

# A comparative in vivo and in vitro L-band EPR study of irradiated rat incisors

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## Abstract

L-band ( $\sim 1$  GHz) EPR has the potential to measure the absorbed radiation dose in human teeth inside the mouth (in vivo analyses). One crucial point in the development of the method is to know if dosimetry evaluation carried out in vivo after accidental exposures can be reliably based on calibration curves built in vitro. The aim of the present work is to specifically address this point. First, we compared L-band in vitro and in vivo analyses in irradiated rat teeth and estimated the possible loss in in vivo experiments due to rat movements and mouth proximity. Second, the lower pair of rat incisors were analysed by L-band EPR before and after irradiation (50 Gy), first on the living rat, then on the same dead rat, finally after extraction of the teeth. X-band powder spectra were also taken after crushing of the two teeth. Irradiations of dead rats and extracted teeth were also carried out. Comparing L-band spectra obtained with living rats and removed heads does not show any significant difference due to possible small rat movements or breathing. Relative standard deviations of the amplitudes of the dosimetric signal are quite high (27–54%). Nevertheless, it seems to be a tendency to have higher signals in irradiated extracted teeth than in irradiated animals.

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## 1. Introduction

Very similar X-band EPR spectra of irradiated teeth are observed whatever the origin: human teeth (Wieser et al., 2000) or teeth of animals, for example elephant (Debuyst et al., 2000), reindeer (Klevezal et al., 1999), walrus (Hayes et al., 1998), cow (Toyoda et al., 2003), rat (Brik et al., 2000; Miyake et al., 2000), mouse (Toyoda et al., 2003; Khan et al., 2003). The EPR spectrum principally consists of

two signals, one due to the stable  $\text{CO}_2^-$  radicals (“dosimetric signal”) and the other to the so-called “native” radicals.

Although EPR dosimetry with human teeth is usually performed at X-band ( $\sim 9.7$  GHz) and with enamel powder (Wieser et al., 2000), it was recently suggested to use L-band ( $\sim 1$  GHz) EPR spectroscopy to measure the absorbed radiation dose in human whole teeth in situ, without removing the tooth from the mouth (Miyake et al., 2000). The L-band spectrum of a whole tooth is reduced to a single composite line, sum of the dosimetric and native signals. In using a surface coil resonator placed on top of the tooth, enamel was found to be responsible of 88% of the total EPR signal (Zdravkova et al., 2002a). This type of resonator was therefore identified as the best choice for in vivo L-band measurements. Different in vitro L-band studies with photon

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and neutron irradiated extracted human molars were then undertaken in the framework of an European project “EPR Dose Reconstruction with Teeth” in order to check on the feasibility and the reliability of the method with the available spectrometers and resonators (Zdravkova et al., 2002a, b, 2003a, b).

A further step in the development of measurement procedures for living objects involves *in vivo* L-band measurements of irradiated teeth. To our knowledge, only the L-band study of Miyake et al. (2000) refers to this subject describing measurements carried out in alive rats. These authors presented a linear dose-response in the dose range 0–40 Gy obtained with X-ray irradiated anaesthetized rats whose upper pair of incisors were surrounded by the twin spiral loops of their L-band resonator. They obtained similar dose responses for rat teeth irradiated *in vivo* and *in vitro*.

On the other hand, following the X-band results obtained by Brik et al. (2000) with enamel powder from X-ray irradiated rat teeth, the efficiency of producing  $\text{CO}_2^-$  radicals by irradiation is different for teeth in living and dead rats. These authors compared EPR spectra from rats sacrificed after irradiation, rats suffocated just before irradiation and teeth extracted from dead rats and subsequently irradiated (70 Gy). While the intensities from sacrificed and suffocated rat teeth enamel produced by the same radiation dose were approximately equal, the signal from extracted teeth was roughly twice as intense.

The crucial point from these controversial observations is to know if dosimetry evaluation carried out *in vivo* after accidental exposures can be reliably based on calibration curves built *in vitro*. The aim of the present work is to specifically address this point. First, we compared L-band *in vitro* and *in vivo* analyses and estimated the possible loss in *in vivo* experiments due to rat movements and mouth proximity. Second, we verified whether the factor of 2 between *in vitro* and *in vivo* irradiations observed by Brik et al. (2000), but not by Miyake et al. (2000), could be reproduced in L-band and X-band. Therefore, the lower pair of rat incisors were analysed by L-band EPR before and after irradiation, first on the living rat, then on the same dead rat, finally after extraction of the teeth. X-band powder spectra were also taken after crushing of the two teeth. Irradiations of dead rats and extracted teeth were also carried out.

## 2. Materials and methods

### 2.1. Animals

About 40 male Wistar rats with body weight 300–400 g were purchased from the UCL animal house facilities unit of the faculty of medicine. Rats were anaesthetized with chloral hydrate (400 mg/kg *i.m.*) for irradiation and subsequent EPR measurements lasting around 3 h.

### 2.2. Radiation

The rats and extracted incisors were X-ray irradiated (Philips 250 RT, 250 kV, 15 mA, 1 mm Cu filter; field size 1.0 cm diameter for the rat mouth) with a dose rate of 1.2 Gy/min. A single dose of 50 Gy (dose in water) was delivered to all teeth using fullface irradiations.

### 2.3. EPR measurements

Whole teeth EPR measurements were carried out using a L-band Magnostech EPR spectrometer (Berlin, Germany) equipped with a low frequency microwave bridge operating at 1.2 GHz and an extended loop resonator (surface coil of 3.5 mm inner diameter and 2 mm thickness, and twin spiral loops which fit closely around the lower pair of the rat incisors) specially designed and built by Dr T. Walczak (Dartmouth Medical School, Hanover, NH, USA) for use in living rat as in Miyake et al. (2000) (see figure 3 of the last reference). A frequency counter (CUB RF minicounter, Optoelectronics, USA) enabled the measurement of the microwave frequency. The spectrometer settings of EPR parameters for measurement of rat teeth were: 95 mW input microwave power, 3 mT sweep field, 1024 data points, 1 min scan time, 0.06 s time constant, 0.34 mT modulation amplitude, 40 scans.

Powder EPR spectra were taken at X-band with a Bruker EMX-8/2.7 spectrometer (100 kHz modulation frequency, 5 mW microwave power, 0.34 mT modulation amplitude, 5 mT field range, 1024 data points, 20.48 ms conversion time, 10.24 ms time constant, 20 scans). The magnetic field was measured by a Bruker ER 036 TM NMR teslameter and the microwave frequency by a Bruker EMX 040-1161.8A frequency meter. The Bruker weak pitch signal was used for signal intensity normalization.

### 2.4. EPR spectra analysis

The spectra deconvolution was done with the DOSIMETRY software package developed by the GSF-National Research Centre for Environment and Health of Munich and the Institute of Metal Physics of Ekaterinburg (Koshta et al., 2000; Zdravkova et al., 2003a). EPR spectra were deconvoluted into one or two isotropic native signals and an anisotropic  $\text{CO}_2^-$  species. The latter signal was approximated either by a linear combination of two Gaussian functions whose line positions, widths and relative contributions can be adjusted, or by a simulated powder spectrum.

### 2.5. Procedures

Following Brik et al. (2000), three different procedures were used:

*Procedure 1:* anaesthesia of the rat; *in vivo* L-band spectrum of the unirradiated lower incisors; 50 Gy irradiation; *in vivo* L-band spectrum; killing of the rat by use of overdose

of anaesthetics and extraction of its head; L-band spectrum; extraction of the two teeth; L-band spectrum; crushing of both teeth; X-band spectrum.

*Procedure 2:* same steps with rats killed by overdose of anaesthetics just before irradiation.

*Procedure 3:* irradiation of extracted teeth followed by L-band (whole teeth) and X-band (powder) spectra.

Several series of five rats were irradiated during the same day. EPR spectra of the living rats were always taken immediately after irradiation, those of the extracted heads the following day and those of the extracted teeth several days after irradiation.

### 3. Results and discussion

#### 3.1. General comments on the L-band EPR spectra of the irradiated rat incisors

For equal dose, the amplitude of the single composite L-band EPR signal of the two lower rat incisors was found to be around ten times weaker than that of a human molar. Therefore, in order to have reliable results, a dose of 50 Gy was used throughout this work. This intensity loss is mainly due to the weight, shape and relative enamel/dentin proportion of the samples: predominant contribution of enamel in the case of human molar (total weight of around 1.5 g, half of which being pure enamel in the crown, around 1 cm diameter in the 1.2 cm diameter, 2 mm thick loop) (Zdravkova et al., 2002a), and of dentin in the case of rat incisors ( $\sim 0.15$  g, 1–2 mm diameter for each incisor in the 3.5 mm diameter, 4 mm thick twin loops). The “open-rooted” incisors of rodents are known to grow throughout life and only the buccal surface has enamel (Hilson, 1986). Dentin is known to be less radiosensitive than enamel (Romanyukha et al., 1996). Moreover, the sensitivity of mouse enamel to gamma radiation was recently found to be lower than that of human tooth enamel (25% lower according to Toyoda et al. (2003); 50% lower in Khan et al. (2003)).

As already discussed previously (Zdravkova et al., 2003a, b), an intrinsic resonator signal interferes with the tooth signal. As the position, shape and intensity of this resonator signal varies with time, it is necessary to take its spectrum before and after that of the tooth and to subtract it from the tooth signal. In this work, a more reliable result is obtained by subtracting from the irradiated incisors spectrum that of the unirradiated incisors, both spectra being taken as close together as possible (a couple of hours with living rats). For extracted heads or teeth, the subtraction was performed with a spectrum of another unirradiated head or teeth recorded the same day.

#### 3.2. X- and L-band spectra analysis

The X-band spectra of unirradiated (Fig. 1a) and 50 Gy irradiated (Fig. 1b) crushed incisors can be simulated by

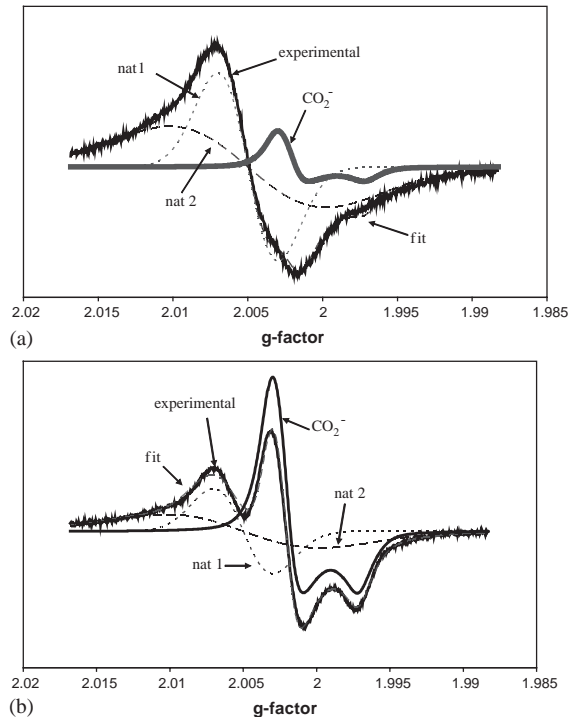


Fig. 1. (a) Experimental and simulated X-band EPR spectrum of unirradiated crushed rat incisors. (b) Experimental and simulated X-band EPR spectrum of crushed rat incisors extracted from a 50 Gy *in vivo* irradiated rat. Each simulated spectrum (“fit”) is the sum of  $\text{CO}_2^-$  and two native (“nat 1” and “nat 2”) signals.

means of two native isotropic signals at  $g = 2.005$  with different linewidths ( $\sim 0.7$  and  $1.8$  mT corresponding to “nat 1” and “nat 2” respectively in Fig. 1) and an anisotropic  $\text{CO}_2^-$  simulated signal as already used in Zdravkova et al. (2003a). A two-component approximation for the native species has already been used previously (i.a. Ivannikov et al., 2001). The native components were found to be 10–20 times less radiosensitive than the carbonated species. A value of  $\sim 20$  was also found for the ratio of the slopes of the regression lines for the  $\text{CO}_2^-$  and native organic components ( $g = 2.0045$  with  $0.78$  mT line width) in human tooth enamel (Vanhaelewyn et al., 2001). The X-band analysis demonstrated that the spectrum obtained after subtraction of the unirradiated tooth spectrum from the irradiated one is principally due to the  $\text{CO}_2^-$  radicals. This approximation was then considered to be sufficient for analysing the spectra of the less sensitive L-band spectroscopy.

A typical L-band spectrum of 50 Gy irradiated whole incisors, after subtraction of the unirradiated teeth spectrum, is shown in Fig. 2. It represents the most common case where only the  $\text{CO}_2^-$  signal is visible. This  $\text{CO}_2^-$  signal in L-band was simulated by the sum of two Gaussian functions with appropriate parameters able to reproduce the

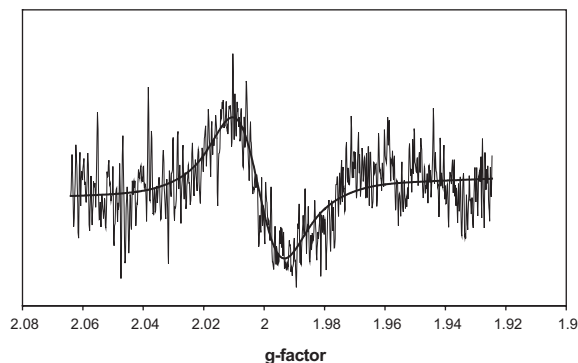


Fig. 2. Experimental and simulated L-band EPR spectrum of the two lower incisors of a 50 Gy irradiated living rat. Only  $\text{CO}_2^-$  radicals are taken into consideration after subtraction of the same unirradiated rat incisors spectrum.

experimental lineshape and width. For this purpose, a reference L-band spectrum of a 875 Gy irradiated human whole molar without its root and crown dentin, with a predominant contribution of  $\text{CO}_2^-$  (Zdravkova et al., 2002a), was taken with the present EPR parameters (0.34 mT modulation amplitude and 95 mW microwave power) and simulated with the DOSIMETRY program.

Table 1 gives L- and X-band results obtained with altogether 20 rats. Mean values for 5 or 10 rats and standard deviations of the distribution of the individual measurements are quoted for the  $\text{CO}_2^-$  and one of the two native signals. The amplitudes obtained in X-band cannot be correlated with those in L-band. No normalization procedure against weight and reference signal was done in L-band, but well in X-band. Subtraction of the unirradiated teeth spectrum was performed in L-band, but not in X-band. All measurements are retained, there are no outliers. In order to compare two experimental means and their standard deviations, statistical t- and F-tests were used for  $P = 0.05$  (Miller and Miller, 2000).

### 3.3. Comparison between *in vivo* and *in vitro* EPR measurements

The possible influence of the movements of the anaesthetized rats and the presence of the nearby head on the L-band EPR spectra was studied by procedures 1 (irradiation of living rats) and 2 (irradiations of rats killed just before). Comparing spectra obtained with whole rats and removed heads does not show any significant role of possible small rat movements or breathing. In the first column of Table 1, the spectra of whole rats and their removed heads are pooled together. A signal height variation related to the age (weight) of the animals and the thickness of their teeth is observed:  $73 \pm 20$  for rats weighting 290–320 g and  $94 \pm 22$  for rats of 350–400 g.

A signal intensity loss due to the presence of the nearby head is a priori expected, even if this effect is probably reduced by the fact that the rat incisors stick out of the mouth. A comparison between whole rats or heads and extracted teeth indeed indicates an intensity loss in the *in vitro* experiment (29%, see second row in Table 1). Opposite results are, however, obtained for the *in vivo* experiments (first row of Table 1).

After the L-band analysis, the entire incisors were gently crushed and X-band spectra taken. The relative standard deviations obtained in X-band for the native species are around 20%, whereas those for  $\text{CO}_2^-$  are around 35% (see two first rows in Table 1). These variations are not that smaller than in L-band (27–54%).

### 3.4. Comparison between *in vivo* and *in vitro* irradiations

The possible difference between *in vivo* and *in vitro* irradiations was studied comparing procedure 1 (living rats) and both procedures 2 (dead rats) and 3 (irradiated extracted teeth). From Table 1, it appears that the means obtained with living rats and rats killed just before irradiation are approximately equal in L-Band (around 80), but those obtained from the extracted teeth (63 and 112) are found to be significantly different at the 0.05 level. On the other hand, the X-band values are not significantly different (34 and 51, 12 and 14).

No significant difference at the 0.05 level is observed between means obtained with irradiated extracted teeth and those of irradiated dead rats (102 and 112, 18 and 14), but well for  $\text{CO}_2^-$  in X-band (74 and 51). However, a significant increase is observed for all cases going from irradiated living rats to irradiated extracted teeth: 63 and 102, 34 and 74, 12 and 18. It is difficult to give a definitive statement because the standard deviations are very big, but there seems to be a tendency to have higher signals in irradiated extracted teeth than in living rats. This is rather in agreement with the X-band results of Brik et al. (2000) who observed that procedure 3 produces  $\text{CO}_2^-$  signals roughly twice as intense. It should be noted that the comparison is not straightforward as L-band results concern rat incisors (predominantly dentin), whereas the X-band results of Brik et al. (2000) concern rat tooth enamel whose origin (incisor or molar) is not specified.

## 4. Conclusions

Comparing L-band spectra obtained with living but anaesthetized rats and removed heads does not show any significant difference due to possible small rat movements or breathing. A signal height variation related to the age (weight) of the animals and the thickness of their teeth is observed in the absence of any mass normalization. A signal intensity loss due to the presence of the nearby head, a priori expected in *in vivo* measurements, was not observed

Table 1

Mean values and standard deviations of the distribution of the individual measurements of the amplitudes of the  $\text{CO}_2^-$  signal (L- and X-band) and of the narrow native signal (X-band) in arbitrary units

	L-band: $\text{CO}_2^-$ in whole rats and heads	L-band: $\text{CO}_2^-$ in extracted teeth	X-band: $\text{CO}_2^-$	X-band: native narrow signal
Irradiation of 10 living rats	$84 \pm 23$ ( $n = 20$ )	$63 \pm 31$ ( $n = 10$ )	$34 \pm 13$ ( $n = 10$ )	$12 \pm 3$ ( $n = 10$ )
Irradiation of five rats killed just before	$80 \pm 43$ ( $n = 10$ )	$112 \pm 35$ ( $n = 5$ )	$51 \pm 16$ ( $n = 5$ )	$14 \pm 2$ ( $n = 5$ )
Irradiation of five extracted teeth		$102 \pm 31$ ( $n = 5$ )	$74 \pm 16$ ( $n = 5$ )	$18 \pm 5$ ( $n = 5$ )

The sample size ( $n$ ) is given in brackets.

probably because the rat incisors stick out of the mouth. The relative standard deviations in L-band are very high (27–54%). These deviations are due to the different weights of the animals, their radiation sensitivities, positioning of the rat during irradiation and of the teeth in the resonator, and spectrometer uncertainties. In X-band, where the spectra were normalized against sample weight and weak pitch reference amplitude, the relative standard deviation still remains important ( $\sim 35\%$  for  $\text{CO}_2^-$ ,  $\sim 20\%$  for the native species) showing a high variability amongst rats. Mixed results are obtained by comparing irradiated living, dead rats and extracted teeth. Despite the huge relative standard deviations, there seems to be a tendency to have higher signals in irradiated extracted teeth, in agreement with the result obtained by Brik et al. (2000).

It could be that these results, valid for evergrowing rat incisors, do not apply to permanent human teeth. The EPR signal in rat incisors mostly originate from dentin, that of human teeth from crown enamel which normally remains constant throughout the life of the tooth. In the case of rat incisors, the resonator can be placed a little bit outside the mouth, whereas, in the case of humans, it should be placed inside, especially for the often studied molars. However, this study can be considered as a development of measurement procedures for living objects. Subtraction of an unirradiated tooth spectrum from the irradiated one should be a good practice also for human teeth. Both spectra, one from the irradiated person, the other from another preferably young person, should be taken as close to each other as possible so that the fluctuating intrinsic resonator signal is removed after the subtraction. Further technical developments are still necessary in order to reduce the noise, the fluctuation and intensity of the intrinsic resonator signal and the scan time, and improve the measurement precision and detection limit.

As there is a possible difference between the intensity of the signal coming from extracted teeth or teeth present in alive people, the final validation in humans should probably be based by comparing teeth extracted, irradiated and put afterwards in a mouth environment for the measurement, and teeth irradiated in people with calibrated dose delivered

to the mouth (patient undergoing a radiotherapy treatment, for example).

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