves is underway in our laboratory.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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