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Clinical and Experimental *In vivo* EPR For the Detection of Free Radicals in Dermatology

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Abstract

Human skin is constantly directly exposed to the air, solar radiation, environmental pollutants, or other mechanical and chemical insults, which are capable of inducing the generation of free radicals as well as Reactive Oxygen Species (ROS) of our own metabolism. Extrinsic skin damage develops due to several factors: ionizing radiation, severe physical and psychological stress, alcohol intake, poor nutrition, overeating, environmental pollution, and exposure to UV Radiation (UVR). It is estimated that among all these environmental factors, UVR contributes up to 80%. UV-induced generation of ROS in the skin develops oxidative stress, when their formation exceeds the antioxidant defence ability of the target cell. The primary mechanism by which UVR initiates molecular responses in human skin is via photochemical generation of ROS mainly formation of superoxide anion ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$), and singlet oxygen ($^1O_2$). The detection of free radicals is performed by nitroxides with SURFER.

The only protection of our skin is in its endogenous protection (melanin and enzymatic antioxidants) and antioxidants we consume from the food (vitamin A, C, E, etc.). The most important strategy to reduce the risk of sun UVR damage is to avoid the sun exposure and the use of sunscreens. The next step is the use of exogenous antioxidants orally or by topical application and interventions in preventing oxidative stress and in enhanced DNA repair.

Introduction

The skin is an ideal target organ for EPR for several reasons. It is thin and detection of nitroxides applied topically does not re- quire large penetration depth of microwaves so one can use the S-band (2.2-3.0 GHz for *in vivo* or even X-band for *in vitro* specimens, which results in an improved sensitivity. Imaging does not require full 2D or 3D imaging; once nitroxides are applied topically a simples pectral - spatial 1 Dimaging with one gradient orthogonal to the skin surface is sufficient to obtain distribution of nitroxides and the redox status in the different skin layers.

The surface loop coils are sufficient, i.e. the whole objects need not to be within the resonator, which allows the EPRI of objects of any size, including humans. This is an important feature in cell opens the possibilities of application of EPR in human (clinical) studies of not only Pharmaco-kinetics of nitroxides, but also of mechanism of action of certain drugs, skin melanoma, oximetry [1-3]. A nice illustration of pharmacokinetics of nitroxides in skin as studied by 1 D spectral-spatial EPR is given by Fuchs et al. [4] studied diffusion of several nitroxides in to skin biopsies of hairless mice. By studying the spatially resolved diffusion of maleimide - PROXYL nitroxide into different skin layers (surface, epidermis, dermis), which chemically reacts with thiol groups, they effectively determined the spatial distribution of thiol groups as well. The analysis of spectral features showed spatially resolved partitioning of the nitroxide Di-Tert-Butylnitroxide(DTBN) between polar and a polar region. They also found that penetration of lipophilic nitroxides is much faster than penetration of more hydrophilic nitroxides. They also studied percutaneous absorption and distribution of anitroxide- labeled drug (estradiol) and a local anesthetic (procaine) finding that the former readily penetrates in to deep skin layers while the latter does not, even in the presence

of a penetration enhancer, such as dimethyl sulfoxide. This paper clearly demonstrated that both the rate and spatial distribution of the physiological processes involved in the metabolism of nitroxides or nitroxide labeled compounds can be studied. Although this research has been performed using X-band EPR and skin biopsies, it set the stage for further extension for *in vivo* applications, which always raises the question of toxicity. Various nitroxide probe shave been tested for bio stability in the skin and possible cutaneous irritation [5,6]. The stability in skin biopsies, homogenates and cell cultures of keratinocytes decrease in the following order imidazoline4 pyrrolidine 4 DTBN4 piperidine 4 oxazolidine [5]. The irritation potency of different nitroxides has been classified as nonirritant to moderately irritant, thus, nitroxide scan be safely employed for *in vivo* EPR studies of skin even at high concentrations (100mM) [6]. The fact that some nitroxides and drugs are simply impermeable to skin, as demonstrated by studying the spin labeled equivalents, prompted another line of research of investigating potential drug carriers. Group of authors [7,8] systematically studied potential applications of liposomes as drug carriers for topical treatment of skin disorders using 1DEPR. They found that entrapment in liposomes promotes the penetration of otherwise impenetrable charged nitroxides in to skin and that the loss of integrity of liposomes in different layers of the skin (indicating the site of delivery) can be assessed. Studies revealed that the size and phospholipid composition of liposomes significantly influence their efficiency, thus providing the base for their application in medicine and cosmetics. Nitroxide pharmacokinetics has been studied in various preparations of skin homogenates and cell cultures [5,9], animal [5,10,11] and human biopsies [5,12] as well as *in vivo* on animal models [10,11,13,14] or on human subjects [10,15]. The principle for measuring distribution and pharmacokinetics is the same as outlined in, except that measurements were performed *in vivo* at lower frequencies and using more sophisticated equipment. The larger part of research has been devoted to the evaluation of the UV irradiation influence on the redox status of skin. UV irradiation of the skin can cause inflammatory processes through several possible mechanisms: (1) direct damage of DNA; (2) generation of different free radicals (ROS, lipid derived radicals - peroxyl or alkyl), and (3) production of inflammatory agents (prostaglandins, histamine). EPR studies are of importance for two main reasons: (1) better understanding of processes under- lying postulated oxidative damage induced by UV irradiation; (2) examining free radical scavenging efficacy of various anti- oxidant sand sun screens for protection. Enhanced production of ROS following UV irradiation of the skin has been demonstrated by spin trapping [16,17] and also by analyzing nitroxide decay kinetics [10-12,13,15]. *In vivo* measurements of UVB influence on rat skin [13] and analysis of the obtained 15N-PDT reduction skin ethics showed the decrease of both penetration and reduction rate in all skin layers after the exposure to UVB light. The decrease of the reduction rate constant was more pronounced in dermis and hypodermis than in epidermis layer. Oximetry measurements showed that pO_2 did not change after treatment with UVB light, hence oxygen concentration and microcirculation were disqualified as possible causes of alterations in nitroxide reduction rates. Results of other authors [10-12], however, showed the opposite trend - the increase of reduction rate of nitroxide after the treatment of skin by UV light. Exposure of the skin not only led to the change in nitroxide signal decay rate but also affected the shape of the kinetic curve [13]. Namely, when three different nitroxides (TEMPO,3-CPand 3-CxP) were topically applied to non-irradiated skin biokinetics corresponded to first order exponential process while upon irradiation by UV light all the kinetics could be fitted by bi-exponential function. It has been assumed that the faster process is due to reduction of nitroxides by radicals produced UV irradiation, while the slower process corresponds to reduction of nitroxides by so called daughter radicals of ROS (for instance lipid carbon centered and lipid peroxyl radicals). The work of Takeshita et al. [11] adds to understanding of a probable cause of nitroxide.

Skin Testing on Large Surface

The incidence of malignant melanoma [18,19,20] the most dangerous form of skin cancer, is rising each year. However, some aspects of the tumor initiation and development are still unclear, and the current method of diagnosis, based on the visual aspect of the tumor shows limitations. For these reasons, developments of new techniques are ongoing to improve basic knowledge on the disease and diagnosis of tumors in individual patients. Discussion and the way that a new measurement method [17] based on Electron Paramagnetic Resonance (EPR) might answer to it will be explained and debated.

The surface loop coils are sufficient, i.e. the whole objects need not to be within the resonator, which allows the EPR of object of any size, including humans. This is some important features since it opens the possibilities of application of EPR in human (clinical) studies of not only pharmacokinetics of nitroxides, but also of mechanism of action of certain drugs (storage in Liposomes), skin melanoma, oximetry. UV-light generate free radicals which were reduced by nitroxides in a different manner. (Figure 1)

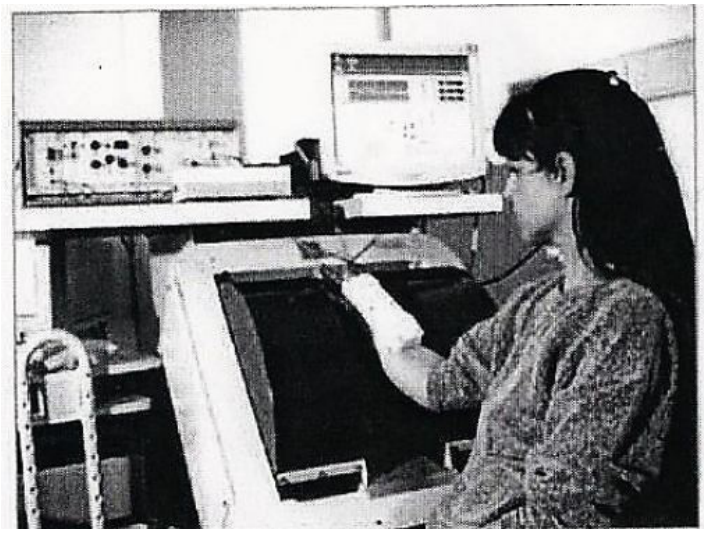

Figure 1: Surface Coil (S-band)-3 GHz)-resonator in a Teflon-retainer made for application on skin.

Using the 1D localized spectroscopy (SURF ERS) we have measured the reduction kinetics of Tempo in human skin. An ethanol/water (50/50) solution of 30 µl with a Tempo concentration of 50 mM were applied on the human forearm. The tempo solution was covered with a Finn chamber (Epitest Ltd Oy Finnland) to prevent an evaporation.

After a treatment time of 20 min. the Tempo solution was completely penetrated into the skin and the measurement could start. The surface coil was fixed on the forearm at the place of the Tempo application. The forearm was positioned together with the surface coil resonator in the center of the electromagnet. The ESR spectra were measured with a sweep time of t_B = 0,5 s and a field sweep of $\Delta B\ \Delta B$ = 9,5 mT to prevent movement artefacts by the volunteers. To increase the sensitivity 80 spectra were accumulated and resulted in a final spectrum and a total recording time of 40 s.

For the determination of the reduction curve 25 spectra were used which measurement required 1000 s. The measured spectra are represented in Figure 2 a. The intensity distribution as a function of time of the mid field peak of tempo is shown in Figure 2 b. The reduction kinetic was best fitted as mono exponential decay (e-curve) which show that the radical reduction corresponds to the following function

$S(t) = S(0) \exp - (kt)$.

With S(0) as the int*e*nsity at t=0.

This reduction curve is determined by the rate constant k and the half time $t_{1/2}$ which can give information about the radical scavenging activity of the patient. The presented reduction curve resulted in a rate constant k = 0,14/min or half life time $t_{1/2}$ =4,8 min.

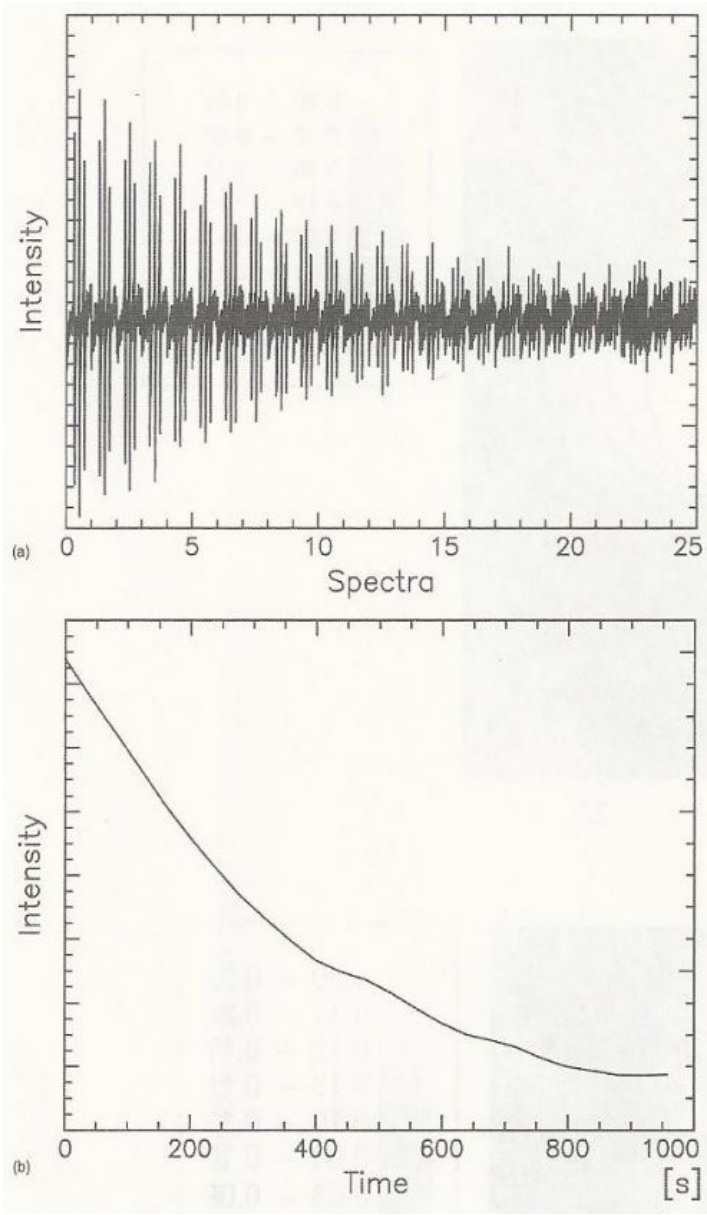

Figure 2(a-b): Reduction of the spin probe Tempo in the skin of a human forearm after 20 min treatment of 30 µl Tempo solution (50 mM solved in ethanol water 50/50) represented by 25 spectra **(a)** in a time period of 1000 s and representation of the corresponding reduction curve **(b)** as the intensity distribution of the mid field peak of Tempo versus time which resulted in a rate constant k = 0,14 /min and a half life time of $t_{1/2}$=4,8min.

Surface Mapping

Scanning the surface (SURF_ERM) of a large sample by mechanical movement of the surface coil can result in a 2D map. As an example, a clover leaf was putted with its stalk in vessel filled with ethanol/water (20/80) solution of the spin probe

Tempyo 100 mM. After a treatment time of 24 h the leaf was removed out of the spin probe solution and was fixed in the x-z plane of the magnet. A scanning area with the dimensions of x =35mm and z = 35 mm with 16 continuous measuring points to each direction resulted in a matrix of M_{xy}=16x16=256 points. The spatial resolution was calculated from the ratio

$$\Delta x,z = \frac{x,z}{16 \text{ points}} = \frac{35 \text{ mm}}{16} = 2{,}2 \text{ mm}$$

Using a measuring time of **T** = 1 s per matrix point the recording time for a 2D image (Figure 3) needed 256 s =4,26 min. The magnetic field was fixed during the measurement at $B=B_o$

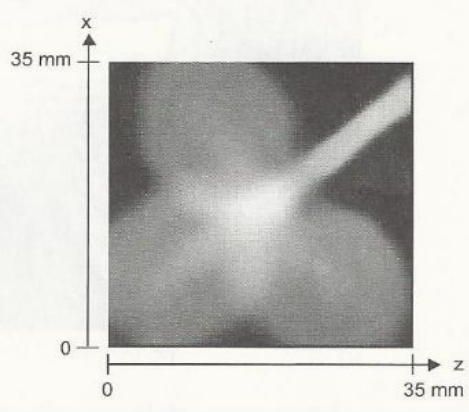

Figure 3: 2D ESR image of a clover leaf.

As practical application of this technique we have tried to answer the question if the spin probe was really reduced in the skin or if it was penetrated transversal over the skin surface. To solve this problem, we have applied a 50 mM Tempyo solution (ethanol/water 50/50) on the skin surface of a human volunteer After a treatment time of 20 min we could measure the lateral 2D spatial distribution of Tempyo in the surface which is represented in Figure 4a. After a waiting time of 2 h a 2D image of the same area was measured. This 2D image is represented in Figure 4b. Analyzing the two 2D images of Figure 4 a and b it is seen that there is no significant change in distribution pattern of Tempyo within 2h. So, the isotropic diffusion and active transport of the spin probe in the tissue liquids can be considered as a minor mechanism. Reduction is therefore the main mechanism responsible for the signal intensity decay.

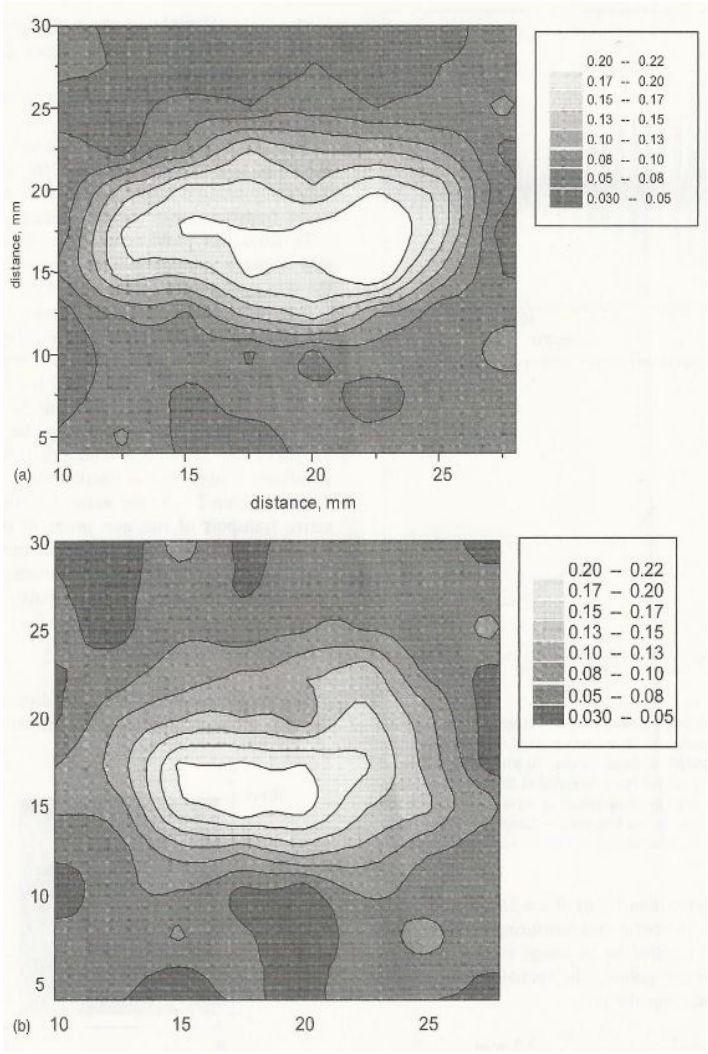

Figure 4(a-b): Lateral distribution pattern of the spin probe Tempyo in the skin surface of human forearm. An ethanol water (50/50) solution of 30µl with Tempyo (50mM) was applied on human forearm and treated for 20 min under an occlusion with a Finn chamber. The first 2D (x,z) image (a) was measured immediately after removing the Finn chamber. Two hours later the second mage (b) was measured showing no significant change in the distribution pattern of Tempyo.

Surface Introscopy

The measurement of the spatial distribution of the spin per-

pendicular to the skin surface needs the additional application of a magnetic field gradient in this direction. The axis of the surface coil which is fixed on the skin surface is oriented in the y-direction which must be also the direction of the field gradient gradyB. This optical examination, even if effective, shows limitations: it does not provide any information about the penetration of the tumor in the skin, which is a crucial factor to determine the growth state of the potential melanoma and to provide an adapted treatment. The use of EPR imaging for the mapping of free radicals trapped in melanin might allow filling in this lack of spatial information in a noninvasive way. This optical examination, even if effective, shows limitations: it does not provide any information about the penetration of the tumor in the skin, which is a crucial factor to determine the growth state of the potential melanoma and to provide an adapted treatment. The use of EPR imaging for the mapping of free radicals trapped in melanin might allow filling in this lack of spatial information in a noninvasive way. The skin surface was arranged in the x-z plane whereby the main magnetic field B0 is oriented in z- direction. A field gradient gradzB= 1,26 T/m was generated by four rectangular permanent magnets which were mounted on the poles of the magnet. The Skin of a human volunteer was treated with a 50 mM Tempyo solution ethanol water (50/50) in the same manner like for spectroscopy measurements of the reduction kinetics. For the measurements of the spatial distribution we have used the middle peak of the Tempyo spectrum which is represented in Figure 5a. After applying the magnetic field gradient, the spectrum was broadened which is caused by the distribution of the spin probe in the different skin layers. The deconvolution of the Introgram IG(B) with the middle line of the Tempyo spectrum resulted the spatial distribution of the introgram R(r) which is shown in Figure 5b. The introgram represent the highest Tempyo concentration in the upper layers of the skin which are the horny layer thickness 16,8µm ± 2µm and the thickness of the epidemis 46,9 µm ± 2,3 µm. The total thickness of the human skin which includes the dermis is about 3 mm. Ten intrograms were measured in a time period of 700 s with a recording time of 60 s for one introgram and a time interval between two measurements of 10s, The 2D spatial-temporal image IM ((y,t) shown in Figure 5c the reduction of Tempyo in the upper skin layer over a time period of 700 s.

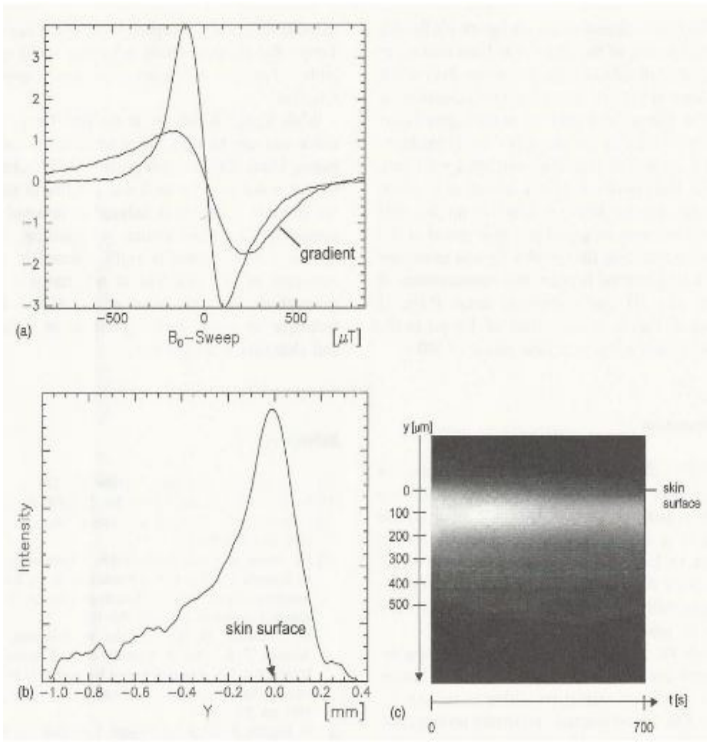

Figure 5(a-b): Longitudinal distribution of Tempyo in the human skin measured for the middle line of the spectrum and the corresponding Introgram IG(B) (a) caused by the field gradient gradyB =12,6 mT/cm in the vertical y direction, Tempyo was applied in the same manner like for the experiment in Figure 2. (b) The deconvolution of the Inrogram IG(B) resulted in the vertical spatial distribution R(y) of Tempo. The time depend reduction of the vertical distributed Tempo is represented by 10 intrograms measured over a time period of 700 s which resulted in the 2D spatial-temporal image IM(x,T) (c).

Conclusion

With the application of the two different spin probes Tempo and Tempyo it was possible to measure the reduction capacity of the skin against Tempo and the penetration behavior of the spin probe Tempyo in transversal and vertical direction. While Tempo is reduced in the skin Tempyo is stable and can be used for penetra-

tion measurements. Using this two spin probes which molecular size is not so different it was possible to solve the question if Tempo is reduced or removed in transversal (x,z) and vertical (y) direction . The answer is that Tempo is really reduced in the epidermis of the skin and is not removed by penetration. The application of the SURF _ER technique to many similar problems in biology and medicine is imaginable. The investigation of melanomas by EPR is a new and famous method for testing skin.

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