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ABSTRACT The biological effects of millimetric waves systems, MMW (e.g. 5G communications, automotive radars, or crowd control systems, ADS) systems are still scarcely investigated. Hairless rats were exposed to 94GHz continuous wave (MMW, CW), under acute high power conditions (3sec at10kW/m^2) and under environmental/professional lower power exposure (4 hours a day, for 5 days a week, for 6 months at 10mW/cm^2). It was found that the 94 GHz acute exposure provoked the disappearance of the epidermis upper corneal layers and the increase of the inflammatory gene expression SOCS-3 after 3Hrs. Besides, neither histological nor genetic modification could be observed after chronic 94 GHz exposure and after an acute and a chronic capsaicin exposure. Conclusions :After a powerful acute exposition a thermal effect is observable, associated with a localized inflammatory response. After chronic, low power exposition, an hypoalgesia effect was found, possibly related with an increase in brain plasticity. Mechanistically, the hypothesis is based on low noise stimulation of thermal pain receptors at subthreshold level. Conversely, neither inflammation nor no direct change in gene expression of the skin cells was found.

KEYWORDS : biological effects, millimetric waves, rat.

INTRODUCTION

Since the second world war, the applications of electromagnetic waves have greatly developed, inducing an ever-increasing environmental exposure of populations. Alongside domestic applications (mainly based on energy transport, 50Hz) and industrial (for example in HF bands, welding, drying ...), the rise of wireless telecommunications, the population is also subject to radio frequencies (broadcasting frequencies mainly ranged between 100 to 800MHz) and especially mobile telephony since the end of the 20th century. Both the need for more dense and faster information transmission, and the generalization of the use of cell phones quickly threatened to saturate the networks, this leading to increase the frequency domain towards higher frequencies (900MHz, 1.8, 2.45, 5.8 GHz). In order to offer higher communication speeds, the manufacturers have upshifted the frequencies in the millimetric waves (MMW). Fifth generation technologies (5G) already implemented, use frequency bandpass up to around 26GHz, and the manufacturers prospects are to offer systems operating at 60GHz. Furthermore, while 5G telephony is only being implemented in Europe and the USA, china is nowadays producing and testing 6G technologies now, which frequency ranges are even higher (up to THz) in order to " improve the speed and power of communications[1]

In the field of automotive radars, other industrial applications of MMWs were started in the 1970s, currently leading to extensive developments of advanced driver assistance systems to detect objects that are difficult to optically observe due to distance or environmental factors. For instance such radars, mainly operating at 24, 77 and 79 GHz can play a crucial role in helping drivers maintaining safe distances[2]. All the systems described here before involve low level power emitting conditions, and only low level continuous human exposure. Besides, higher powered MMW systems devices have been designed as less lethal systems destinated to crowd control (especially in USA, known as ADS, anti-denial systems, non-lethal weapons[3]) in military/police applications. These systems operate by emitting electromagnetic radiation in millimeter band at 94 GHz. A this frequency; the depth penetration of millimeter remain superficial (80% of the energy is absorbed in the range pf 0 to 0.5 mm), leading to a specific stimulation of the free nerve).

As the energy involved is too low to be deleterious, MMW exposure results in an intense burn sensation without tissue damage itself inducing in the escape of the exposed person. Finally, behind the notion of major technical progress the crucial question of MMW biological effects-deleterious or not- and human safety arise. The aim of this study was to investigate possible the biological risks associated with acute high power (accidental or deliberate use) or chronic low power exposures to MMW, using hairless rats as model and 94GHz continuous wave transmitter as MMW, due to its low atmospheric attenuation. A preliminary step was to set and characterize the emitting system in order to obtain both a good dosimetry and homogenous field conditions.

MATERIAL AND METHODS

Rats : Hairless male rats, 16 weeks old (250g), were purchased

by Charles River labs, RN 13, Route de Pacy, 27930 Miserey, France. All experiments were submitted to animal ethics committee (number2019/13.0). During the exposure, artificial light was lit in the chamber in order to preserve the nychthemeral cycle of rodents. The animals were placed either alone anesthetized in a plexiglass cage (electromagnetically neutral) for acute exposure, or by groups of 5 in a large-volume cage for chronic exposure (see figure 1A).

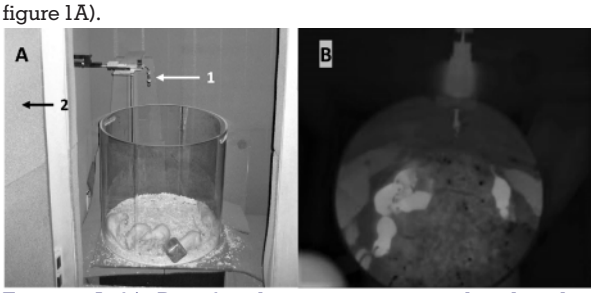


Figure 1:*Left*/ Rats for chronic exposure in the plexiglas cage inside the anechoic chamber ; arrow shows : 1/the end of the wave guide and horn antenna; 2/The MMW absorbent foams covering the door and walls of the exposure chamber. *Right*/ Infrared temperature image from FLIR camera of the same system

Exposure systems and dosimetry. The 94 GHz transmission VZB-2788A1 94 GHz EIK Subsystem consisted of an EIK model number VKB2463T1, 80 watt CW, 94 GHz air cooled EIK, integrated power supply RF source, RF input and output components, and integral cooling and protective device system was from Communications & Power Industries Europe Ltd, Walton Lodge, Bridge Street, Walton-on-Thames, Surrey KT12 1BT, United Kingdom. It was connected to a waveguide bearing a horn antenna placed in the anechoic chamber. Horn was different following a narrow exposure of a localized part of an anima (32 dBi gain focused horn), or a large aperture cone, allowing low power exposure of several animals (Wide aperture scalar horn antenna with a gain of 19 dB). This set up allowed simultaneous whole body exposure of a group of up to 8 animals.

The temperature of the chamber was kept constant (20 ± 1 °C) via air conditioning system and a ventilator. In the millimetric band (frequency above 30GHz) the power density (**S,** in W/m^2) is the main dosimetry criterion[4]. Two exposure conditions were used *i)* an acute high power exposure during 3s at **S**=10kW/m^2 resulting an superficial increase in skin temperature to 53 ° C. Each animal was exposed either to MMW on a side or to capsaicin (positive control, 2 mg/cm^2, 30 minutes occlusive patch), activator of thermal Vaniloid pain receptors. Skin biopsies were taken after 3, 6 and 24h on anesthetized (n=10) rats for each biopsy collection time).

ii) Chronic experiments, using exposure of 4 hours a day, 5 days a week, for 6 months, at a power density **S**=10mW/cm^2 (twice the ICNIRP professional permissible exposure level [3,5]**). S** was then assessed using classical calculation methods and confirmed by the surface thermal rise measurements. The estimation of E-field levels was performed by numerical resolution of Maxwell's equations, i.e. the finite difference method in the time domain (FDTD method[6]) which is chosen because it is a powerful tool allowing the digitization and resolution of spatio-temporal differential equations These simulations were validated by temperature measurements (FLIR camera, infrared thermography, see figure 1B). At this frequency, far field conditions were fulfilled for a 1m distance between the horn antenna and the exposure plane.

Methods: ***Acute exposure*** **:** 3, 6, or 24 hours after the end of exposure the animals were euthanized by decapitation. Eight skin biopsies with a diameter of 5mm were taken from the exposed 94 GHz or capsaicin side. Eight control biopsies were performed on each animal on the contralateral side at an identical location. For each animal, the resulting pairs (exposed side" and "contralateral side" biopsies) were dispatched as follows : 2 "kept in a formalin bath for histological study, 2 kept in a formalin bath for histological study, 2 frozen in liquid nitrogen for immunohistochemical labelling, 2 frozen in liquid nitrogen for study of the genome by Micro-array technique, and 2 stored in a "RNA later" type preservation solution for study by RT-qPCR of the transcriptome. Statistical analyses used non parametric Mann-Whitney (MW) or Kushal-Wallis (KW) tests. The data are compared to a positive control, known to be an identical pattern of reaction (intense burning sensation, without injury), capsaicin. Capsaicin is the active ingredient in chili peppers, its action appears specific to thermal pain receptors Transient Receptor Potential Vanilloid (TRPV)[7].

Chronical exposures **:** The experiments were carried out in two separate identical campaigns. For each campaign, the animals were divided into 2 groups of 16 animals(Exposed /sham). The power density of 10mW/cm^2 required were obtained using a large aperture scalar horn antenna, with a gain of 19 dB making it possible to obtain a homogeneous exposure spot in far fields at a distance of 1 m from the source. These conditions allow simultaneous whole body exposure of a group of up to 8 animals. Due low power -painless- exposure conditions the rats were left free to move around in the plexiglass enclosure. During the experiment (exposed or sham-exposed 4 hours a day, 5 days a week over 6 months) , the animals underwent the behavioral "plantar test[8]" at 2, 4, 6 months after the initiation of the first exposure. The animals were euthanized by decapitation 24 hours after their last exposure. For each animal blood collections were done : 1 ml for corticosterone dosage and 1 ml for circulating endorphins assay (sample taken before euthanasia, 20 minutes after the last exposure). Skin biopsies were also performed : 2 stored in a formalin bath for histological study, 2 frozen in liquid nitrogen for study of the genome by micro-array technique, 2 stored in an "RNA later" type preservation solution for PCR study of the transcriptome. Besides, Cortex Somatosensorial, Thalamus, Hypothalamus, Hippocampus were collected in a preservation solution type "RNA later" for study by PCR of the transcriptome.

RESULTS

Acute high level exposures.

Histology

As at 94 GHz, all the radiation energy is absorbed in the epidermis and dermis, the histological skin study looked for potential tissue damages. Each formalin biopsy was included in paraffin, then blocks were cut with a microtome in serial section of 15 µm. The biopsies were oriented so as to have on the same section, the epidermis, the dermis and the hypodermis. The sections were then stained by classical staining of the saffron hematoxylin phloxin type[9].

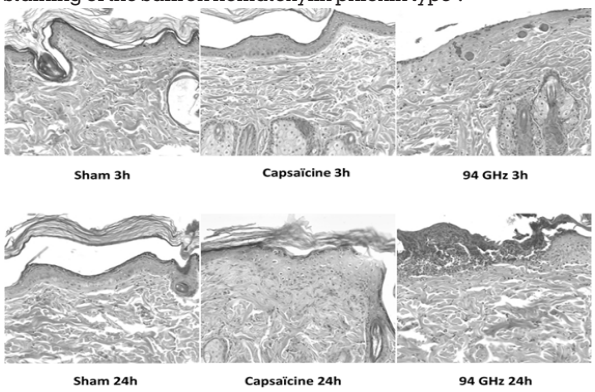


Figure 2: Histological section of the skin 3 hours and 24

hours after capsaicin exposure or 94 GHz exposure. Hematoxylin phloxin saffron staining. Magnification x 20

The results (see figure 2) obtained by classical histology made it possible to demonstrate the absence of effect during capsaicin exposure, both for an early (3 hours) or more delayed (24 hours) period. All the structures were well preserved, no cellular modification was observed.

For 94 GHz exposures, a marked modification of the epidermis was observed, with a disappearance of the upper horny layers of the epidermis after 3 hours, and their reorganization at 24 hours resulting in crust formation. No other changes was observed either in the deeper layers structures or in cells morphology. Such exclusive lesions of the epidermis, could be related with scratching lesions (this behaviour was observed in exposed animals) or with blister formation.

Immunohistochemistry

A specific immunohistochemical labelling of langerine was performed on frozen biopsies in order to identify Langerhans cells of the epidermis as described in the literature[10]. Counting the number of cells makes it possible to estimate the prevalence of the number of these cells present and can be an indicator of a local inflammatory reaction[11] (figure 3).

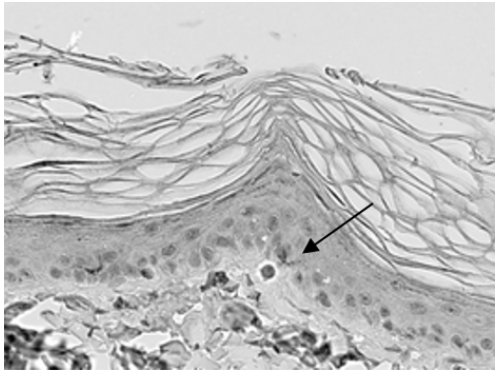
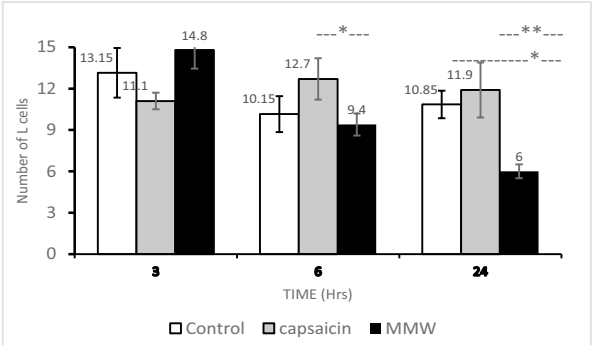


Figure 3. Immunohistochemical labelling of Langerine. A Langerhans cell (dark cells) can be seen in the middle of the image. Magnification X 40.

A showed on the figure 4, in the control and capsaicin groups, the number of Langerhans cells present in the epithelium remained constant within the whole observation time (mean values around 12/slide with approximate SEM ranged between 0.5 and 2. A similar cell density was found in the samples subjected to capsaicin and the small fluctuations did not reach significance. Such was not the case for MMW exposed samples. After initial 3Hrs variations, significant decrease in the Langherans cell densities were measured with control samples after 6Hrs evolution ($p<0.05$) and especially after 24Hrs ($p<0.01$).



□Control; ■capsaicin; ■□; MW ; *: p < 0.05; **p < 0.01□
Figure 4. Time evolution of Iangherans cell count/ slide. (average and SEM) after 3,6,24Hrs

This observation strongly suggests the recruitment of these cells, which leave the epidermis to go to the lymph nodes [10]. and could be related to the development of a localized inflammatory reaction. Morreover such type of response is commonly present after "sunburn" type lesions [11, 12].

Study of gene expression at the skin level by Micro-Array technics.

The study of gene expression by microarray (Micro-Array) is a method of high throughput screening of the transcriptome, allowing simultaneous visualization of 40,000 genes expression. This first approximation approach, was in the present case seeking for the coarse disturbances induced by MMW (i.e. metabolic modification, inflammatory reaction, DNA repair processes or transduction pathways)[12]. As no definitive results -only tendances- could be extracted from these experiments, we decided to observe gene expression by RT-qPCR method. The first step was to identify the genes of interest to be studied. Hence, in the absence of being able to rely on the Micro-Array results, it was decided to rely on the histological observations highlighting the likely development of a localized inflammatory reaction. In addition to the operating principle of the device, other genes of interest have been identified. The genes selected were:

- HSP 70: Gene encoding the heat shock chaperone protein HSP 70, indicating an increase in temperature.
- TRPV-2: Gene encoding the thermal pain receptor.
- ICAM-1: Gene encoding a continuous intercellular adhesion protein present in low concentration in leukocyte membranes and endothelial cells : it marks the action of pro-inflammatory cytokines.
- IL1-β: Gene encoding Interleukin 1 beta, an early pro-inflammatory cytokine.
- SOCS-3: Gene encoding the suppressor of cytokine signaling 3 protein. Used to sign that an inflammatory reaction has taken place.

The results are presented on the figure 5 : The variations in gene expression induced by MMW exposure were considered as significant when exceeding a factor of 2 (Wilcoxon tests did not provided more result) . These results showed that the overall effects have to be related with a thermal effect as revealed by HSP70 expression variations [13].

This early thermal effect (ratio of 11.6 at 3Hrs) did not resulted to a longer term (6 or 24Hrs) change in the expression of HSP 70, signaling a return to a normal situation. However the development of an inflammatory reaction was probably present as suggested by the limited increase in ICAM-1 and IL1-β expressions after 94 GHz exposures. Note that whereas at the observation time selected only small variations were noted, a peak in secretion of these factors would happened earlier. However, the SOCS-3 ratio of 7 found at 3Hrs confirmed that an inflammatory phenomenon had occurred [14].

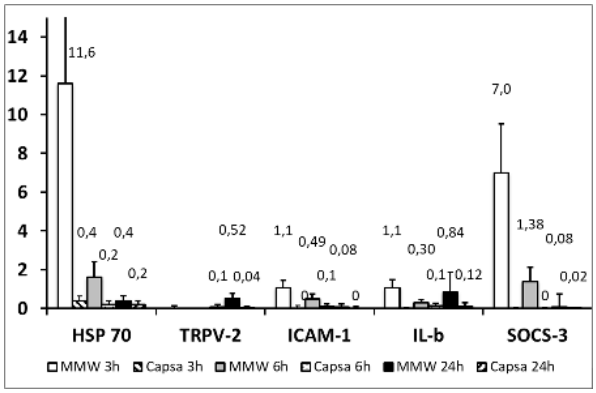


Figure 5 : Relative gene expression compared to the contralateral control side (ratios exposed to MMW or

capsaicin versus contralateral control side ± SEM). Numerical values are presented beside histograms.

Besides, the slight increase in the expression of the TRPV-2 gene 24 h after exposure to MMW would also be in agreement with the mediation of the effect of radiation through this receptor [15, 16] . By the way of contrast, similar experiments performed using capsaicin exposure did not resulted in any modification of gene expression.

Study of gene expression in the brain.

This part of the study was performed by RT-qPCR. The objective of this action was to determine whether the systemic effect observed in animals resulted in changes in the gene expression of genes involved in the development of an antidepressant or analgesic-type response. The measurements were performed on gathered samples from thalamus and hypothalamus.

This led to selected the following genes of interest:

- **ARC**: gene encoding the activity-regulated cytoskeleton-associated protein. It is a marker of neuronal plasticity, which has a key role in antidepressant effects or the onset of chronic pain
- **cFOS**: gene encoding a protein corresponding to a nuclear transcription factor, the main function of which is the induction of transcription of a gene. It is a marker of neuronal activity
- **Keap 1**: gene encoding an antioxidant regulatory protein
- **Nurr 1**: gene involved as a protector of neuroinflammation and protector of dopaminergic activity

Direct observations are presented on figure 6A/. Whereas several differences in gene expression were observed between control and exposed groups (increase in cFOS, Nurr 1, ARC) could be observed, none of them were statistically significant due to intragroup dispersion. This led to perform an individual observation within the different groups, this resulting in the separation of 2 subgroups within the MMW exposed group, labeled here as responder groups (MMW-R, 70% of the exposed population) and non responder group (MMW-NR, 30%) . This distribution was at the origin of self averaging effect, itself precluding the detection of any significant result.

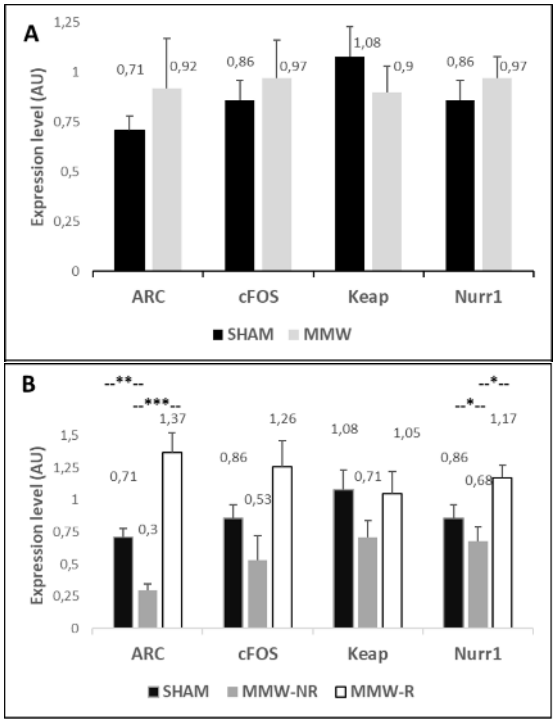


Figure 6 : A/ Global level of gene expression by group according to the gene considered + SEM : SHAM versus MMW-exposed populations ; B/ Same as in A/ after separation of the responder (MMW-R) and non-responder (MMW-NR) subpopulations; (KW *: p<0.05; **p<0.01; ***p<0.005.

After this redistribution, a very significant difference in the expression of the ARC, cFOS and Nurr1 genes in responder animals exposed to 94 GHz was observed. The cFOS gene confirms the onset of a brain response induced by exposure. This suggests an increase in brain plasticity, evidenced by the increased expression of ARC. This increase in neuronal plasticity is close to the response obtained with the use of antidepressants. The exposure conditions used in this section corresponded to accidental overexposure or deliberate aggressive use. The following part is representative of environmental circumstances that may be encountered in the context of communications or 5G mobile telephony (or future predictable 6G). The small increase in Nurr1 also indicates an action on the dopaminergic pathways. Like the effect on corticosterone level, these would suggest an increased secretion of endorphins.

Chronical low-level exposures

Behavior, stress and endorphins

Plantar test : The "plantar test" behaviour tests were carried out at 2, 4 and 6 months after the beginning of MMW exposure period. Based on the provocation of an unpleasant sensation, this test is a benchmark test in assessing pain perception and potential analgesic effect. Briefly, the animals were placed individually in a plexiglass cage.

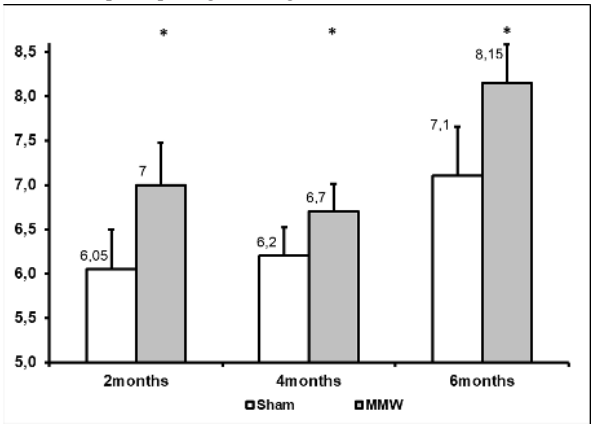


Figure 7: Mean response time (sec) to the plantar test ± SEM after 2 months, 4 months and 6 months MMW exposure (KW : * : p<0.05)

After a period of habituation, an infrared pod was positioned under a hind leg. The animal removed its paw when the sensation became unpleasant for it. The time between the onset of infrared exposure and the time of removal was measured. This delay is usually considered as correlated with the threshold of pain perception. As shown on the figure 7, MMW exposure resulted in a significant increase the withdrawal time after 2 months MMW exposure consistent with an overall hypoalgesia effect. Moreover the similar differences observed after longer experimental time i.e. after 4 and 6 months suggested that no attenuation or habituation occurred with time.

Determination of circulating endorphins and plasma corticosterone level.

Endorphins are endogenous opioids involved in pain control [27]. This family of neurotransmitters is naturally produced by the body, including during exertion [28] or after injury [29]. The analgesic action of these molecules is particularly important. They bind to the same receptor as morphine and have an action estimated to be between 20 and 30 times greater than it [30]. Plasma endorphins were assayed by ELISA technique, 20 minutes after the last exposure as described in the

"methods" section .

As shown on the figure 8A, the animals exposed to MMW exhibited a significant increase in the concentration of circulating endorphins (37.1 +/-11 versus 77.2 +/-20pg/mL.

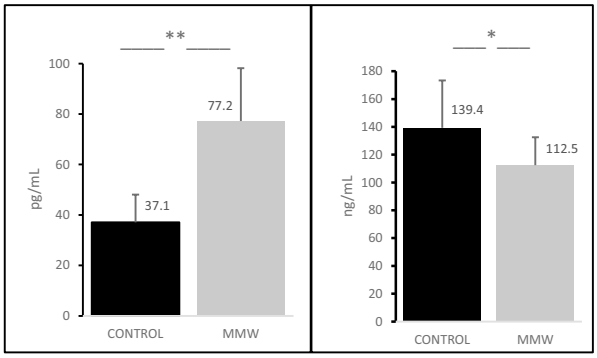


Figure 8 A/ Mean plasma endorphin ± SEM concertation B/ Mean plasma corticosterone concentration ± SEM ; Mann Wittney tests : (KW * : p<0.05 ; ** : p<0.01).

This feature strongly supports that decrease in pain perception observed by plantar test could be related to this increase in circulating endorphin.

Plasma corticosterone dosage : Plasmatic corticosterone level is a classic marker of stress in animals [31, 32]. This assay was performed by radioimmunoassay using Coat-a-Count kits(Coat-A-Count Cortisol_PITKCO-9, Siemens, Meditechno : www.meditechno.pt). As shown on the figure 8B, the exposed animals showed a lower plasma corticosterone concentration than the control animals, possibly indicating a lower state of stress in these animals. This observation is agreement with the previous findings and might be related with an increased secretion of endorphins.

gene expression in the skin cells of hairless rats in vivo. As these results have been published elsewhere in collaboration with our group (Habauzit et Al., 2020)[13]., they are only summarized here below. Briefly, the modifications to the whole gene expression profile were analyzed with a gene expression microarray on RNA extracted from skin explants (see methods) Expression of Heat Shock Protein (HSP, small, 40,70 and 90 families), 18 genes involved in skin inflammation, wound repair and tissue remodeling were investigated. This work revealed that, without modification of the animal's temperature, long-term chronic 94 GHz-MMW exposure did not significantly modify the gene expression of the skin on either the young or adult rats.

DISCUSSION

Our work focused on MMW exposures, acute high power and chronic low power exposure. Between the two situations, the stakes and the targets to be observed differ greatly: indeed, the power levels cover 3 power decades (from 10mW / cm^2 to 100W / cm^2, i.e. intermediate power levels corresponding to the authorized exposure levels defined by the various international bodies[3-5] (IEEE, ICNIRP), i.e. much higher than public environmental levels (μW / cm^2), up to powers corresponding to high power weapon systems[14].

For acute exposure, except professional accident (contact with 5-6G sources weather radars transmitters), the levels that we have chosen constituted an extreme, except to induce deliberately burns (it would need about 100W/cm^2); they are similar as the levels used as defined by the American doctrine for use of such non-lethal weapons[15,16,17,18]that is to say a "brief reversible and exclusively superficial effect.

The results of the experiments carried out here in vivo under high intensity acute exposure showed that the observed biological effect were not limited to the sole sensation of heat. Indeed, unlike the positive control, capsaicin, which specifically activates thermal pain receptors, high power exposures at 94 GHz caused the development of a localized inflammatory reaction, as shown from the recruitment of cells responsible for skin immunity and through the activation of cytokines, messengers of inflammation.

In terms of chronic or repeated exposure, several studies report biological effects, not only deleterious, but therapeutic, (used in former Soviet Union) ; these powers are of the order, with the exception of 10-20mW/cm^2. Power densities used here were twice the levels of the ICNIRP standards (i.e. 10mW/cm^2), to avoid a thermal effect and be consistent with potentially encountered levels (5-6G). "Public" exposure did not fall within this framework because of the powers involved (several tenths of μW) and are unlikely to produce any general effect, except if considering specific signalling properties, or indeed effects linked to coding/pulse modes, or to multi-frequency exposures (5 -6G) used in communications, or even in association with other physical or chemical exogenous agents.

Chronical low level MMW exposures (twice those recommended by ICNIRP standards, i.e. 10mW/cm^2, in the absence of temperature modification) showed no deleterious effect on skin *in vivo* in terms of gene expression. As mentioned by Habauzit et Al[19]., « *Without modification of the animal's temperature, long-term chronic 94 GHz-MMW exposure did not significantly modify the gene expression of the skin on either the young or adult rats*". As these works focused on skin, the first target for MMW, if one considers the small depth penetration of MMW at this frequency (0.4mm at 94GHz), direct biological effects resulting from all body exposure were very unlikely. However, several results mentioned general responses after superficial exposure [20,21,22], that would by the way of nervous receptors stimulation and/or via general mediator secretion[23,24]. Our results obtained from plantar tests and from corticosterone /endorphin measurements clearly support this hypothesis. This is in agreement with past publications[25,26,27] and animal experimentations[28,29,30,31]: hence, stimulation of endorphin secretion is almost the best hypothesis to explain hypoalgesia effect and stress diminution[32], as classically used in former Eastern-Bloc countries in the late 20th century. Finally, high frequency technologies miniaturization would allow easy-to-use devices that could be used on an outpatient basis, in order to relieve various painful pathologies[33], as especially described in the Pakhomov 's review[34]

CONCLUSIONS

High intensity whole body MMW exposure in rats may induce thermal effects -burns-, and inflammatory responses, directly following the power density used, e.g. close to sunburns. By the way of contrast, low level chronical (repeated) exposures result in hypoalgesia properties, that would result from endorphin release after peripherical nervous receptors stimulation.

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