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COMBINED EXPOSURE TO DIFFERENT NON-IONISING RADIATIONS ON THE HUMAN SKIN *IN VITRO*

PhD thesis

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LIST OF ABBREVIATIONS

AD	Adaptive dose
AE	Additive effect
AR	Adaptive response
CD	Challenge dose
CW	Continuous wave
DSB	Double-strand break
ELF	Extremely low frequency
EMF	Electromagnetic field
FPG	Formamidopyrimidine DNA glycosylase
IARC	International Agency for Research on Cancer
ICNIRP	International Commission on Non-Ionising Radiation Protection
IF	Intermediate frequency
IR	Ionising radiation
LTE	Long Term Evolution (4G mobile technology)
MF	Magnetic field
NDI	Nuclear Division Index
PC	Positive control
RF	Radiofrequency
ROS	Reactive oxygen species
SAR	Specific absorption rate
SD	Standard deviation
SED	Standard erythema dose
SSB	Single-strand break
TNF	Tumour necrosis factor
UMTS	Universal Mobile Telecommunications System (3G mobile technology)
UV	Ultraviolet

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1. INTRODUCTION

1.1. Non-ionising radiations

Humans are exposed to various types of radiation all over the world. Different radiations can be distinguished, e.g. ionising radiation and non-ionising radiation. The fundamental difference between ionising and non-ionising radiation is their physical properties, basically manifested in their ionising ability. The 0–3 PHz frequency of electromagnetic spectrum is called non-ionising radiation. The electromagnetic field (EMF) includes the static electric and magnetic fields (0 Hz), the extremely low frequency (ELF, 0.001–300 Hz), the intermediate frequency (IF, 300 Hz – 100 kHz), the radiofrequency (RF, 100 kHz – 300 GHz), the infrared range (300 GHz – 375 THz), the visible light (375 – 750 THz) and the ultraviolet (UV) radiation (750 THz – 3 PHz) (Figure 1). Electromagnetic radiation is an energy flow that spreads from a source to any direction; it spreads in space as a wave, delivering energy and impulse.

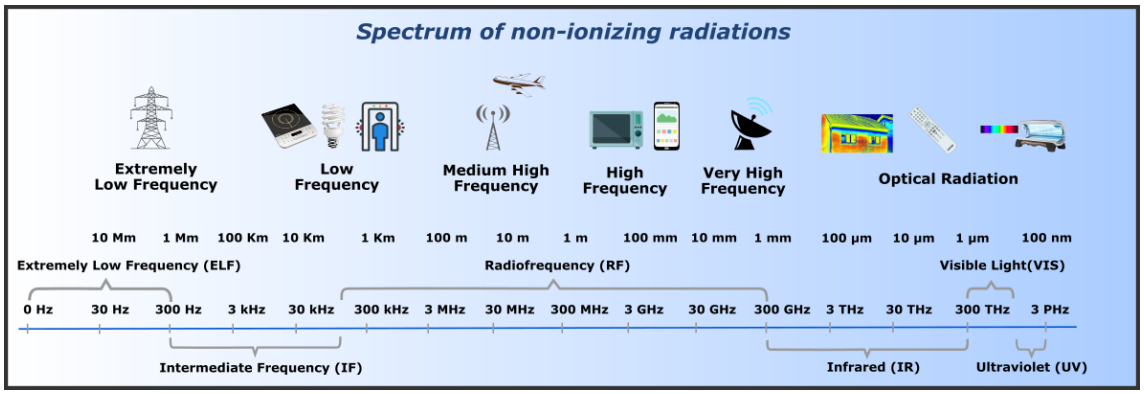

Figure 1. The spectrum of non-ionising radiation. Unpublished figure made by Péter Pál Necz based on the author's instructions.

The rapid technological development of the past centuries – by more intensive usage of instruments producing EMF − resulted in humans being more exposed to non-ionising radiation in all areas of life. The effect of ionising radiation is well-known. Nevertheless, the possible interactions between non-ionising EMF and biological systems are not yet fully understood. Although these radiations do not have ionising effect, their electrical, magnetic and thermal effects can influence the human body. During everyday life, humans are surrounded by various EMF sources; the most significant of them is the 50/60 Hz ELF – created around all devices that work with electricity –, the high-voltage transmission lines and transformer stations. The worth highlighting RF sources are the

TV and radio broadcast stations, the mobile phone base stations, and the microwave ovens. Due to their increased everyday usage and the high presence of artificial sources, the WHO is focusing on research into the effects of extremely low electromagnetic fields and radiofrequency radiation as a high priority. Based on the epidemiological studies on the correlation to childhood leukaemia the International Agency for Research on Cancer (IARC) classified ELF to Group 2B as "possibly carcinogenic" to humans, just like RF based on an increased risk for glioma, a malignant type of brain cancer, associated with wireless phone use (1,2). In order to protect the population against the possible adverse effects of non-ionising radiation, the International Commission on Non-Ionising Radiation Protection (ICNIRP) has issued guidelines (3), and the European Commission has published recommendations for the exposure limits of EMF (4).

1.1.1. Radiofrequency

The rapid development of wireless communication in the last few decades has resulted in increased exposure of humans to RF. Most of these − unlike other types of radiation − are of artificial origin. In the human environment, several sources generate RF, the best known are the radio broadcast, mobile phone base stations and mobile phone devices, but the emerging wireless technologies (e.g. Wi-Fi, car radars in millimetre wave range) also generate them. Within the range of the RF radiation (3 MHz – 300 GHz) the millimetre wave band is defined from 30 GHz to 300 GHz. The new generations of wireless technologies (3G, 4G, 5G, Wi-Fi) operate at higher frequencies than the earlier digital mobile phone system known as second generation (2G), which usually operated at 900 MHz.

The specific absorption rate (SAR) is a standard metric that shows the amount of absorbed RF power in the body or biological object, usually expressed in watt per kilogram (W/kg). In general, as the frequency increases, the wavelength of radiation and the penetration depth into the human body decrease. Moreover, higher frequency leads to higher SAR, which means that RF exposure does not penetrate deeper layers, it is absorbed only in the skin and cornea, but with a higher absorption efficiency (5–7).

Safety standards and recommendations, which limit the exposure to RF of the human body in residential and working areas, are based on the well-established thermal effects. These restrictions set different limits for the most exposed body parts; the head,

the body and the limbs (8,9). Regarding the international guidelines, thermal effects may occur when the radiation exposure causes more than 1°C temperature increase in the human body. Athermal (compensated) effects occur when the temperature does not increase due to thermoregulation (between 0.5−2 W/kg). Non-thermal effect is, when the intensity of RF radiation is so low that the amount of energy involved does not significantly increase the temperature of a cell, tissue or organism and thermoregulation is not involved in this either (10). Nevertheless, these non-thermal effects may be able to induce certain physical or biochemical changes, but this has not been fully confirmed yet. Thermal effects therefore also depend on the thermoregulatory capacity of the given biological system and organ. Above 4 W/kg SAR of whole body EMF exposure results a >1°C temperature increase of the body core, which is potentially harmful. Without the thermoregulation process (i.e., *in vitro* systems), above 1 W/kg SAR the temperature may increase depending on the exposure conditions (11). Nevertheless, to the best of our knowledge, the potential adverse health effect of RF fields is caused by thermal effects. The adverse nonthermal effects of EMF have not been established so far; however, the exponential increase in RF exposure raises questions about their possible health impacts. Therefore, there is an ongoing concern in the public regarding the potential adverse effects from such exposures, as taking steps into modern technologies (i.e. 5G and 6G mobile systems) will result in increasingly high levels of RF exposure being predicted in modern society.

1.1.2. Intermediate frequency

Between the extremely low and RF frequencies, the intermediate frequency magnetic field (IF MF) ranges from 300 Hz to 10 MHz. Not just RF but also IF MF exposure has significantly increased in the last few decades due to the development of consumer and industrial devices. Several emerging technology products generate IF MF, varying widely in frequency and field strength (12). There are many consumer devices in households that use IF MF. Devices using inverter technology − e.g., refrigerators, microwave ovens, laundry machines or air-conditioning systems − operate in the 19−70 kHz range (13). The most typical household device is the induction cooker, which has been replacing traditional gas stoves in many kitchens due to its better energy efficiency and the lack of open flame and leakage risks. However, the impact of the human body

exposure induced by induction cooktops is inconclusive in the vicinity of a magnetic environment. The most vulnerable exposed persons are pregnant women and young children because the fetus and children's heads are at the same height as the cooking tops (14,15). Further devices in households, such as energy-saving bulbs and fluorescent lamps, operate from 1.2 to 100 kHz. Anti-thief gates, electronic article surveillance systems and proximity readers − commonly installed in shops, hospitals or libraries − operate in a wide range, from 20 Hz to 2.45 GHz (16–18). IF sources, e.g., touchscreens and smartboards, occur for education purposes, portable hearing units and electrosurgical units for healthcare (19). Inductive battery chargers are becoming increasingly commonly used and operate at IF frequencies as well. Although due to the sustainable, environmentally friendly road transport needs, the electric and hybrid cars have become a common usage, there is a widespread public concern about the EMF exposure that they generate. Studies showed reassuring results regarding the EMF exposure of electric and hybrid cars, when IF MF exposure is less than 20% of the reference levels for general public exposure set by ICNIRP (20,21).

1.1.3. Ultraviolet radiation

Solar radiation is the main natural source of human exposure to ultraviolet (UV) radiation. Around 95% of UV radiation that reaches the Earth's surface is UV-A (315–400 nm), most of the solar UV-B (280–315 nm) and the whole range of UV-C (100–280 nm) are blocked by the stratospheric ozone (22). Epidemiological and laboratory studies have established that UV radiation plays a significant role in the incidence of all major types of skin cancer (23). Based on these studies, UV radiation is classified by Group 1, as "carcinogenic" to humans by IARC (24).

The biological effectiveness of UV radiation mainly depends on its wavelength (25). The dose of UV radiation is expressed in effective radiant exposure (J/m^2), which is weighted by the spectral curve of biological effectiveness and exposure time, and it is integrated over wavelengths. The standard erythemal dose (SED) is expressed for dermal usage, when 1 SED is equivalent to 100 J/ m^2 of effective radiant dose.

UV radiation is clearly associated with DNA mutagenicity and apoptosis, furthermore, it is responsible for inducing sunburn and several skin disorders, e.g., skin cancer, including melanoma, basal-cell carcinoma and squamous cell carcinomas (26).

UV can cause direct DNA damage by the creation of cyclobutane pyrimidine dimers (CPDs) and pyrimidine-pyrimidone (6-4) photoproducts (6-4 PP) (27,28). Furthermore, it can cause indirect DNA damage via oxidative stress, generating reactive oxygen species (ROS) (29,30). The reactions of the body are inflammation, gene mutation and photoimmunosuppression, which are in correlation with photoaging and photocarcinogenesis (30–32).

1.2. The human skin

The skin is the largest organ and at the same time, the first defence of the body. Its most important function is the protection from external factors, but it also plays an important role in regulation and sensation. Skin consists of three layers, the epidermis, the dermis and the hypodermis. The epidermis is the top layer, provides a barrier from the outside, consists mostly of keratinocytes, but melanocytes, Merkel cells, Langerhans cells and inflammatory cells also appear (33). The dermis is the second layer of the skin, connected to the epidermis by the basement membrane. Fibroblasts, macrophages and mast cells are the main components.

The skin also plays a crucial role in immunity, thus also in inflammation. Inflammation is a physiological response of the body against various insults, which has many potential causes, such as infection, pathogens, radiation or physical damage. Due to the inflammation, the cytokine (e.g., interleukin and tumour necrosis factor) production is activated in the skin.

ROS occur naturally in the whole body, in all cell types. The increased level of ROS could disturb many vital cellular processes, such as proliferation and differentiation, inflammation or even DNA damage as well. If the elevated ROS level stays persistent or occurs repeatedly, it can lead to genetic and epigenetic mutations that can indirectly cause cellular malfunctions. The most common ROS products – such as superoxide ($\bullet O_2^-$) and hydroxyl (•OH) radicals, hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and nitric oxide (•NO) – serve as a protection against pathogens and also as signalling molecules. Although these reactive molecules can potentially damage cells, their presence and production are not necessarily harmful, as ROS are normally present in all cells, but usually they are kept in balance (34). Nevertheless, the imbalance in oxidative stress level plays a crucial role in pathogenesis and in the progression of many diseases (35). The 8-

oxoguanine is a modified form of the DNA base guanine caused by oxidative damage. It is one of the most common and well-studied oxidative lesions in DNA caused by ROS, widely used as a biomarker to assess oxidative stress level in cells (22).

1.2.1. Skin exposed to non-ionising radiation

By now, we know for sure that UV radiation causes inflammation in the skin. UV radiation induces lipid peroxidation and prostaglandin production by cyclooxygenase2; furthermore, UV-induced mediators like tumour necrosis factor (TNF) and interleukins (IL) increase, leading to skin inflammation. UV radiation triggers epidermal keratinocytes to elaborate specific cytokines such as interleukin 1 alpha (IL-1α), a key inducer of the inflammatory cascade (36). IL-1 diffuses to the adjacent dermis, and fibroblasts secrete secondary cytokines, such as IL-6 and IL-8. Moreover, UV radiation causes protein oxidation and induces matrix metalloproteinases (MMPs) (37). Matrix metalloproteinase-1 (MMP-1) induced by UV is a crucial biomarker of photoaging (38–40), because MMPs can degrade the extracellular matrix. MMP-1 cleaves the collagen, which is completely degraded by MMP-2,3,9 (41).

Several studies are investigating the different potential effects of EMF on the skin but investigating the inflammation due to EMF are very scarce. Kubat *et al.* found changes in the relative amount of (m)RNAs encoding the enzymes involved in heme catabolism and removal of ROS in skin cells due to 27.12 MHz of pulsed EMF (42). In another study, Kim *et al.* showed that exposure to 1760 MHz RF significantly induced ROS generation, leading to MMP activation and FoxO3a and ERK1/2 phosphorylation in HaCaT cells (keratinocyte cell line) (43). Massaro *et al.* (44) investigated the RF-induced non-thermal effects on human fibroblast cells. Pulsed and continuous 1.6 GHz RF exposure (0.4 W/kg) modified the expression of different heat-shock proteins (HSP) (i.e. HSP-90, HSP-60, HSP25). Furthermore, the RF exposure caused different changes (i.e. increased number of lysosomes, modified cytoskeletal organisation and mitotic spindle anomalies) in the ultrastructure of fibroblasts.

However, how does non-thermal RF affect the skin at the cellular level? The results on this topic are controversial. Choi *et al.* exposed different cells (adipose tissue-derived cells, liver cancer cells, HeLa cells, etc.), including normal fibroblasts to 1.7 GHz RF-EMF for 72 hours. Their results strongly suggest that the RF exposure decreased cell

proliferation and increased senescence by increasing intracellular ROS levels (45). On the other hand, Jin *et al*. evaluated the impact of 1.762 GHz LTE (Long Term Evaluation 4G mobile technology) RF exposure alone and in combination with 10 Gy ionising radiation on two types of skin cells, the murine melanoma cell line B16 and the HaCaT. They found that 8 W/kg LTE exposure reduced cell viability in melanoma cells but did not in HaCaTs. Furthermore, EMF reduced the DNA double-strand breaks (DSB) priorly induced by ionising radiation in both cell types. According to this result, we can assume that LTE-RF may promote DNA DSB regeneration and illuminate the potential beneficial effects of RF as well (46).

Regarding the IF MF, the most popular issue is whether induction cooktops have an effect, especially on pregnant women. The most important question is whether IF MF has toxicity on the embryo (47). Because of this, *in vitro* studies are carried out on ovary-derived cells (15,48,49), glial cells (50,51) or retinal-derived cells (52,53). Unfortunately, investigations of IF MF effect on skin-derived cells are lacking and just a limited number of publications have investigated its effect in fibroblast cells (54). Each of these papers found that IF MF did not cause cellular genotoxicity *per se*.

1.3. Combined exposures

The possible outcomes of the combined exposure to different agents have always been a focus of scientific research. In the last few decades, several studies have been conducted related to ionising radiation (55), UV radiation (56–59), other physical agents (60) and EMF exposure (61,62) − mainly to RF exposure (63). In these studies, several working hypotheses were proposed, according to which combined (or consecutive) exposure to RF and other radiation might enhance or mitigate the biological effects of exposure to known toxicological agents (64). Although several studies agree that RF exposure alone is non-toxic or non-genotoxic below the internationally accepted exposure limits.

However, one phenomenon is worth highlighting within combined exposures, called the *'adaptive response'*. This phenomenon can be observed when cells which were pre-exposed to low and non-toxic doses of a genotoxic agent (as adaptive dose, AD) become resistant to the damage induced by subsequent exposure to a higher and toxic dose of the same, similar or another genotoxic agent (as challenge dose, CD) (65). The

adaptive response is a well-described phenomenon in the field of ionising radiation (55,66–69), however, there are some studies which extended it to non-ionising radiation (70), where the low (adaptive) dose of radiation was substituted with either RF (63,71), IF MF (72) or UV exposure (73).

The evidence for ionising radiation-induced *adaptive response* has been documented several times. Several mechanisms have been validated when many signalling pathways and molecules are triggered. At molecular level, ionising radiation influences various signalling pathways, which lead to the activation of genes and proteins. One of the main signalling pathways involved in the ionising radiation-induced adaptive response is the DNA repair pathway. The activation of certain DNA repair enzymes and proteins triggers the cell cycle as well. Some genes can be activated related to the cell cycle, such as cyclins (i.e. cyclin D, E, A, B), cyclin-dependent kinases (CDKs) (i.e. CDK1A, CDK2A) and proteins (i.e. p53, p21) or can be activated related to the apoptosis (74).

According to the current scientific knowledge, the RF exposure is non-genotoxic but may induce some extremely small amount of DNA damage (e.g., via ROS), which is undetectable with the current available methods. This DNA damage may act like a stressor which presumably activates similar pathways to ionising radiation-induced *adaptive response* (70).

At the very first time, Sannino and their colleagues (75) presented a study for EMF-induced adaptive response on human blood lymphocytes, when RF pre-exposure significantly decreased the micronuclei incidence induced by mitomycin-C. They used 900 MHz RF exposure at an average absorption rate of 1.25 W/kg, up to a peak value of 10 W/kg SAR. Although the 10 W/kg SAR value is far above the safety exposure limits, it was considered as a worst-case scenario of maximum local exposure of the head in a mobile phone user. The temperature was controlled at 36.9 ± 0.5°C to exclude the thermal effect. Later, they investigated the influence of the cell cycle, when their results confirmed their previous investigation and suggested that the timing of AD exposure to RF is important to elicit AR, it could be induced only at the S-phase of lymphocytes (76).

Sannino *et al.* later extended their investigations and published that 1950 MHz RF exposure can also induce adaptive response against ionising radiation (71) in human

lymphocytes or against mitomycin-C in Chinese hamster lung fibroblast cell line (77). Their study with 3-aminobenzamide - an inhibitor of poly (ADP-ribose) polymerase enzyme - suggests that DNA repair mechanisms are possibly involved in RF-induced adaptive response. He *et al.* (78) also confirmed that poly (ADP-ribose) polymerase enzyme has an impact on RF-induced AR in bone marrow stromal cells.

A study of Ji *et al.* (79) showed that 900 MHz RF exposure influenced the DNA repair mechanism of gamma-irradiated bone marrow stromal cells. Their evidence indicated a faster kinetics of DNA strand break repair in 900 MHz RF combined ionising radiation-exposed cells than gamma-irradiated cells alone.

Cao *et al.* (80) examined whether RF pre-exposure could induce adaptive response against gamma irradiation in mice. They found that 120 $\mu W/cm^2$ power density of 900 MHz led to the highest protection of hematopoietic tissue damage induced by gamma irradiation. Moreover 120 $\mu W/cm^2$ of 900 MHz RF increased further the expression of cyclin-D1, cyclin-E and CDK4 subjected to gamma irradiation. Another *in vivo* study also confirmed that RF exposure could induce adaptive response in mice (81).

These papers contribute to proving that EMF pre-exposure could promote a protective effect against DNA damage induced by a higher dose of consecutive exposure to chemicals or ionising radiation on *in vitro* cell models or *in vivo* animal models. These papers assume that the DNA repair and the cell cycle could be involved in EMF-induced adaptive response. Since the EMF-induced adaptive response scientific topic has recently not been investigated, further investigations – expanding to different frequencies, timing of challenge dose and endpoint - are needed to confirm these assumptions.

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2. OBJECTIVES

The aim of this thesis was to investigate whether non-ionising radiation alone or in combination with other radiation can cause non-thermal effect on the human skin. The research was divided into two parts for better understanding and due to the differences in the experimental approach.

2.1. Part I.: The inflammatory effect of consecutive non-ionising exposures of RF and UV on 3D skin models

Our aim was to observe whether two physical agents − which have been classified in different carcinogenicity categories by IARC −, such as the RF (Group 2B, as possibly carcinogenic to humans) and the UV radiation (Group 1, as carcinogenic) in different consecutive combinations do have any effects on viability, inflammation and photoaging process of human 3D skin model.

We focused on two kinds of exposure within the RF:

- 1950 MHz third generation (3G) wireless mobile technology (Universal Mobile Telecommunications System: UMTS) and
- 2422 MHz Wi-Fi,

while regarding UV radiation, we used a solar simulator.

- Our first hypothesis was that the *adverse* effects of UV radiation on viability and inflammatory processes of 3D skin tissue may be *enhanced* by a subsequent RF-exposure.
- Our second hypothesis was that pre-exposure to RF could *prevent* the 3D skin from the inflammation caused by UV irradiation, through the *adaptive response*.

  - The first objective was to investigate the effect of consecutive exposure to UV radiation and RF exposure on viability and inflammatory process of 3D skin model.
  - The second objective was to investigate the possible adaptive response manifested through induced inflammation of 3D skin model.

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2.2. Part II. The genotoxic effect of consecutive exposure of (non-ionising) IF MF and ionising radiation on human dermal fibroblast cells

Our aim was to observe whether IF MF exposure alone and in combined with ionising radiation exposure has any effect on genotoxicity and co-genotoxicity of human dermal fibroblast cells.

- First, based on the literature, we hypothesised that IF MF radiation alone does not have any effect on human dermal fibroblast cells.
- Our second hypothesis was that IF MF – through the *adaptive response* phenomenon – can prevent the fibroblast cells from the genotoxic effect of ionising radiation.
  - Our first aim was to investigate whether 22 kHz or 250 kHz IF MF could cause any genotoxic effect on fibroblasts.
  - Our second aim was to investigate whether IF MF has any preventive effect against the harmful effects of ionising radiation on human fibroblast cells.

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3. METHODS

3.1. Part I.: 3D skin model exposed to radiofrequency (UMTS or Wi-Fi) and ultraviolet radiation

3.1.1. The 3D skin model

The development of *in vitro* models provides an opportunity to study the biological processes in the skin even more comprehensively. 3D skin models are innovative products, cultured to form a multilayered model of human dermis and/or epidermis. The full-thickness models are developed for skin research in which fibroblast-keratinocyte cell interactions are important. In these models, the dermal-epidermal junction is presented by a well-developed basement membrane. These manufactured skin models are ideal for *in vitro* research because they are uniform and highly reproducible. However, due to this relatively new innovation, these models are more expensive than the conventional cell cultures. Consequently, there are few scientific studies investigating on 3D skin models.

We used a full-thickness 3D skin model, EpiDermFT from MatTek corporation (EFT-300, MatTek, Ashland, USA), to investigate the inflammation processes and photoaging. The EpiDermFT skin system consists of normal human epidermal keratinocytes and normal human dermal fibroblast cells. The same 5 main layers of the epidermis can be found in EpiDermFT (e.g., basal layer, spinous and granular layers, lucidum layer, and cornified layer) like in those found *in vivo*. The dermal layer is composed of viable dermal fibroblasts. Both epidermal and dermal layers are metabolically and mitotically active and form an *in vivo*-like morphology (Figure 2).

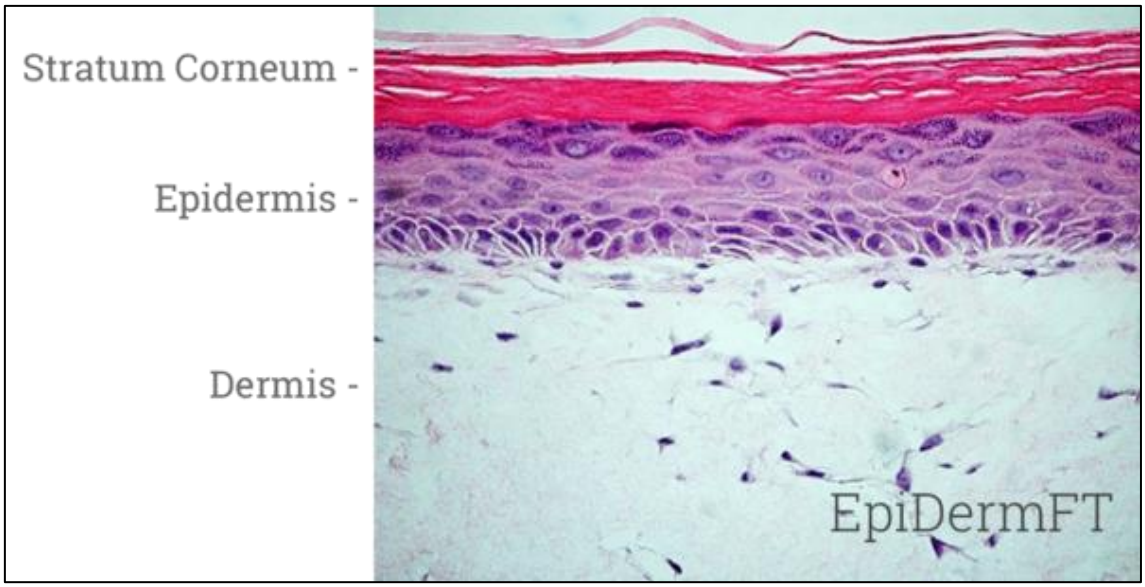

Figure 2. Morphology structure of EpiDermFT. Figure sourced by MatTek. Retrieved 2025 Jan 9 from MatTek's website (82).

EpiDermFT tissues were maintained according to the manufacturer's handle protocol. The tissues were shipped as single-well tissue culture plate inserts at 4°C. After arrival, the inserts were transferred into 35 mm Petri dishes filled with 1 ml medium and maintained at 37°C with 5% CO_2 during the experiments. Two tissues were handled as parallel samples for each condition.

3.1.2. Radiofrequency exposure systems

1950 MHz UMTS exposure system

A short-circuited WR-430 resonant waveguide exposure system at 1950 MHz RF field (Figure 3) was described earlier in our paper (83). The short-circuited waveguide exposure system was adopted from previous studies to optimise efficiency and SAR uniformity inside the *in vitro* tissue samples (84,85). Two waveguides were used, one for effective 1950 MHz RF exposure, one for sham exposure. The sample installation was parallel in both waveguides. Simulations were performed by CST MicrowaveStudio® (Computer Simulation Technology, Darmstadt, Germany, 2016) to evaluate scattering parameters, as well as SAR distribution inside the samples. Both real and sham exposed short-circuited waveguide systems were placed in CO_2 incubator. Air ventilation inside the waveguide was provided, and the temperature of the samples was monitored by optical probes. Temperature elevation of the tissues was kept between +/- 1°C. The 3G

UMTS systems used WCDMA (Wideband Code Division Multiple Access) modulation, which is a non-periodic cocktail of signals with a 5 MHz bandwidth. The UMTS WCDMA signal was generated by a specific UMTS synthesiser (Bonn-Hungary Ltd., Hungary) according to the requirements of RF bioelectromagnetic studies (86). Since the output RF power level of the UMTS synthesiser was limited, an RF power amplifier (Bonn-Hungary Ltd., Hungary) was connected to guarantee the necessary RF power up to 4 W/kg SAR level in the samples.

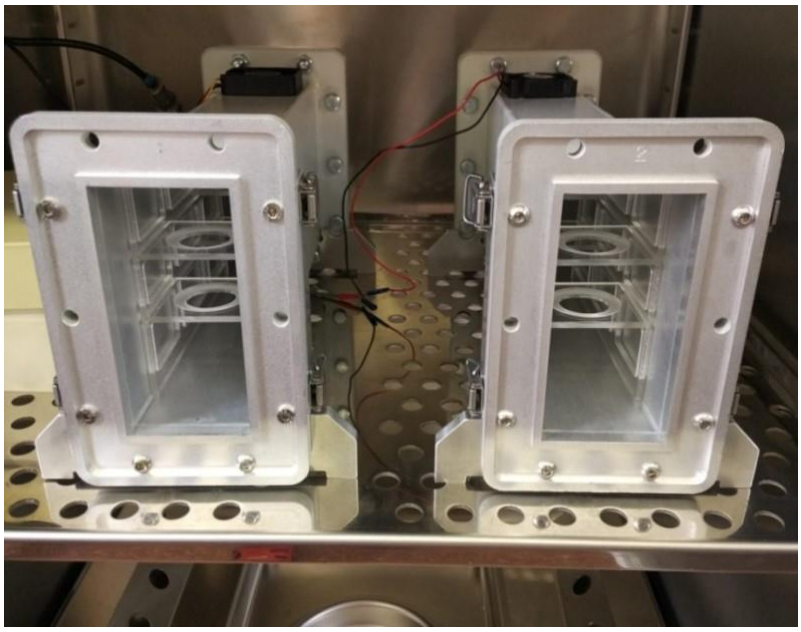

Figure 3. Exposure system for 1950 MHz UMTS exposure. Figure adapted from the author's original publication (83).

2422 MHz Wi-Fi exposure system

The Wi-Fi RF exposure system was built in our laboratory. For this purpose, a wire patch cell (WPC) structure was chosen, described first by Laval *et al.* (87) at 900 MHz, and later modified by Paffi *et al.* (88) operating at Wi-Fi 2.4 GHz.

The WPC was placed in a CO_2 incubator to provide all the necessary environmental conditions needed for *in vitro* experiments (Figure 4). The temperature of the samples was controlled using two plate water jackets placed on the external side of the WPC, which was connected to a thermostatic bath. The disturbances in the electronic instrumentation of the incubator may influence the power density, therefore the WPC was put inside a metal grid shielding cage equipped with RF-absorbing material (20 dB of attenuation) to avoid RF-field reflection of metallic walls. The Wi-Fi signal was generated

by an access point (AP) and a client unit (CU) operated at 2422 MHz frequency. A timer and an RF switch relay were used to achieve the on/off RF exposure (89). The Wi-Fi signal generated by AP/CU units was amplified by a solid-state amplifier (Milmega, Ryde, UK) up to the requested RF power level.

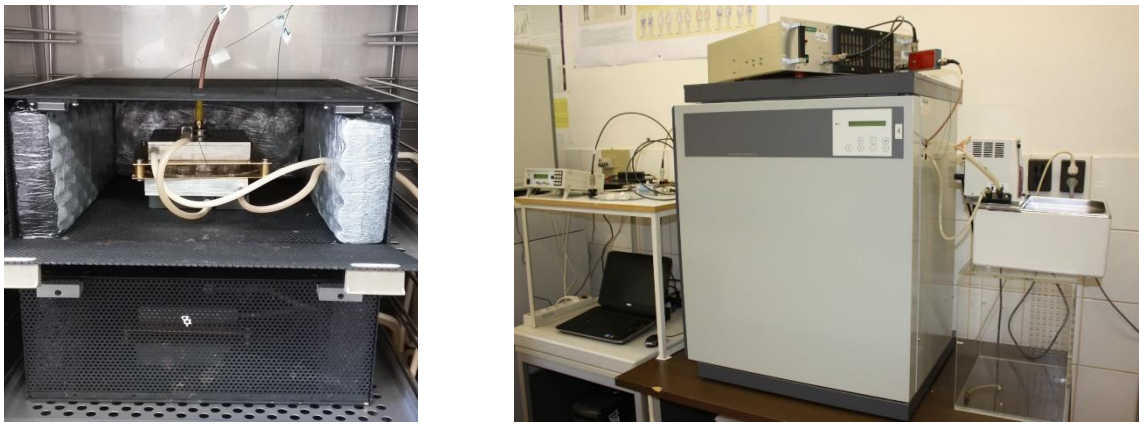

Figure 4. Wi-Fi exposure system**.** The exposure system was placed in an incubator to provide all necessary environmental conditions for *in vitro* experiments. Figure adapted from the author's original publication (90).

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3.1.3. Ultraviolet exposure system

The aim of experimental UV irradiation of these samples was the simulation of natural solar UV exposure of human skin. Thus, we used a sun simulator lamp, which is dedicated by international standards for this purpose. For the UV exposure, a SOL-500 solar simulator (Hönle UV Technology, Gilching, Germany) was used, equipped with a H2 filter which transmitted wavelengths above 290 nm (UVB, UVA, visible light) (Figure 5.).

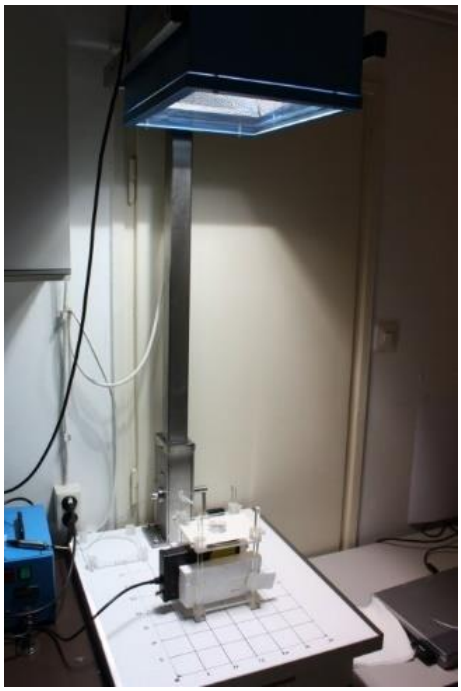

Figure 5. Hönle SOL 500 solar simulator was set for UV exposure. Figure adapted from the author's original publication (83).

The light spectrum of the SOL 500 unit was generated by gas discharge lamps containing metal halides. These light sources emit an almost continuous spectrum that closely resembles natural sunlight and includes both UVA and UVB spectrum. The spectral distribution is shown in Figure 6.

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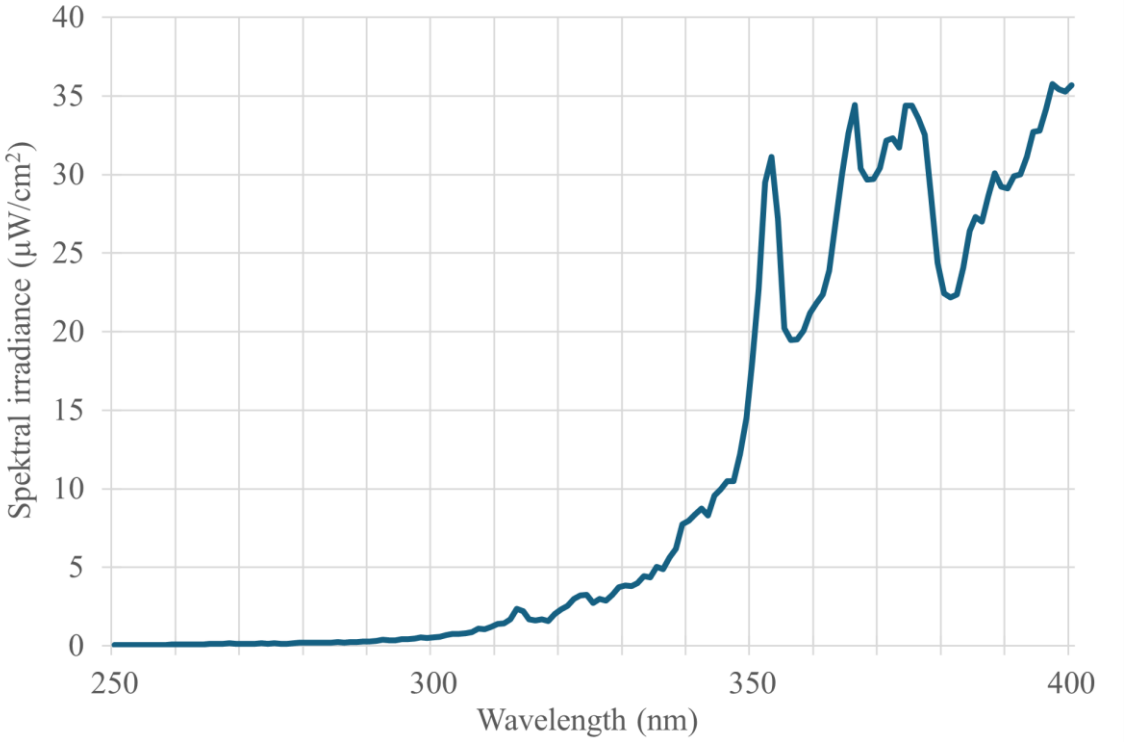

Figure 6. Spectral irradiance of SOL 500 solar simulator. Unpublished figure made by the author.

The radiometric intensity at the Petri dishes was monitored by a calibrated spectroradiometer (ILT-900, International Light, Peabody, MA, USA) (Figure 6) and this value was weighted with erythemal function according to ICNIRP (91). The UV dose of the samples was controlled by defining the exposure time and the distance between the lamp and the samples. The UV dose was determined in Standard Erythema Dose (SED) units. 1 SED is equivalent to 100 J/m^2 erythemally effective radiant exposure (H_{eff}), which was calculated from the integral of the effective irradiance (E_{eff}) with respect to exposure duration (Δ_t).

$$H_{eff} = E_{eff} \times \Delta_t$$

where:

H_{eff} = erythemally effective radiant exposure (J/m^2)

E_{eff} = effective irradiance (W/m^2)

Δ_t = exposure duration (s)

and

$$E_{eff} = \sum_{250}^{400} E_\lambda S_\lambda \Delta_\lambda$$

where:

E_{eff} = effective irradiance (W/m^2)

E_λ = spectral irradiance from measurements (W/m^2 * nm)

S_λ = relative spectral effectiveness

Δ_λ = measurement interval in nanometers (nm)

The effective irradiance was set to 0.111 W/m^2. Therefore, the exposure duration was adjusted to the value of erythemally effective radiant exposure. Petri dishes were exposed for 30 minutes to 2 SED and 60 minutes to 4 SED.

UV doses were adapted to our hypothesis and our preliminary results on viability and cytokine production. During exposures, the medium in Petri dishes was replaced by Dulbecco's Phosphate Saline (DPBS) to avoid the absorption of UV radiation in the medium. The temperature of DPBS was monitored and kept below 37°C by a water bath during the exposures.

3.1.4. Experimental protocols

The order of the UV and the RF exposures varied depending on the aim of the study. In all cases, so-called 'sham exposure' conditions were created to compare the results of exposed and non-exposed samples and to exclude false results. In each experiment, two tissue samples were used as parallels.

Additive Effect protocol

In the case of the first protocol, our hypothesis was that RF exposure might enhance the adverse effects of UV radiation. To test this hypothesis, 2 SED of UV exposure (as first exposure) was followed by a 24-hour RF irradiation (as second exposure) at 4 W/kg of SAR. In both RF conditions (1950 MHz UMTS or 2422 MHz Wi-Fi), the exposure was switched on and off every 20 minutes. For better understanding, we will refer to this protocol as *Additive Effect protocol*, which is shown in Figure 7.

Each experimental run included the following conditions:

- sham exposed, as negative control (SH),

- RF exposed, in a switched on/off manner at 4 W/kg (RF),
- 2 SED of UV irradiated and consecutive 4 W/kg RF exposed in an on/off manner (UVRF),
- 2 SED of UV irradiated (UV).

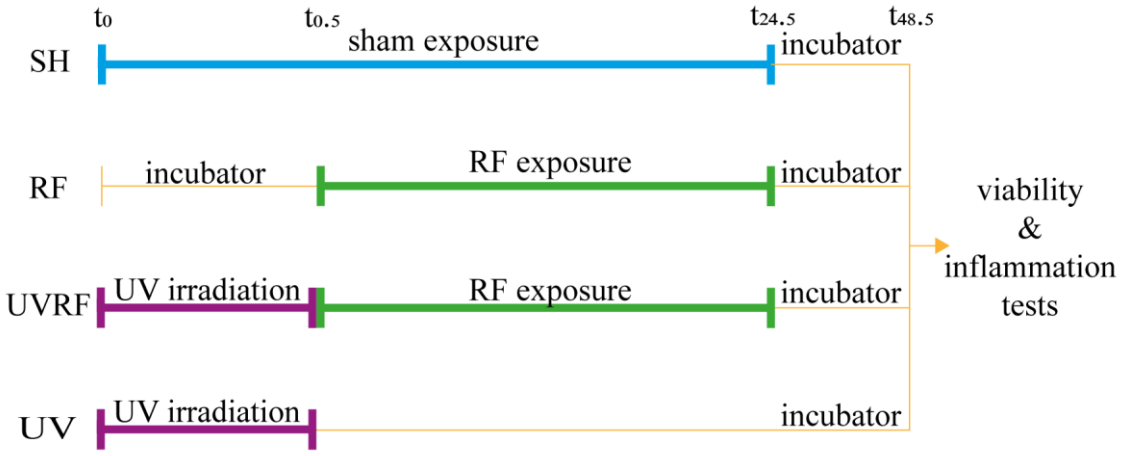

Figure 7. Schematic representation of the *Additive Effects protocol* applied in different types of exposure conditions. The samples were evaluated 24 hours after the exposures by viability and inflammatory tests. SH: sham exposed sample, RF: sample exposed to 4 W/kg RF on/off, UVRF: sample exposed to 2 SED UV and a consecutive 4 W/kg RF on/off, UV: 2 SED UV irradiated sample. The time scale is represented in hours. Figure made by Bertalan Pintér based on the author's instructions.

Adaptive Response protocol

In the case of the second protocol, our hypothesis was that pre-exposure to RF could prevent the skin from the adverse effects of UV irradiation; in other words, RF exposure could induce adaptive response. In this case, a 24-hour continuous RF exposure at 1.5 W/kg (as AD) was followed – after a 4-hour incubation – by a 4 SED of UV exposure (as CD). For better understanding, we will refer to this protocol as *Adaptive Response protocol,* which is shown in Figure 8.

Each experimental run included the following conditions:

- sham exposed, as negative control (SH),
- 1.5 W/kg RF exposed continuously and 4 hours later irradiated with 4 SED of UV (RFUV),
- 4 SED of UV irradiated (UV).

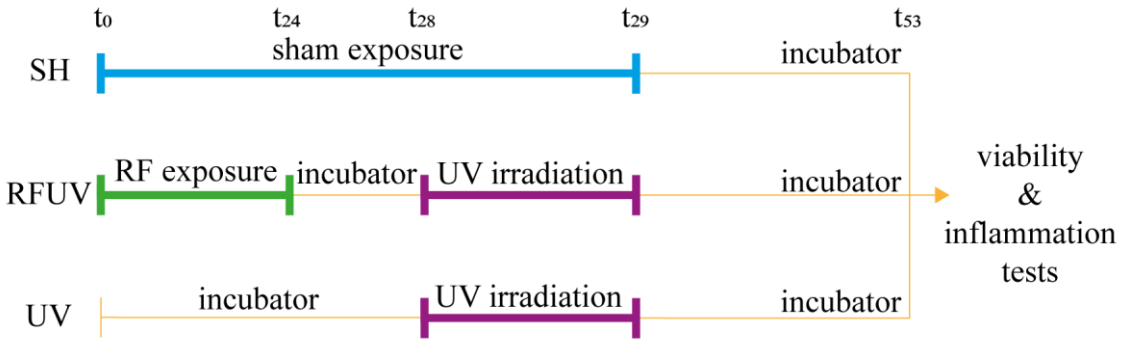

Figure 8. Schematic representation of the *Adaptive Response protocol* applied in different types of exposure conditions. The samples were evaluated 24 hours after the exposures by viability and inflammatory tests. SH: sham exposed sample, RFUV: sample exposed to 1.5 W/kg RF continuously and a consecutive 4 SED UV irradiation, UV: 4 SED UV irradiated sample. The time scale is represented in hours. Figure made by Bertalan Pintér based on the author's instruction.

We adjusted different UV doses and RF modulation accordingly (adapted) to the hypothesis. In the case of the first hypothesis, we used a lower UV dose (2 SED) in order to ensure that we could detect the additional (presumably low) effects of RF exposure – if it occurs at all. Therefore, in the *Additive Effects protocol*, we chose a higher dose of 20 minutes switched on/off modulated RF exposure (4 W/kg) in order to detect possible effects. By switching RF exposure on/off, we aimed to mimic a realistic human exposure to UMTS and Wi-Fi.

In contrast, to verify the second hypothesis, namely the presence of adaptive response, the protective effect of RF exposure – if any at all – would be more likely using lower RF exposure (as AD), and a higher UV dose (4 SED, as CD). Continuous exposure to low dose RF is widely used in medical therapies, we aimed to mimic it. For the *Adaptive Response protocol,* we chose continuous exposure to RF radiation. Since the timing of RF exposure as AD is important to elicit adaptive response (76), we selected

the incubation period between AD and CD (4 hours) based on previously described publications (71,79).

Both protocols were carried out at WCDMA-modulated 1950 MHz UMTS RF exposure and 2422 MHz Wi-Fi exposure. Three independent experiments were carried out at each frequency, exposure protocol and assay endpoint. Viability, cytokine (Il-1α, IL-6, IL-8) and MMP-1 enzyme secretion were investigated on real and sham exposed tissue samples. According to our preliminary results, a 24-hour incubation was necessary after UV exposure for the interleukins to be secreted, and the MMP-1 concentration to reach the highest value.

3.1.5. Assay procedures

Tissue viability

MTT assay is used to measure cellular metabolic activity as an indicator of cell viability, as well as cell proliferation. This assay is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan crystals by metabolically active cells. The viable cells contain NAD(P)H-dependent oxidoreductase enzymes which reduce the MTT to formazan resulting a colorimetric change from yellow to blue (92). MTT kit (MTT-300, MatTek, Ashland, USA) was applied according to the manufacturer's protocol. The percentage of viability of each tissue was determined via spectrophotometry, above 50% were considered as viable cells according to the manufacturer's instructions, which follows the OECD Guideline No. 439 (93,94).

Inflammation and photoaging

At the end of the exposure period, the culture media from Petri dishes were collected and stored at -20°C. Interleukins (IL-1α, IL-6, IL-8) and MMP-1 enzyme secretions were measured by ELISA kits (Thermo Fisher Scientific, Waltham, MA, USA).

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3.1.6. Statistical analysis

The data were analysed using RStudio software, version 1.1.463. All data were subjected to analysis of variance (ANOVA) test. The residuals were also plotted to ensure that all groups had similar variances with approximately normal distribution to fulfil the assumptions of the ANOVA. For pairwise comparison, Tukey's test was conducted. The relevant comparisons of the *Additive Effect protocol* were SH vs. RF and UVRF vs. UV. In the *Adaptive Response protocol,* the relevant comparison was RFUV vs. UV. The *P* value of < 0.05 was considered as significant difference between groups.

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3.2. Part II.: Human dermal fibroblasts exposed to IF MF and ionising radiation

3.2.1. Cell culture

Human Dermal Fibroblast cells from adult skin (Gibco, Thermo Fisher Scientific, Waltham, USA) were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Thermo Fisher Scientific, Waltham, USA) containing 10 % fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific, Waltham, USA) at 37°C in a humidified incubator (Binder, Tuttlingen, Germany) with 5% CO_2. The cells were reseeded into 35 mm Petri dishes (Corning®, NY, USA) at 3–6×10^3 cells/ cm^2 density one day before starting the experimental protocol. The fourth passage of the cell culture was used for the experiments.

3.2.2. Intermediate frequency magnetic field exposure system

The exposure system consisted of a function generator to produce the IF signal, a power amplifier and a solenoid coil cylinder, which was described earlier by our research group (72,95) (Figure 9). The entire coil system was placed into a CO_2 incubator (HETO-HOLTEN A/S, Cellhouse 154, Allerod, Denmark) where the background (50 Hz) magnetic field was negligible, ranged between 0.5 and 1.5 μT (96). Petri dishes were placed into the coil using a plastic holder. The coil operated at resonant mode, and the magnetic flux density was 100 μT (~ 80 A/m) at 22 kHz or at 250 kHz, respectively. The temperature of the sample was maintained at 37°C by the water flow system. In this exposure system, fibroblast cells were exposed to IF MF for 24 hours. Sham exposed samples were kept in another CO_2 incubator, in the same milieu but in the absence of the exposure coil.

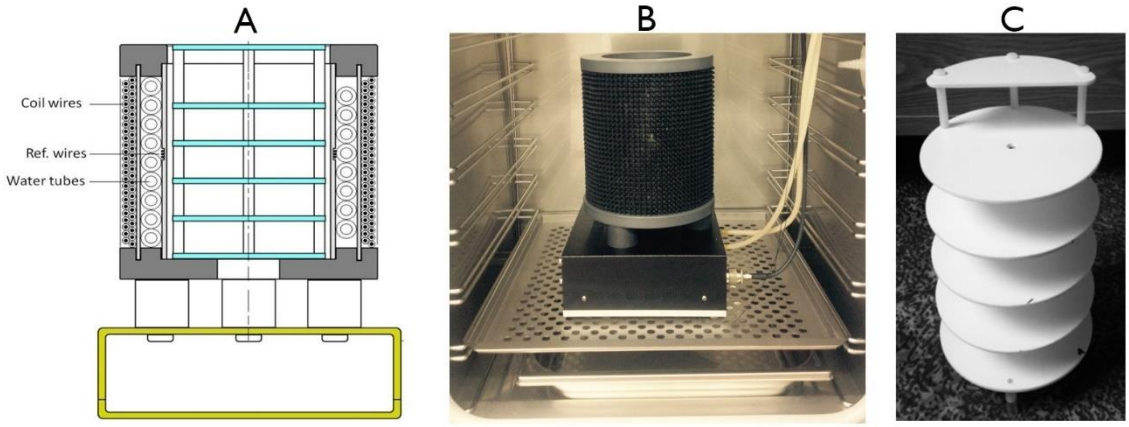

Figure 9. Intermediate frequency (IF) magnetic field (MF) exposure system. **A:** The scheme of the solenoid IF MF exposure system. **B:** The solenoid coil was placed in a CO_2 incubator. **C:** Sample holder within the solenoid coil. Figure adapted from the author's original publication (97).

3.2.3. Ionising irradiation

Ionising irradiation was applied – as CD − to investigate the adaptive response phenomenon. X-RAD 225/Xli X-ray source (Precision X-ray, North Branford, USA) was used for X-ray irradiation at a dose rate of 0.5 Gy/min or 1 Gy/min. This X-ray source is a fully automated system; it has a large internal X-ray chamber, equipped with a motorised turntable and adjustable, programmable specimen shelf. Automated or fixed collimators with integrated filter recognition provided focused irradiation, while an in-built software ensured dose control.

Cells were exposed to ionising radiation in the Petri dishes at room temperature. Each genotoxicity assay has been developed to detect different endpoints − such as single-strand breaks (SSBs), DSBs, oxidative stress or genetic aberration − thus, they differ in their sensitivity. Consequently, the appropriate dose of ionising radiation had to be chosen according to the type and sensitivity limit of the assays, which are shown in Figure 10.

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3.2.4. Experimental protocols

The experimental protocol was carried out at two different frequencies, 22 kHz and 250 kHz. Three independent experiments were performed at both frequencies, each of them evaluated with three different methods: *FPG-modified alkaline comet assay*, *cytokinesis-block micronucleus assay*, *γH2AX assay*.

The following 5 exposure conditions were examined:

- sham and sham exposure as negative control (SH+SH)
- IF MF and sham exposure (IF MF+SH)
- IF MF exposure and challenged with ionising radiation 4 hours later, as CD (AR)
- ionising irradiation alone, according to the selected CD (IR)
- ionising radiation as a positive control (PC).

Two Petri dishes were handled as parallels per conditions at each endpoint. Sham exposed samples received exactly the same environmental conditions that other samples had at that time, but in the absence of the actual IF MF or ionising exposure. All Petri dishes were kept in the same milieu and handled simultaneously.

We select 4-hour incubation time between IF MF (as AD) and ionising radiation (as CD) to investigate the adaptive response, based on previously published studies as described above in section 3.1.4.

The *alkaline comet assay* protocol (with FPG modification) is presented in Figure 10(A). Since the oxidative stress and SSBs of the DNA usually regenerate rapidly – within 4 hours by DNA repair – the *FPG-modified alkaline comet assay* protocol was completed with two additional exposure conditions (‘SH’ and ‘IF MF’ condition) to examine the possible genotoxic effect of IF MF independently. In this case, cells were exposed to sham or IF MF for 24 hours and immediately processed for analysis with the comet assay. Figure 10 also shows the protocol of the *cytokinesis-block micronucleus assay* (B) and the *γH2AX assay* (C). To minimise individual biases, all experiments for each endpoint were assessed in a blind manner.

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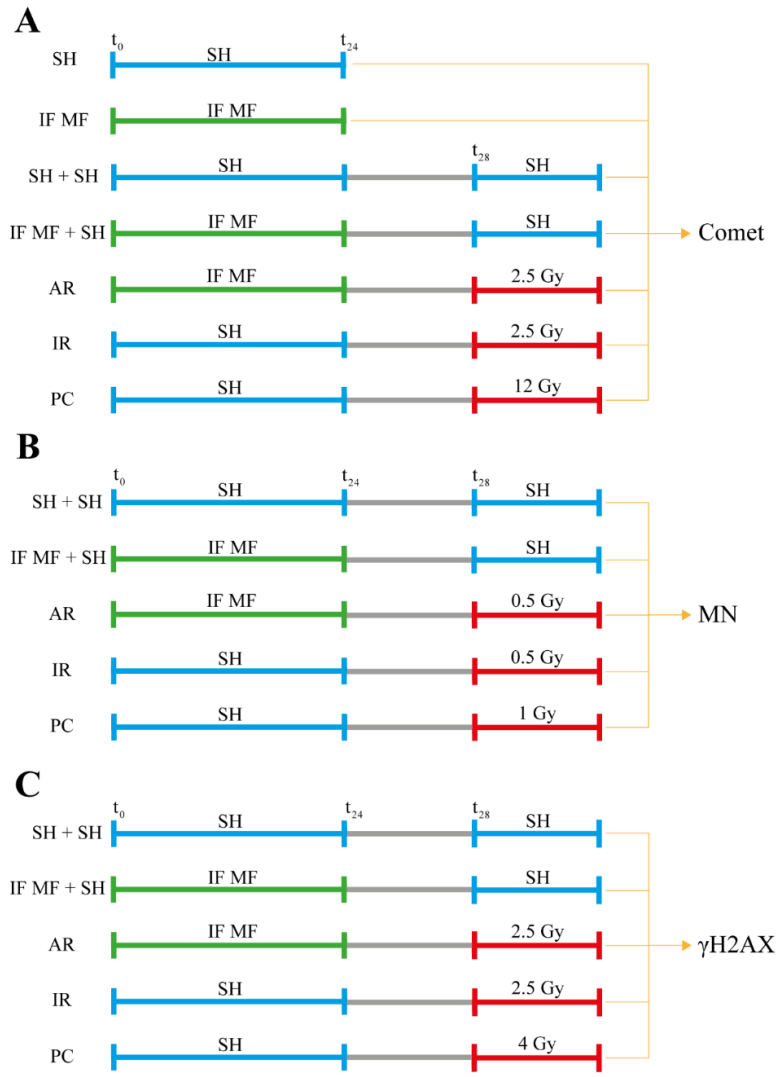

Figure 10. Schematic representation of the experimental protocols. The protocol and irradiation conditions allowed us to investigate the direct genotoxic effects of exposure to IF MF and the adaptive response (AR), respectively. Human dermal fibroblast cells were exposed to 22 kHz or 250 kHz IF MF for 24 h (at t0 - t24) and/or four hours later were exposed to ionising irradiation (at t28). The time of ionising irradiation was a maximum of 15 min. **(A):** The protocol and irradiation conditions used for *FPG-modified alkaline comet assay*. SH: 24 h of sham exposure alone, IF MF: 24 h of IF MF exposure alone, SH+SH: sham and sham exposure, IF MF+SH: IF MF and sham exposure, AR (adaptive response): IF MF exposure and 4 h later challenged with 2.5 Gy ionising radiation, IR: 2.5 Gy ionising irradiation only, PC (positive control): 12 Gy as a positive control. **(B):** The protocol and irradiation conditions used for *cytokinesis block micronucleus assay*. SH+SH: sham and sham exposure as a negative control, IF MF+SH: IF MF and sham exposure, AR: IF MF exposure and 4 h later challenged with 0.5 Gy ionising radiation, IR: 0.5 Gy ionising irradiation only, PC: 1 Gy as a positive control. **(C):** The protocol and irradiation conditions used for the *γH2AX Assay*. SH +SH: sham

and sham exposure as a negative control, IF MF+SH: IF MF and sham exposure, AR: IF MF exposure and 4 h later challenged with 2.5 Gy ionising radiation as CD, IR: 2.5 Gy ionising irradiation only, PC: 4 Gy as a positive control. Figure adapted from the author's original publication (97).

3.2.5. Assay procedures

FPG-modified alkaline comet assay

The *alkaline comet assay* (a single-cell gel electrophoresis technique) was used to detect the SSBs of DNA damage (98). The *FPG-modified alkaline comet assay* protocol introduced by Dušinská and Collins (99) was applied to detect 8-oxoguanine in DNA. The formamidopyrimidine DNA glycosylase (FPG) from *E. coli* releases damaged purines, generating an apurinic/apyrimidic (AP) site. The advantage of this method is that the FPG enzyme cleaves the AP sites, creating nucleotides that appear in the *FPG-modified alkaline comet assay* added to the SSBs, thus also allowing the measurement of oxidative stress as well.

The cells were trypsinised and the cell suspension from a Petri dish was mixed with 1% low-melting point agarose and pipetted into slides pre-coated with 1% normal-melting point agarose. All slides were immersed in freshly prepared cold lysis buffer (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH; 1 % Triton X-100) and lysed at 4°C. Up to this point, all slides were treated according to the *alkaline comet assay* procedure. Those slides, which were not treated with FPG-modification *(Lysis condition)* – measuring SSBs only –, were run with electrophoresis after lysis.

Those slides, which were treated with FPG modification (*Buffer condition* and *FPG condition*) were washed with enzyme reaction buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM Na_2EDTAH_2O, 0.2 mg/ml BSA, KOH, pH 8.0), then were treated with 50 µl enzyme reaction buffer *(Buffer condition)* or FPG enzyme solution *(FPG condition)* in 1:1000 dilutions and incubated at 37°C for 30 min. All slides (*Lysis, Buffer and FPG condition*) were run with agarose gel electrophoresis in cold electrophoresis solution (1 mM Na_2EDTA, 300 mM NaOH, pH 13). After 20 minutes of unwinding, the electrophoresis lasted for 22 minutes. The voltage and the running time were set up by the following: V/cm*min = 20. The prepared slides were stained with Sybr Gold (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) and evaluated using the

Comet Assay IV software (Instem, UK). 100 comets were examined per slide and the tail DNA% parameter was calculated to describe each comet. (Figure 11), which indicates the amount of DNA fragments in the nucleus.

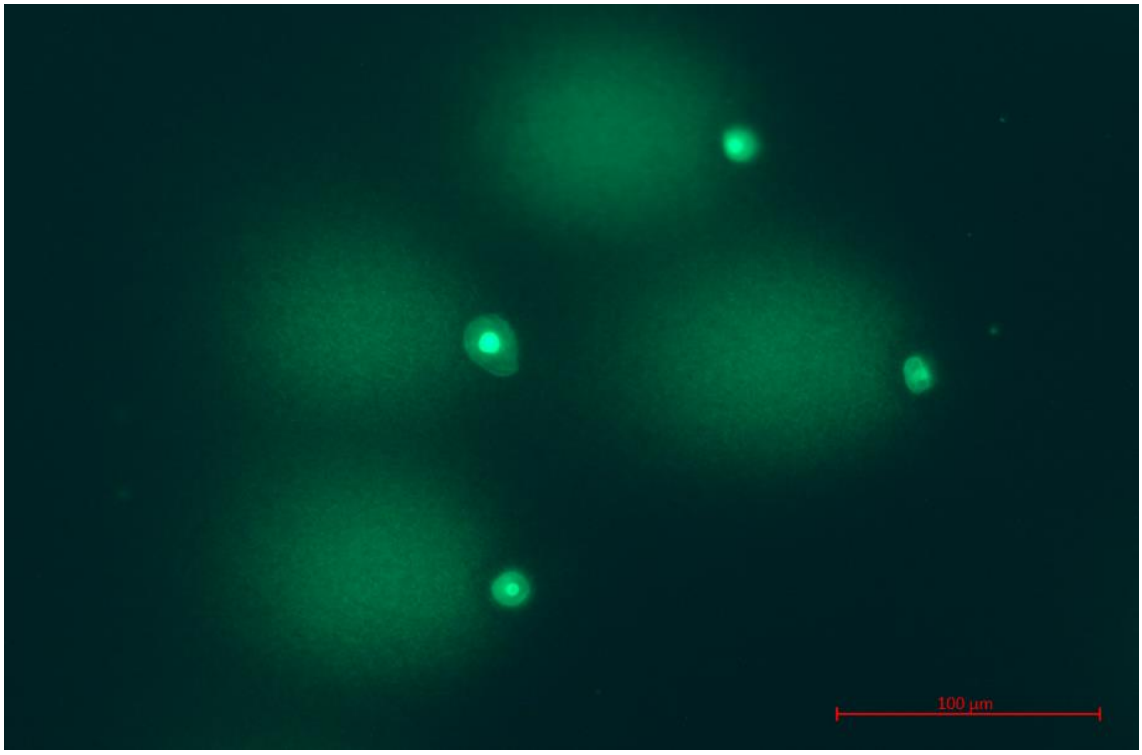

Figure 11. *FPG-modified alkaline comet assay* measures 8-oxoguanine added to SSBs, as oxidative stress. Figure created by the author with Zeiss Zen Microscopy Software.

Cytokinesis-block micronucleus assay

The *cytokinesis-block micronucleus assay* is a preferred method for detecting DNA damage, aberrations and chromosome fragmentations. The method is based on the inhibition of the last stage of cell division: the cytokinesis. Cytochalasin-B blocks the division of the cytoplasm, resulting in binucleated cells. DNA damage results chromosome fragments or whole chromosomes – that lag behind at anaphase – form micronuclei in binucleated cells. The examination of the number and the formation of micronuclei provides a comparative assay for DNA damage detection.

The *cytokinesis-block micronucleus assay* was performed according to standard procedures (100,101). Fibroblast cells were cultured on 20x20 mm coverslips in 35 mm Petri dishes. After exposure, 1 µg/ml cytochalasin-B (Sigma-Aldrich, St. Louis, MO USA) was added to the culture medium and fibroblasts were incubated for 72 hours further. Hypotonic potassium chloride (0.075 M) solution was added for 5 minutes and

fixation was performed by adding methanol. Fixed cells were stained with 10 μg/ml Acridine Orange (Sigma-Aldrich, St. Louis, Missouri, USA) in PBS and examined at 63× magnification objective using a Zeiss AxioPlan fluorescence microscope (Oberkochen, Germany) (Figure 12). According to Fenech *et al.* (100), scoring was based on visual inspection. 100 cells in each sample were measured for Nuclear Division Index (NDI) calculation and cytotoxicity scoring. Micronuclei formations (MNi) were evaluated in 500 binucleated (BN) cells. The frequency of BN cells containing MNi (BN with MNi) and the frequency of MNi in BN cells (MNi in BN) were counted.

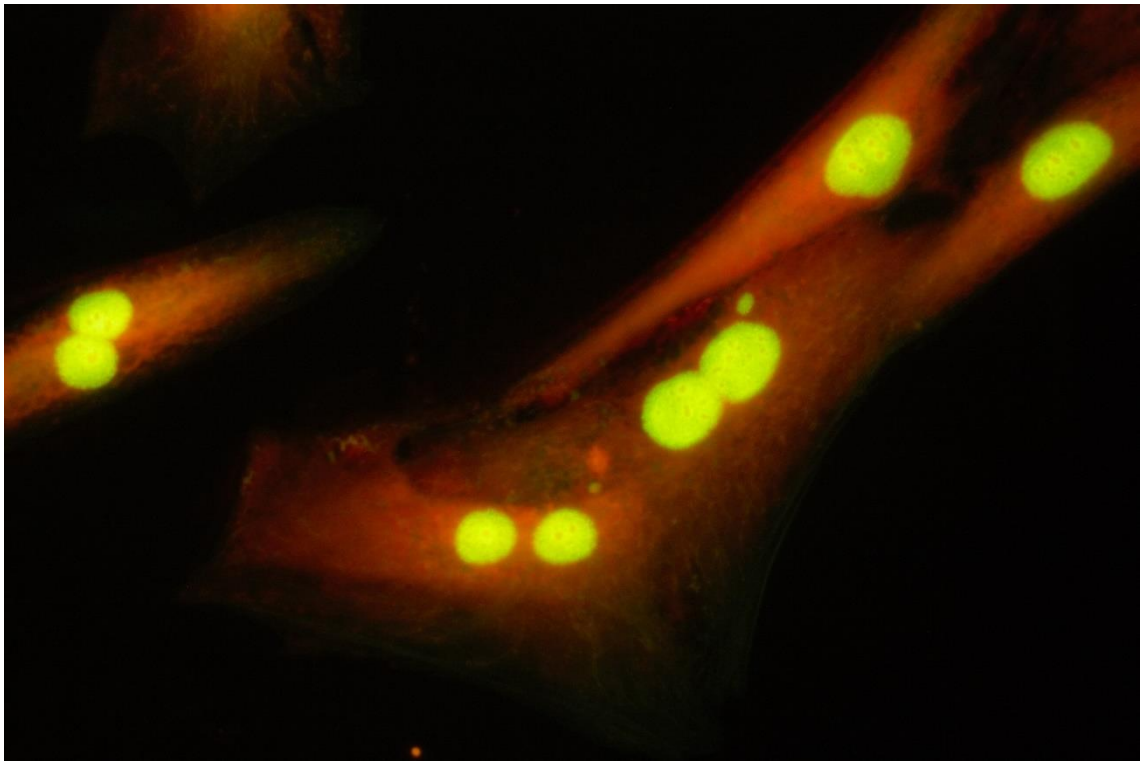

Figure 12. *Cytokinesis-block micronucleus assay* detects the DNA damage, aberration and chromosome fragmentation, which form micronuclei in binucleated cells. Figure created by the author.

γH2AX assay

In eukaryotic cells, DNA is packaged into nucleosomes. A nucleosome is a "beads on a string" structure in which histone octamers – two from each of four histone protein families, H4, H3, H2B and H2A – wrap around 146 base pairs (102). The nucleosomes are further coiled into chromatin and then into chromosomes. H2AX protein, as a subfamily of H2A histone, is a key element in DNA repair by identifying the DSBs in the DNA. H2AX histone phosphorylates at serine 139 around DSBs, wherethe

phosphorylation occurs in the gamma position, thus the phosphorylated histone form is called γH2AX (103). Since γH2AX phosphorylation is rapidly induced by DNA DSBs, it is one of the earliest markers of DSBs. There are several possibilities to visualise γH2AX, the most used is staining by antibodies, when the specific antibody binds to γH2AX, visualising a “focus” in the nucleus.

The cells were cultured on 20x20 mm coverslips in the 35 mm Petri dishes. Based on our preliminary studies, a 24-hour incubation period after the ionising radiation was necessary to detect a manageable number of γH2AX foci. After this incubation, the cells were washed with PBS and fixed by adding freshly prepared ice-cold methanol/acetone solution (1:1) at 4°C for 10 minutes. Cells were rewashed, and blocking was performed with 5% BSA in PBS for 30 minutes at room temperature. Primary γH2AX antibody (Phospho-histone H2A.X, Ser139, 20E3, Rabbit, mAb, 1:4000, Leiden, Netherlands) were added to the cells and incubated overnight at 4°C. The cells were washed, then reacted with goat–conjugated secondary antibodies (Alexa Fluor 488 goat anti-rabbit IgG (H+L), Invitrogen®, Thermo Fisher Scientific, Waltham, MA, USA) and incubated for further 2 hours at 4°C. The cells were stained, and coverslips were stuck onto glass slides with Fluoroshield Mounting Medium containing DAPI (abcam® TM, Cambridge, UK). 200 cells from each slide were examined at 63–100 × magnification objective by using a Zeiss AxioPlan fluorescence microscope (Oberkochen, Germany) (Figure 13). Foci containing cells and the foci number in these cells were counted.

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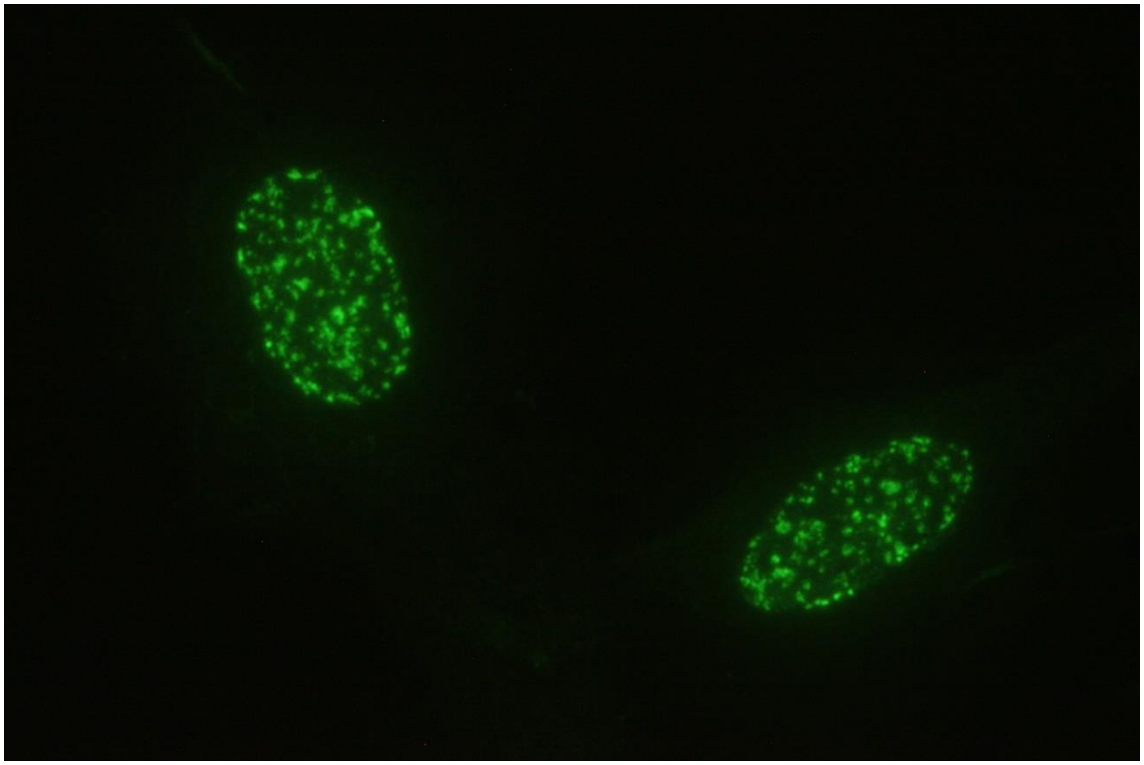

Figure 13. *γH2AX assay* measures the DSBs in the nucleus, which glow green in stained nuclei. Figure created by the author.

3.2.6. Statistical analysis

Analyses were carried out with R Studio software version 1.4.1106 (Software Foundation Inc., Boston, MA, USA). Statistical significance was considered for the p-value < 0.05. The following comparisons were tested: SH + SH vs IF MF + SH, AR vs IR, SH + SH vs IR. A further comparison of SH vs IF MF was relevant for *FPG-modified alkaline comet assay* method.

For *FPG-modified alkaline comet assay*, the median of Tail DNA% was calculated for each slide of the three experiments and analysed by Linear Mixed-Effects Model. For *cytokinesis-block micronucleus assay* the NDI data were analysed with ANOVA. The number of binucleated cells with micronuclei (BN with MNi) and the number of micronuclei in binucleated cells (MNi in BN) were analysed by fitting a negative binomial model. The average number of foci was calculated for the statistical analysis of the *γH2AX assay*. Statistical comparisons were carried out by fitting a linear model using Generalized Least Squares.

4. RESULTS

4.1. Part I.: 3D skin tissue exposed to radiofrequency (UMTS or Wi-Fi) and ultraviolet radiation

4.1.1. 3D skin viability

If the reduction of the viability value reached 50% compared to the sham exposed (non-treated) sample, treated samples were considered as non-viable and excluded from the evaluation. The viability results of the 1950 MHz UMTS exposed protocols are shown in Table 1. and Table 2. shows the 2422 MHz Wi-Fi exposed protocols. UV exposure significantly decreased tissue viability in any case. There were no significant changes between relevant treatments (SH vs RF, UVRF vs UV).

Table 1. Viability of cells according to the MTT assay of 1950 MHz UMTS exposed protocols. Data represent the mean and the standard deviation (SD) of the three independent experiments normalised to SH exposed samples (100%). In the *Additive Effect protocol*, samples were exposed to 2 SED of UV exposure and/or RF irradiation at 4 W/kg SAR. In the *Adaptive Response protocol*, samples were exposed to 4 SED of UV exposure and/or RF irradiation at 1.5 W/kg SAR.

Additive Effect protocol		
	Viability %	
Treatment	**Mean ± SD**	***p*-value**
SH	100 ± 0.0	0.5875
RF	95.89 ± 3.29	
UVRF	85.49 ± 7.76	0.9221
UV	83.61 ± 3.48	

Adaptive Response protocol		
	Viability %	
Treatment	**Mean ± SD**	***p*-value**
SH	100 ± 9.21	
RFUV	83.34 ± 9.35	0.6194
UV	79.23 ± 6.39	

Table 2. Viability of cells according to the MTT assay of 2422 MHz Wi-Fi exposed protocols. Data represent the mean and the standard deviation (SD) of the three independent experiments normalised to SH exposed samples (100%). In the *Additive Effect protocol*, samples were exposed to 2 SED of UV exposure and/or RF irradiation at 4 W/kg SAR. In the *Adaptive Response protocol*, samples were exposed to 4 SED of UV exposure and/or RF irradiation at 1.5 W/kg SAR. Table created by the aurhor.

Additive Effect protocol		
	Viability %	
Treatment	**Mean ± SD**	***p*-value**
SH	100 ± 0.0	0.992
RF	99.15 ± 3.17	
UVRF	91.95 ± 3.89	0.873
UV	89.54 ± 8.16	

Adaptive Response protocol		
	Viability %	
Treatment	**Mean ± SD**	***p*-value**
SH	100 ± 0.0	
RFUV	72.93 ± 12.23	0.778
UV	68.73 ± 8.15	

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4.1.2. Secretion of inflammatory cytokines and enzyme

Effect of UMTS exposure in the Additive Effect protocol on 3D skin model

There were no significant changes between treatments in IL-1α, IL-6 and IL-8 concentrations, but a slight (not significant) increase in IL-1α concentration due to UVRF treatment was observed. The concentration of MMP-1 enzyme was significantly ($p = 0.01$) decreased in UVRF samples compared to UV samples (Figure 14).

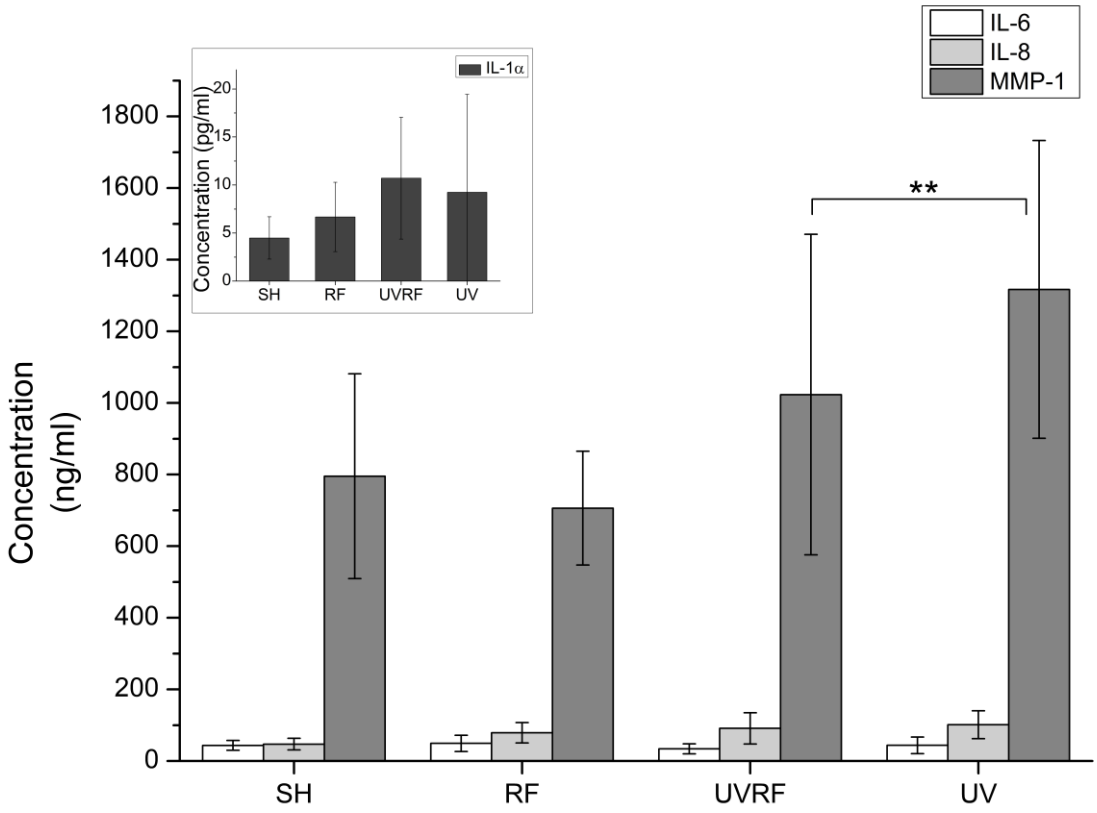

Figure 14. Interleukins and MMP-1 concentration of the UMTS exposed *Additive Effect protocol*, when tissue samples were exposed to 2 SED of UV radiation and/or 24-hour 1950 MHz WCMDA UMTS exposure at 4 W/kg SAR. Data represent the mean concentrations of cytokines and MMP-1 enzyme ± SD of the three independent experiments. Figure created by the author with Origin software based on the author's original publication (83).

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Effect of UMTS exposure in the Adaptive Response protocol on 3D skin model

4 SED of UV exposure significantly increased the interleukins and MMP-1 enzyme concentrations compared to SH exposure. A tendency for decreased values of all interleukins and MMP-1 enzyme concentrations due to RFUV was observed compared to UV radiation (Figure 15). The difference was significant in IL-1α concentration ($p = 0.004$) and in IL-8 concentration ($p = 0.02$).

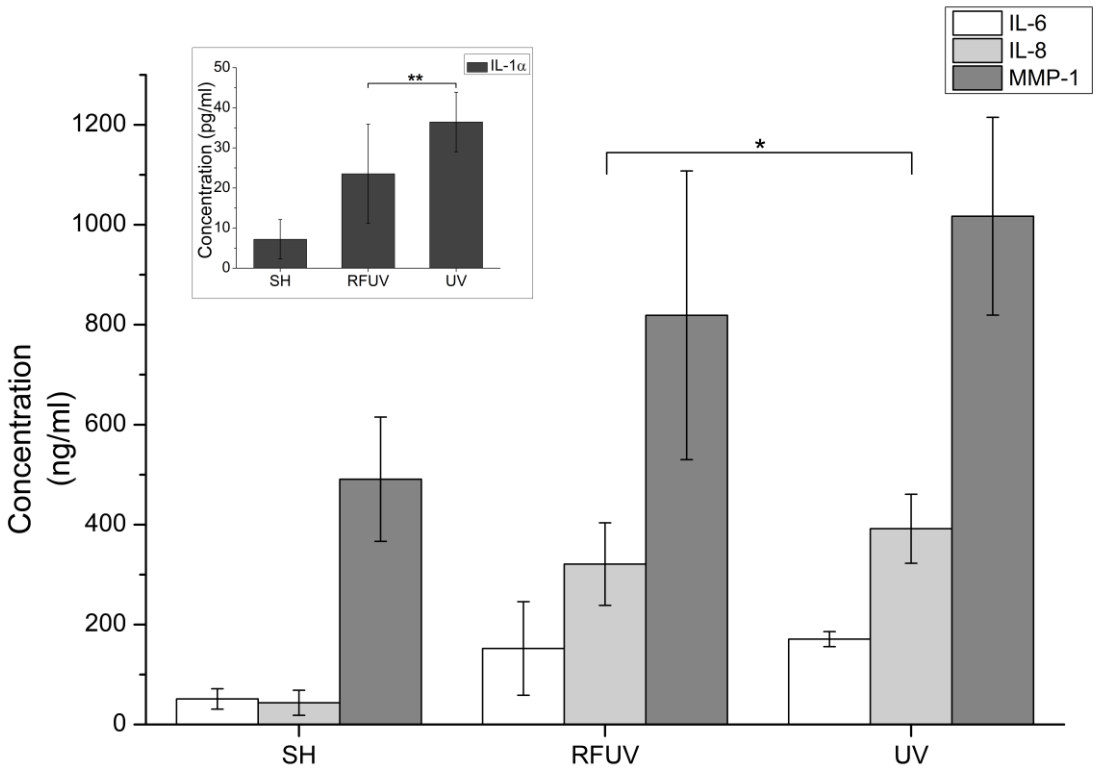

Figure 15. Interleukins and MMP-1 concentration of the UMTS exposed *Adaptive Response protocol*, when tissue samples were exposed to 24-hour 1950 MHz WCMDA UMTS at 1.5 W/kg SAR, then 4 SED of UV radiation. Data represent the mean concentrations of cytokines and MMP-1 enzyme ± SD of the three independent experiments. Figure created by the author with Origin software based on the author's original publication (83).

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Effect of Wi-Fi exposure in the Additive Effect protocol on 3D skin model

We found no significant differences between relevant groups. RF exposed samples were not significantly different from any other samples. Moreover, UVRF exposure slightly (but not significantly) increased the IL-1α, IL-6 and IL-8 concentration compared to UV exposure (Figure 16).

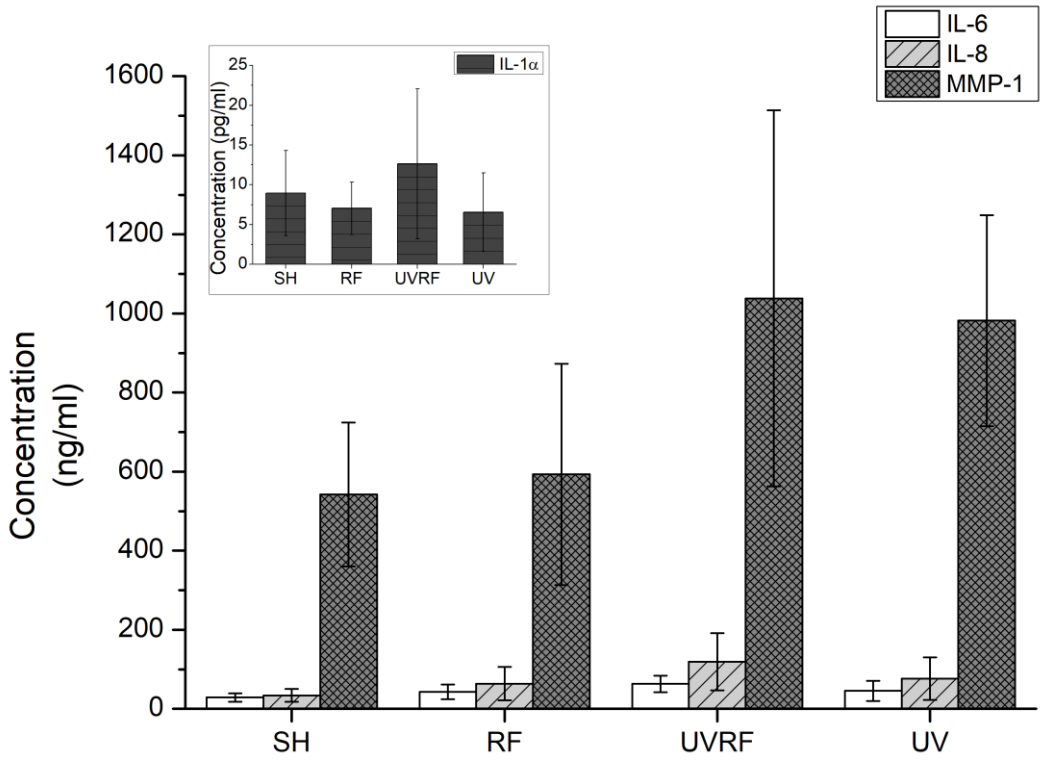

Figure 16. Interleukins and MMP-1 concentration of the Wi-Fi exposed *Additive Effect protocol*, when tissue samples were exposed to 2 SED of UV radiation and/or 24-hour 2422 MHz Wi-Fi at 4 W/kg SAR. Data represent the mean concentrations of cytokines and MMP-1 enzyme ± SD of the three independent experiments. Figure created by the author with Origin software based on the author's original publication (90).

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Effect of Wi-Fi exposure in the Adaptive Response protocol on 3D skin model

UV exposure caused significantly increased interleukins and MMP-1 concentrations compared to SH ($p < 0.01$). RFUV and UV treatments were not significantly different at any concentration. However, a slight trend of the protective effects of RF exposure, namely adaptive response, was observed in IL-6 and IL-8 production (Figure 17).

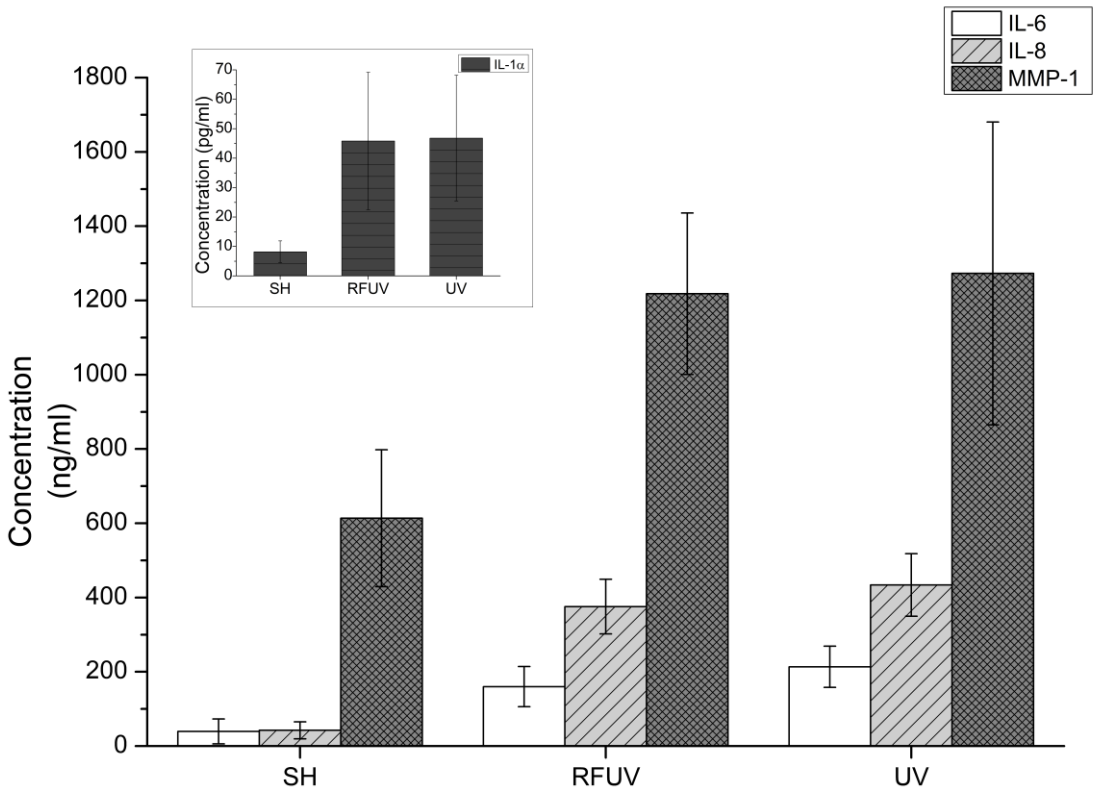

Figure 17. Interleukins and MMP-1 concentration of the Wi-Fi exposed *Adaptive Response protocol*, when tissue samples were exposed to 24-hour 2422 MHz Wi-Fi at 1.5 W/kg SAR, then 4 SED of UV radiation. Data represent the mean concentrations of cytokines and MMP-1 enzyme ± SD of the three independent experiments. Figure created by the author with Origin software based on the auhor's original publication (90).

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4.2. Part II.: Fibroblasts exposed to intermediate frequency magnetic field and ionising radiation

4.2.1. SSBs and oxidative stress observed by FPG-modified alkaline comet assay

We investigated the SSBs with *alkaline comet assay* and oxidative stress with the *FPG-modified alkaline comet assay*. The results are shown in Figure 18 and Figure 19. The ΔTail DNA% values derived from the difference between FPG and Buffer Tail DNA% is shown in the secondary Y-axis. ΔTail DNA% represents the amount of 8-oxoguanine as oxidative stress in the tail.

Overall, the enzyme buffer (Buffer) did not differ from Lysis, however, FPG enzyme treatment caused significantly higher Tail DNA% values. In both frequencies (22 kHz and 250 kHz), FPG enzyme treatment showed significant differences neither between SH+SH vs IF MF+SH, nor between AR vs IR (2.5 Gy). However, a significant increase in Tail DNA% ($p < 0.001$) occurred in IR (2.5 Gy) exposed cells compared to SH+SH exposed cells (Figure 18).

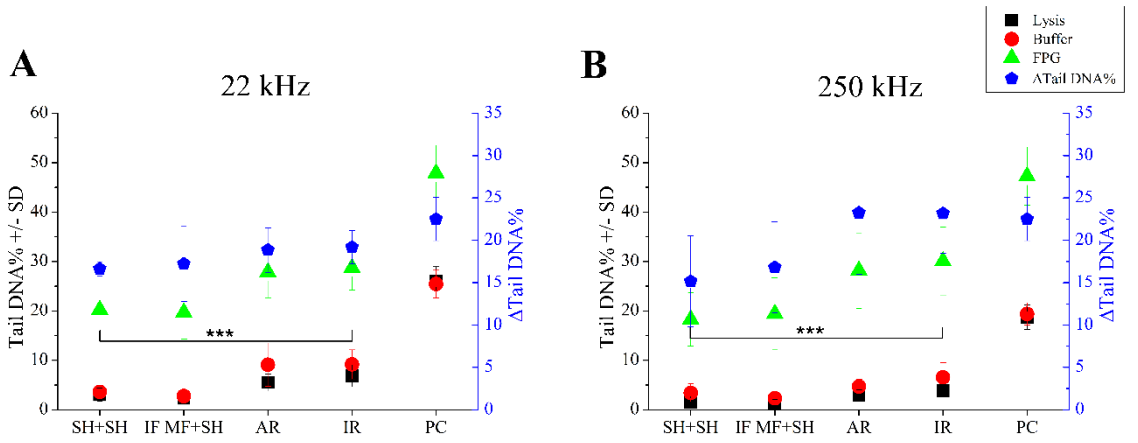

Figure 18. Results of the *FPG-modified alkaline comet assay* investigating the adaptive response. The mean of the median Tail DNA% value of 22 kHz **(A)** and 250 kHz **(B)** IF MF exposed cells ± SD. The ΔTail DNA% values (on the secondary right Y-axis) derived from the difference between FPG and buffer Tail DNA% represent the amount of 8-oxoguanine as oxidative stress level. SH+SH: sham and sham exposure, IF MF+SH: IF MF and sham exposure, AR: IF MF and 2.5 Gy of ionising radiation, IR: 2.5 Gy of ionising radiation, PC: 12 Gy of ionising radiation. ***: $p < 0.001$. Data represent three independent experimental replicates at each experimental condition. Figure adapted from the author's original publication (97).

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Regarding to the additional two conditions determined after 24 hours of exposure, 22 kHz IF MF condition did not differ from SH exposure (Figure 19/A). On the other hand, 250 kHz IF MF exposure showed significantly increased Tail NDA% ($p = 0.033$) compared to SH exposure (Figure 19/B).

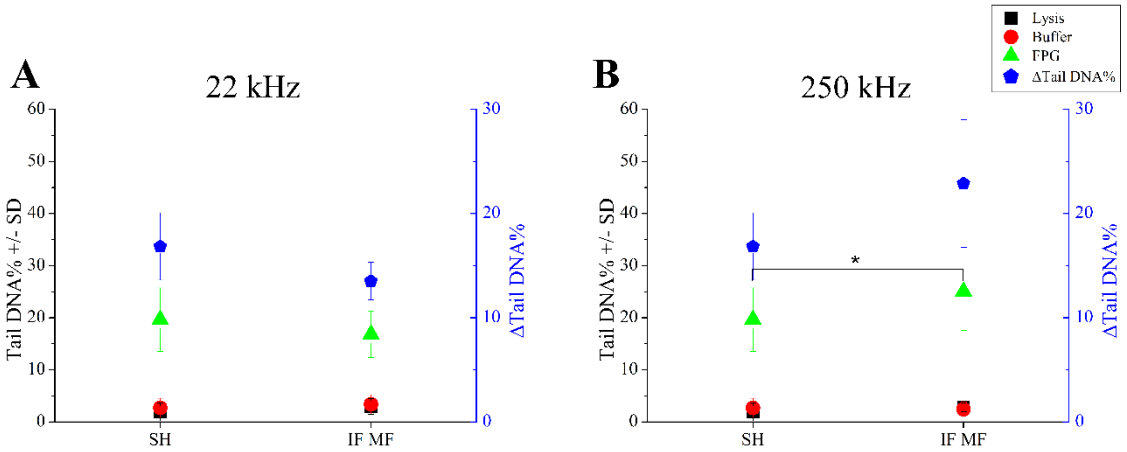

Figure 19. Results of the *FPG-modified alkaline comet assay* investigating the IF MF alone. The mean of the median Tail DNA% value of 22 kHz **(A)** and 250 kHz **(B)** IF MF exposed cells ± standard deviation (SD). The ΔTail DNA% values (on the secondary right Y-axis) derived from the difference between FPG and buffer Tail DNA% represent the amount of 8-oxoguanine as oxidative stress level. SH: 24 h of sham exposure, IF MF: 24 h of IF MF exposure, *: $p < 0.05$. Data represent three independent experimental replicates at each experimental condition. Figure adapted from the aurhor's original publication (97).

4.2.2. Micronuclei analysis of cytokinesis-block micronucleus assay

Table 3. shows the results of 22 kHz IF MF protocol. Nuclear Division Index (NDI) was significantly different ($p < 0.001$) between SH+SH and IR groups. The number of binucleated cells with micronuclei (BN with MNi) significantly decreased in IF MF+SH group ($p = 0.035$). Furthermore significantly increased in IR group ($p < 0.001$) compared to SH + SH group. The difference in the number of micronuclei in binucleated cells (MNi in BN) was marginally significant between SH+SH vs IF MF+SH ($p = 0.069$) and strongly significant between SH+SH vs IR groups ($p < 0.001$). A slight, non-significant reduction in BN with MNi and MNi in BN appeared between AR and IR groups.

Table 3. Results of the *cytokinesis-block micronucleus assay* at 22 kHz IF MF. These data show the average Nuclear Division Index (NDI), the number of binucleated cells with micronuclei (BN with MNi) and the number of micronuclei in binucleated cells (MNi in BN) +/- SD. Data represent three independent experimental replicates at each experimental condition. SH+SH: sham and sham exposure, IF MF+SH: IF MF (22 kHz) and sham exposure, AR: IF MF (22 kHz) and 0.5 Gy of ionising radiation, IR: 0.5 Gy of ionising radiation, PC: 1 Gy of ionising radiation. *: significantly different from SH (p-value < 0.05), ***: significantly different from SH (p-value < 0.001). Table adapted from the author's original publication (97).

	Per 100 cells	Per 500 BN cells	
Treatment	Number of NDI +/- SD	Number of BN with MNi +/- SD	Number of MNi in BN +/- SD
SH+SH	1.51 +/- 0.04	10.17 +/- 5.34	10.67 +/- 5.79
IF MF (22 kHz)+SH	1.48 +/- 0.03	5.67 +/- 1.75 *	6.33 +/- 2.14
AR	1.27 +/- 0.05	35.83 +/- 7.25	39.83 +/- 10.34
IR	1.27 +/- 0.06 ***	41.00 +/- 10.47 ***	47.50 +/- 11.06 ***
PC	1.18 +/- 0.03	86.67 +/- 14.08	108.83 +/- 20.41

The results of the 250 kHz IF MF protocol are shown in Table 4. The Nuclear Division Index (NDI) was significantly different ($p < 0.001$) between the SH+SH and IR groups. The number of binucleated cells with micronuclei (BN with MNi) was significantly higher in IR exposed samples ($p < 0.001$) compared to SH+SH. The difference in the number of micronuclei in binucleated cells (MNi in BN) was highly

significant between SH+SH vs IR groups ($p < 0.001$). A slight, non-significant reduction in BN with MNi and MNi in BN appeared between AR and IR groups.

Table 4. Results of the *cytokinesis-block micronucleus assay* at 250 kHz IF MF. These data show the average Nuclear Division Index (NDI), the number of binucleated cells with micronuclei (BN with MNi) and the number of micronuclei in binucleated cells (MNi in BN) +/- SD. Data represent three independent experimental replicates at each experimental condition. SH+SH: sham and sham exposure, IF MF+SH: IF MF (250 kHz) and sham exposure, AR: IF MF (250 kHz) and 0.5 Gy of ionising radiation, IR: 0.5 Gy of ionising radiation, PC: 1 Gy of ionising radiation. ***: significantly different from SH (p-value < 0.001). Table adapted from the author's original publication (97).

Treatment	Per 100 cells	Per 500 BN cells	
	Number of NDI +/- SD	Number of BN with MNi +/- SD	Number of MNi in BN +/- SD
SH+SH	1.51 +/- 0.02	8.40 +/- 2.88	9.20 +/- 3.11
IF MF (250 kHz)+SH	1.51 +/- 0.08	8.50 +/- 5.43	9.67 +/- 6.47
AR	1.27 +/- 0.06	41. 67 +/- 9.22	49.83 +/- 12.42
IR	1.30 +/- 0.09 ***	46.50 +/- 15.66 ***	57.17 +/- 20.47 ***
PC	1.18 +/- 0.03	86.67 +/- 14.08	108.83 +/- 20.41

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4.2.3. Foci analysis of γH2AX assay

Figure 20 shows the results of the *γH2AX assay*. The 22 kHz IF MF irradiation experiments (Figure 20/A) and 250 kHz IF MF irradiation experiments (Figure 20/B) show similarities. SH+SH vs. IF MF+SH and AR vs IR comparisons were not significant. IR conditions were significantly different from SH+SH.

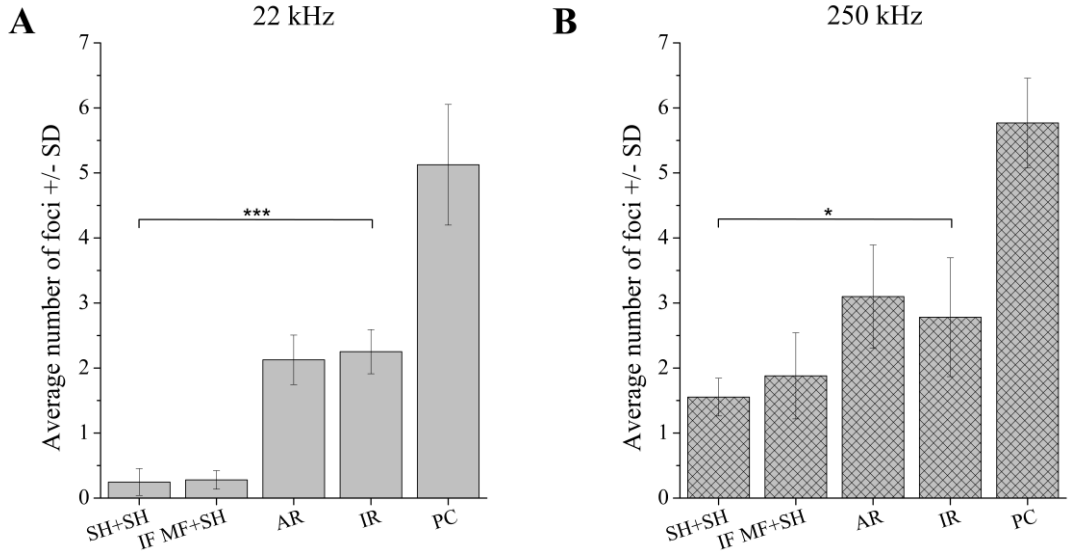

Figure 20. The average number of foci per 22 kHz **(A)** and 250 kHz **(B)** IF MF exposed cells (± SD). Data represent three independent experimental replicates at each experimental condition. SH+SH: sham and sham exposure, IF MF+SH: IF MF irradiation and sham exposure, AR: IF MF irradiation and 2.5 Gy of ionising radiation, IR: 2.5 Gy of ionising radiation, PC: 4 Gy of ionising radiation. ***: $p < 0.001$. Figure adapted from the author's original publication (97).

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5. DISCUSSION

The modern human exploits the entire non-ionising spectrum. The use of the lower range of EMF has already become a part of our everyday life during the last two decades, although modern development has required the creation of newer technologies which tend to operate at higher frequencies. Telecommunications are more efficient using frequency bands higher than GSM 900 MHz (e.g., 5G technology). Nevertheless, the way how the human body is affected by environmental EMF is still to be discovered.

Fortunately, in the last few years, several studies have been published discussing the effects of RF exposure on humans from different perspectives. Many different combinations of research endpoints have been used to study. Starting with the subject of the study (e.g. human-, animal- or *in vitro* studies), continuing with the frequency (from 10 MHz – 300 GHz), the modulation (CW, GSM, UMTS, LTE, Wi-Fi) and the duration (from a few minutes to several weeks) and finally the amount of radiation absorbed in the body (up to 10 W/kg). The wide range of exposure conditions makes it difficult to draw general conclusions (104). RF radiation has been studied extensively for its potential to cause biological effects; its impact remains a subject of debate due to controversial findings in different studies. For example, Regalbuto *et al.* (105) performed a comparative study on different modulations of 2.45 GHz RF EMF. They investigated both continuous and pulsed signals on human fibroblasts at SAR of 0.7 W/kg. They found no induction on biological effects at cellular and molecular levels.

We aimed to perform studies that mimic real-life exposure situations. In the first part of the thesis, we examined the RF radiation in two aspects. In the first, the *Additive Effect protocol* imitates the “worst case” situation from the RF exposure perspective, as after a low dose (2 SED) of UV exposure, the 3D skin tissues were exposed to a high dose of RF, in a switched on/off irradiation manner. However, the irradiation of 4 W/kg SAR is higher than the currently valid safety limits (2 W/kg for local exposure), the switching on and off exposure imitates a real-life situation, since we are not constantly on the phone or surfing on the internet. The second aspect was carried out by the *Adaptive Response protocol*; the tissues received a high dose (4 SED) of UV radiation after a continuous RF irradiation with an SAR value of 1.5 W/kg – it is below the recommended limit. This protocol investigates the well-known ‘*adaptive response’* phenomenon.

On the contrary with the findings of Kim *et al.* (43) – in which the concentration of MMP-1 was increased due to 4 W/kg 1760 MHz exposure –, in our experiments, the MMP-1 concentration increased neither after the exposure to UMTS (1950 MHz), nor to Wi-Fi (2422 MHz) radiation. However, we found that 1950 MHz UMTS irradiation reduced the UV-induced MMP-1 concentration. This finding is similar to the results indicated by Jin *et al.* (46), where human keratinocyte cell line (HaCaT) was exposed to a high dose (8 W/kg) of 1.7 GHz RF-EMF. In this paper, although the thermal effect was ruled out by controlling the temperature, they found that 1.7 GHz radiation might have a protective effect against ionising radiation-induced DNA DSB damage. Although the MMP-1 concentration decreased due to UMTS exposure in our experiment, we could not detect this phenomenon due to Wi-Fi irradiation in the same protocol (*Additive Effect protocol*). The difference between the two results is unclear, perhaps it can be explained by the different modulation of the RF exposure.

Nearly all artificially generated RF radiation involves some form of modulation. This means that the amplitude or frequency of the carrier wave is modulated by a signal of lower frequency than the carrier itself. From the perspective of biological mechanisms of action, amplitude modulation appears to be of particular importance. For example, in the case of 2G GSM mobile phones, the emitted RF power is not continuous but generates pulse modulation with a repetition frequency of 217 Hz (a 0.577 ms pulse width within a 4.615 ms time slot). Similarly, the Wi-Fi signal also represents a pulse modulated RF radiation with rapid change of RF power level. In the case of 3G phones, the modulation scheme significantly differs from that of GSM (2G) and Wi-Fi technology. In 3G systems, the RF radiation is not characterised by a regularly repeating pulsed modulation, but rather by a stochastically varying amplitude modulation with a fluctuation rate of approximately 1500 times per second. This variability is due to the fact that the transmitted power depends on the nature and volume of the transmitted information (106). Moreover, mobile phones are equipped with automatic power control (APC) technology, which can rapidly vary the emitted power in the time domain.

It is presumed that the intensity and the modulation characteristics together may determine the possibility of potential harmful effects. In the case of modulated radiation, the type and depth of modulation become critical factors. A major question arises as to whether biological effects might occur at non-thermal levels due to modulation. Another

issue is how sub-thermal exposures correlate with radiation intensity and modulation. For instance, should a short-term temperature increase caused by pulsed exposure be considered a thermal effect? The modulation types that may cause a potential biological risk include very low-frequency modulation or extremely short pulse modulation (pulses shorter than a few nanoseconds). Low-frequency modulation may have biological relevance due to its overlap with physiological time scales, whereas nanosecond-range pulses involve wide frequency spectra and deeper penetration capabilities. Of course, the intensity of the RF field is also an important determining factor (107).

Although, our finding for the beneficial effect of RF in this aspect (when tissues first received UV, then RF irradiation; in *Additive Effect protocol*) does not support our hypothesis − namely that UV plus RF exposure cause an additive effect − a slight (non-significant) tendency for adverse effect was measured in IL-1α concentrations in both UMTS and Wi-Fi RF-exposures.

We could detect the protective effects of RF − namely the *adaptive response* phenomenon − only in 1950 MHz UMTS exposed IL-1α and IL-8 concentration, but a slight (non-significant) tendency was observed in IL-6 and MMP-1 concentration as well. These results demonstrate that 3G UMTS exposure might have a protective effect when RF exposure precedes the UV exposure. The *adaptive response* was not observed by 2422 MHz Wi-Fi irradiation. Our finding agrees with Sannino *et al.* (77), who demonstrated that 1950 MHz, UMTS pre-exposure could reduce the damage induced by Mitomycin-C. Further investigations also indicated that RF exposure on different frequencies could induce adaptive response (108–111).

Our study is a pioneer in EMF research, as we were the first to publish a combined effect of UV and RF performed on 3D skin tissue culture. Later, Patrignoni *et al.* (112) investigated on ROS production in a combined exposure study. They investigated how the 5G-modulated 3.5 GHz RF radiation impacts human fibroblast and keratinocyte cells. They exposed the cells for 24 hours to 0.25, 1 and 4 W/kg of RF. Furthermore, they combined the exposure with UV-B radiation. They found that 1 W/kg 5G signal reduced the mitochondrial ROS concentration in fibroblasts, on the contrary, 0.25 and 1 W/kg 5G signals enhanced the UV-induced ROS production in keratinocytes.

Several studies suggest that RF at higher frequencies may generate oxidative stress. The study of Cermak *et al.* (113) focused on the oxidative stress evaluation induced by the non-thermal effect of 1800 MHz RF radiation. Their results suggest that 1.6 W/kg RF exposure indicates a transient oxidation-reduction imbalance and ROS activation in fibroblast cells. Choi *et al.* (45) found that a relatively long, 72 hours of LTE exposure with 1 and 2 W/kg SAR values − near the exposure limit − decreased cell proliferation in all cells. Furthermore, intracellular ROS was increased in those cells (adipose tissue-derived stem cells and Huh7 cell line), where cell senescence was observed. However, 1800 MHz RF EMF did not induce ROS in fibroblast cells according to Xu *et al.* (114) and a combined exposure to 837 MHz CDMA and 1950 MHz WCDMA RF radiation did not constantly alter ROS production in different cell types according to Kang *et al.* (115).

In the second part of this thesis, we addressed the effects of 22 kHz and 250 kHz IF MF on human fibroblast cells; among other investigations, we examined whether IF MF influences the oxidative stress. Our results show that 22 kHz IF MF did not, but on the contrary, 250 kHz IF MF induced significant 8-oxoguanine level in human fibroblast cells, which is a common DNA damage via ROS. Therefore, we rightly assume that 250 kHz IF MF generates ROS in fibroblast, however, further studies are recommended to ensure this. As mentioned above, it is proven that RF EMF could induce oxidative stress, but IF MF probably does not have enough energy to cause major changes other than the heating effect. Sundram *et al.* (116) detected a decreased sperm motility of male rats induced by 150 kHz IF MF. They assumed that this could be due to oxidative stress via thermal effects. In our study, the thermal effect was clearly excluded by *in vitro* temperature controlling, hence our results were definitely not caused by thermal effects. Due to the lack of studies that investigate exactly how IF MF effects on ROS production, it is difficult to compare our results with other papers. However, we assumed that the possible ROS induced by 250 kHz IF MF might regenerate fast, since we could not detect increased 8-oxoguanine level 4 hours after the exposure. Furthermore, the question arises why 22 kHz IF MF did not cause increased 8-oxoguanine levels, while 250 kHz did. We assume that the difference lies on the characteristics of the wavelength. The energy of the radiation increases with the frequency. Although there is consistent evidence in experimental studies for EMF-induced ROS formation, a complete picture and scientific

consensus regarding the epidemiological relationship and potential negative and long-term consequences on health have not yet emerged (34).

Aberrant MMP-1 expression is commonly linked to many senescence-associated diseases, such as photo-induced skin wrinkling, premature ageing syndrome or tumour progression. Many of the signals propagated by MMP-inducing stimuli are associated with the production of ROS, which also contributes to the ageing process (117). In the future, we would like to carry out some further experiments to investigate how the relation between MMP-1 enzyme and ROS changes under the influence of EMF exposure.

Furhermore, it is scientifically proven that there are certain substances that can have a protective role agains ionizing- and UV radiation. The treatment of vitamin C may induce a protection agains UV radiation and improve the ROS imbalance (118). Another protective material agains radiation is the melatonin. Melatonin hormone from pinal glans plays an important role in metabolism regulation (e.g., inflammation and apoptosis) and it is also a potent antioxidant, acts like a free radical scavenger. It couls alter on oxidative stress level by reducing ROS (119), and could modulate the indirect harmful effects of radiation by reducing DNA damages (120). Melatonin successfully counteracts DNA damages induced by UV radiation (121).

Due to the contradictrory results of recent scientific publication – whether EMF induce or reduce oxidative stress – I believe that it will be worthwhile to pay scientific attention to certain substances that reduce oxidative stress levels. To examine how these ROS-protective substances (e.g., vitamin C and maletonin) interact with EMF. More scientific research should be conducted, such as Wang *et al.* (122). This study showed that melatonin could potetntially improve the 2.45 GHz RF-induced reproductive damage in male mice.

According to micronucleus formations, we found that the cell division (NDI) was significantly decreased, the number of binucleated cells with micronuclei (BN with MNi) and the number of micronuclei in binucleated cells (MNi in BN) were significantly increased as a result of ionising radiation. These results concur with the study of Litvinchuk *et al.* (123), which reports a linear increase in binuclear cells containing micronuclei for absorbed doses from 1 to 5 Gy. Our findings regarding to sham exposure were similar to Elbakrawy *et al.*(124) and Yoshioka *et al.* (125). However, our results

also showed that 22 kHz IF MF exposure slightly decreased the number of binucleated cells with micronuclei (BN with MNi) and marginally decreased the number of micronuclei in binucleated cells (MNi in BN). 250 kHz IF MF exposure caused no changes in micronucleus formations, which is comparable to the studies of Miyakoshi *et al.* (49) and Sakurai *et al.* (15) at 23 kHz IF MF. Based on our controversial results – namely, the oxidative stress increased due to 250 kHz IF MF while micronucleus formation decreased due to 22 kHz IF MF –, these findings should be interpreted with caution and further studies are needed.

Furthermore, to investigate the phenomenon of adaptive response, we examined whether IF MF exposure has any effect when combined with ionising radiation. Based on other studies, which detected the adaptive response after exposure to ionising or non-ionising radiation (71,126,127), we hypothesised that exposure to low level of IF MF will reduce the harmful damage of ionising radiation. Although the adaptive response is a described and published phenomenon in the field of non-ionising radiation (71,127), similar to other investigations on carcinoma cell line H295R (72), we could not observe the protective effect of IF MF exposure against ionising radiation on skin fibroblast cells. As a result, we were unable to detect the adaptive response under the exposure conditions of the present study. Our study is mainly focused on investigating the adaptive response phenomena, but it could also be relevant to investigating co-genotoxicity. In this perspective, our findings show not just the lack of adaptive response, but also the lack of co-genotoxicity induced by IF MF.

The upcoming technologies, such as 5G and 6G will use the millimetre-range of RF EMF. As frequency increases, the penetration depth into the human tissue decreases, thus the radiation in the millimetre wavelength range is absorbed only in the skin and cornea. Although there are differences between 4G LTE and 5G signals, the differences in the modulation are minimal. The transmitted radio frequency signals in both technologies appear noise-like, within a limited frequency range. However, the larger range of frequencies available in 5G justifies the need for further health research, especially in the 5G millimetre-wave band. Investigations on the possible health effects of higher frequencies will become necessary and should be observed. Hence, it is necessary to carry out comparative studies like this thesis (128).

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Not just RF, but other EMF exposures are omnipresent too in modern society. Therefore, humans are exposed from different sources in their everyday life. The investigation of EMF is a current topic of research, in my opinion, researchers should pay attention to the frequencies of future technologies (i.e. millimetre range) and frequencies with gaps in knowledge (i.e. IF MF). Many publications concentrate on the worst-case scenario, but it would be more worthwhile to put non-thermal measurements in focus that mimic an everyday life exposure. In the future, we would like to continue the study of the present PhD thesis by examining the relationship between MMP-1 enzyme and ROS in the human skin. Furthermore, RF could influence certain DNA repair pathways, as described by others, it would be important to examine the protein expression patterns due to combined exposures.

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6. CONCLUSIONS

Within this thesis, we investigated the effects of different EMF radiations on human skin, both alone and in combination with other radiations (ionising and UV radiation). This thesis mainly examined the phenomenon of the additive effect and adaptive response.

We found that RF exposure generated by 3G UMTS mobile systems at 1950 MHz under the described exposure conditions did not induce an inflammation process in the skin tissue *in vitro*. On contrary with our hypothesis, we found that 24-hour UMTS exposure significantly reduced the MMP-1 enzyme concentration of prior UV exposure. Another important finding is that UMTS exposure did not enhance other inflammation processes (IL-1α, IL-6, IL-8 concentration) induced by UV radiation. On the other hand, we could detect the adaptive (protective) response on IL-1α and IL-8 concentrations, as the pre-exposure to UMTS reduced the effect of UV radiation.

We cannot detect either the additive effect or the adaptive response due to Wi-Fi exposure. The difference between the effect of Wi-Fi and UMTS RF exposure is assumed to be due to the difference in modulation between the two wireless systems. Therefore, the investigations of the possible adverse and protective effects on the skin due to the high frequency electromagnetic fields become more important before the deployment of 5G mobile systems. With the introduction of the 5G and 6G technologies, further investigations on the skin will become fundamentally crucial.

In our laboratory environment, during a 28-hour exposure protocol, we could detect a decreased number of micronucleus formations in 22 kHz exposed fibroblast cells, although we found an increased oxidative stress upon 24 hours of 250 kHz IF MF exposure alone. Furthermore, we could not observe an IF MF-induced adaptive response or co-genotoxic effect on ionising irradiated fibroblast cells. The industrial development results that there are more and more devices using IF MF (e.g., induction cooktops, induction chargers, electric vehicles) in the human environment. Due to the lack of literature, investigations of IF MF-generated possible health effects are crucially important.

Overall, this study provides no evidence for a significant impact of EMF, particularly for non-thermal effect, however, it shows some results for adverse and

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protective effects of EMF as well. Based on our studies, we assume that EMF radiation does not cause direct DNA damage but might induce some cellular and molecular changes in human skin cells.

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7. SUMMARY

The wireless communication technology has made the life convenient and efficient for humans. Nevertheless, we are continuously exposed to EMFs, which raises questions about their possible biological and health effects. Therefore, there is a public concern regarding the potential for adverse human health effects. This thesis was aimed to investigate whether non-ionising radiation alone or in combination with other radiation can affect human skin.

The first part of the thesis aimed to investigate whether two physical agents, the RF exposure and UV radiation – both classified by the IARC – in combination have any effect on the inflammation of human skin. We found that 4 W/kg 1950 MHz UMTS exposure significantly decreased MMP-1 concentration pre-induced by 2 SED of UV radiation. A slight (insignificant) tendency for additive effect was measured in IL-1α concentration via both UMTS and Wi-Fi frequencies. Furthermore, we detected the adaptive response phenomenon, when the protective effect of 1.5 W/kg 1950 MHz UMTS exposure significantly decreased IL-1α and IL-8 concentrations followed by 4 SED of UV radiation.

The second part of the thesis aimed to investigate whether IF MF in itself could affect the DNA integrity and whether it could possibly induce a protective effect against ionising radiation by the adaptive response phenomenon. Overall, we could detect a decreased number of micronucleus formations in 22 kHz exposed fibroblast cells, although we found increased oxidative stress upon 24 hours of 250 kHz IF MF exposure alone. Furthermore, we could not observe an IF MF-induced adaptive response or co-genotoxic effect on ionising irradiated fibroblast cells. Based on our controversial result, these findings should be interpreted with caution and further studies are needed.

Overall, this thesis provides no evidence of significant impact of EMF, especially for UMTS, Wi-Fi RF or IF MF exposure in the skin. Based on our studies, we assume that EMF radiation does not cause direct DNA damage but might induce cellular and molecular changes in skin cells.

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9. BIBLIOGRAPHY OF CANDIDATE'S PUBLICATIONS

Candidate's publications related to the theme of the thesis:

Szilágyi Zs, Németh Zs, Bakos J, Necz P P, Sáfár A, Kubinyi Gy, Selmaoui B, Thuróczy Gy. Evaluation of Inflammation by Cytokine Production Following Combined Exposure to Ultraviolet and Radiofrequency Radiation of Mobile Phones on 3D Reconstructed Human Skin *In Vitro*. Int. J. Environ. Res. Public Health 2020; 17:4401. https://doi.org/10.3390/ijerph17124401.

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Candidate's other publications:

Németh Zs, Laczkovich-Szaladják E, Brech A, **Szilágyi Zs**, Kubinyi Gy, Thuróczy Gy. Intermediate frequency magnetic field at 250.8 kHz does not induce DNA damage or "Adaptive Response" *in vitro*. Genetics&Applications 2019; 3:1. https://doi.org/10.31383/ga.vol3iss1pp42-50.

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