Radiofrequency Radiation at 40 kHz Induces Hepatic Injury in Long-Evans Cinnamon (LEC) Rats, an Animal Model for Human Wilson Disease

Hiroshi SAKAI1, Noboru HORIGUCHI1, Daiji ENDOH1, Kenji NAKAYAMA2 and Masanobu HAYASHI1)*

1)Department of Basic Veterinary Radiology, School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu 069–8501 and 2) Hokkaido Institute of Public Health, Sapporo 060–0819, Japan

(Received 11 June 2010/Accepted 29 September 2010/Published online in J-STAGE 13 October 2010)

ABSTRACT. In the present study, we examined effects of radiofrequency (RF) radiation at 40 kHz on hepatic injury in Long-Evans Cinnamon (LEC) rats, an animal model for human Wilson disease, which is a heritable disease of copper metabolism in the liver. The activities of ALT and AST in serum of LEC rats exposed to RF radiation for 2 weeks were approximately 3.8-fold and 2-fold higher than those in serum of sham-exposed rats, respectively. Although there were no significant differences in hepatic copper contents between LEC rats exposed to RF radiation for 2 weeks and sham-exposed rats, copper contents in the kidney and serum of exposed LEC rats were approximately 4.2-fold and 12.9-fold higher than those in sham-exposed rats, respectively. Relative O2–-scavenging activities in the S-100 fraction of the liver of LEC rats exposed to RF radiation for 2 weeks were 1.6-fold higher than those in sham-exposed rats. No significant differences were observed in activities of AST and ALT in serum and relative O2–-scavenging activity in the S-100 fraction of the liver of normal control WKAH rats that were sham-exposed and exposed to RF radiation. No significant differences were observed in copper contents in the liver, kidney and serum of WKAH rats that were sham-exposed and exposed to RF radiation for 2 weeks. The results show that RF radiation at 40 kHz induced hepatic injury in LEC rats.

KEY WORDS: animal model for human, copper, hepatic injury, LEC rat, RF radiation.

The question of whether electromagnetic fields in the radiofrequency (RF) range constitute a health hazard in exposed individuals has gained broad public interest because of the widespread applications of RF-radiation-based technology [9]. RF radiation is a portion of the electromagnetic spectrum with frequencies ranging from 3 kHz to 300 GHz, below that of visible light and above that of extremely low-frequency fields. RF radiation is produced by many man-made sources, including mobile phones and base stations, television and radio broadcasting facilities, radar, medical equipment, microwave ovens and radiofrequency heaters as well as a diverse assortment of other electronic devices within our living and working environments [9].

Sufficiently intense RF radiation can cause heating of materials with finite conductivity, including biological tissues. A number of well-established biological effects and adverse health effects from acute exposure to intense RF radiation have been documented [1, 9]. It is thought that these effects are related to localized heating or stimulation of excitable tissue from intense RF radiation. Stress proteins including heat-shock proteins (HSPs) are one group of proteins that have been reported to be affected by RF radiation. French et al. [10] hypothesized that repeated exposure to RF radiation may act as a repetitive stressor, leading to continuous over-expression of HSPs in exposed cells and tissues. However, despite a large number of studies, there have been conflicting results with respect to exposure to RF radiation and HSP expression [4, 5, 23, 26]. Biological effects of non-thermal RF radiation also remain unclear. Although Leszczynski et al. [20] suggested that non-thermal RF radiation induced a cellular stress response, the majority of the research does not show a role for non-thermal RF radiation in inducing a generalized cellular stress response [23]. Furthermore, there is no convincing evidence of differential gene expression in either cell culture or rodent tissues after exposure to non-thermal RF radiation [5].

Based on a large amount of historical knowledge, national and international exposure limits have been established to protect the general public against adverse effects associated with exposure to acute RF radiation [15, 16]. However, the safety of exposure to non-thermal RF radiation remains controversial. Furthermore, biological effects of RF radiation lower than 1 MHz are unknown.

An inbred strain of the Long-Evans Cinnamon (LEC) rat was established as a mutant strain that spontaneously develops fulminant hepatitis associated with severe jaundice at about 4 months of age [28]. The LEC rat has a deletion mutation of at least 900 bp in the 3’-terminal region of Atp7b [25, 34], the rat homologue of the human Wilson disease gene, ATP7B. ATP7B encodes P-type ATPase, which is important for transport of copper into the Golgi apparatus, in a combination of copper and ceruloplasmin, and for excretion of copper from hepatocytes to the biliary tract [3, 29]. A defect in the Atp7b gene results in abnormal copper metabolism, which is characterized by hepatic copper accumulation in LEC rats [21]. Therefore, LEC rats provide a useful experimental model for human Wilson disease [2, 33]. Copper accumulates in the liver of LEC rats and copper can efficiently produce reactive oxygen species (ROS). It is
considered that ROS induce tissue and DNA damage [17, 31, 32, 36]. Since it has been suggested that RF radiation at 40 kHz generated by medical equipment showed protective effects against ROS [14], we examined the biological effects of RF radiation on LEC rats. However, in the present study, we found that exposure to RF radiation at 40 kHz induced hepatic injury in LEC rats.

MATERIALS AND METHODS

*Rats*: Inbred strains of LEC and WKAH/Hkm (WKAH) rats were obtained from Charles River Japan Inc. (Yokohama, Japan) and the Institute for Animal Experimentation, School of Medicine, Hokkaido University, respectively. LEC rats were bred in our breeding facility. All research protocols were approved by the Animal Research Committee of Rakuno Gakuen University. The rats were housed at a temperature of 25 ± 2°C and exposed to a daily cycle of 14-hr light and 10-hr darkness. A solid diet (MF-Food, Oriental Yeast Co., Ltd., Tokyo, Japan) and water were provided *ad libitum*. Twenty male LEC and WKAH rats were used in the present study. WKAH rats were used as normal control rats.

The liver, kidney and serum were obtained from three to four rats as indicated in the results. The tissues were washed repeatedly in cold saline solution, frozen with liquid nitrogen, and stored at –80°C until used.

*Radiofrequency (RF) radiation*: The system used in the present study consisted of a signal generator (TR10-1N/100/DDPM, Gamma Ltd., Tokyo, Japan), signal amplifiers (DUA-1A and DF1906, Ekurea Ltd., Osaka, Japan), oscillator (AG2030, Kenwood Ltd., Tokyo, Japan), and oscilloscope (TDS 2002, Ekurea Ltd., Osaka, Japan). Forty kHz of RF radiation at 4 V was generated (Fig. 1a). The exposed rats were first trained stepwise to reduce stress due to confinement in acrylic boxes during exposure for 20 min (Fig. 1b). Restless behavior (frequent urination and bowel movement) that was seen in untrained rats had vanished when exposure started. Rats at 9 weeks of age were exposed to RF radiation at 3 W/kg for 20 min/day on 7 or 14 successive days. Control rats were sham-exposed.

*Measurements of copper*: The rat tissues were digested with nitric acid and perchloric acid as described previously [11]. Copper contents in the rat liver, kidney, and serum were determined using an atomic absorption spectrophotometer (Perkin Elmer AAnalyst 800, Perkin Elmer Life Sciences, Shelton, U.S.A.) with an air/acetylene flame and with a graphite furnace.

*Measurements of AST and ALT activities in serum*: AST and ALT activities in serum of rats were determined spectrophotometrically in an autoanalyzer (Toshiba TBA-120FR, Toshiba Medical Ltd., Tokyo, Japan).

*Measurements of relative superoxide radical (O$_2^-$)-scavenging activities in the liver*: O$_2^-$-scavenging activities in the S-100 fraction of the liver were assessed by ESR and spin trapping, using the spin trap 5,5-dimethyl-1-pyrroline-N oxide (DMPO), according to the method described by Kuwabara et al. [18]. The S-100 fraction from the rat liver was prepared as described previously [12]. Relative O$_2^-$-scavenging activity was calculated as follows:

\[
\text{Relative O}_2^\text{-scavenging activity} = \left( \frac{[\text{ESR intensity}]_O - [\text{ESR intensity}]_R}{[\text{ESR intensity}]_O} \right) \times 100 \%
\]

where [ESR intensity]$_O$ and [ESR intensity]$_R$ represent the maximum ESR signal intensities in the absence and presence of the S-100 fraction, respectively. Protein concentrations of the S-100 fraction were determined by the method of Lowry et al. [22].

*Statistical analysis*: All data were expressed as means ± standard deviation. Differences between means were analyzed statistically by two-factor repeated measures ANOVA. Values of $P<0.05$ were considered significant.

RESULTS

*AST and ALT activities in serum*: To examine the effects of RF radiation on hepatic injury in LEC rats, we measured the activities of serum AST and ALT. There was no evidence of stress behavior in the rats during exposure to RF radiation. Body weight increased throughout the experiment, and no statistically significant differences were found between sham-exposed and exposed groups (data not shown). Although no significant changes were observed in AST and ALT activities in serum of LEC rats exposed to RF radiation for 1 week, compared with those of sham-exposed rats, the activities in serum of LEC rats after exposure to RF radiation for 2 weeks were significantly higher than those in sham-exposed and 1 week-exposed rats (Fig. 2a and b). Activities of ALT and AST in LEC rats exposed to RF radi-
EFFECTS OF RF ON HEPATIC INJURY IN LEC RATS

Activities of ALT and AST in serum of sham-exposed LEC and WKAH rats (S) and LEC and WKAH rats exposed to RF radiation at 40 kHz for 1 week and 2 weeks (E). Each bar represents the average from four separate experiments with standard deviations (SDs) of mean values. * represents significant differences at $p<0.05$ when compared with sham-exposed and exposed rats.

Copper contents: Since it is thought that copper that has accumulated in the liver is released from severely damaged hepatocytes into the blood and deposited in other organs, including the kidney [13, 27], we examined the effects of RF radiation on copper contents in the liver, kidney and serum of LEC rats (Fig. 3). There were no significant differences in hepatic copper contents between LEC rats exposed to RF radiation for 2 weeks (240.4 ± 11.2 ppm) and sham-exposed rats.

Fig. 2. Effects of RF radiation on activities of ALT and AST in rats. Activities of ALT (a) and AST (b) in serum of sham-exposed LEC and WKAH rats (S) and LEC and WKAH rats exposed to RF radiation at 40 kHz for 1 week and 2 weeks (E). Each bar represents the average from four separate experiments with standard deviations (SDs) of mean values. * represents significant differences at $p<0.05$ when compared with sham-exposed and exposed rats.

Fig. 3. Effects of RF radiation on copper contents in the liver, kidney and serum of rats. Copper contents in the liver (a), kidney (b) and serum (b) of sham-exposed LEC and WKAH rats (S) and LEC and WKAH rats exposed to RF radiation at 40 kHz for 2 weeks (E). Each bar represents the average from four separate experiments with SDs of mean values. SDs are within symbols at some bars. * represents significant differences at $p<0.05$ when compared with sham-exposed and exposed rats.
were approximately 200-fold higher than those in the liver. Copper contents in the livers of sham-exposed LEC rats exposed to RF radiation at 40 kHz for 1 week and 2 weeks. Rats at 9 weeks of age were 16 weeks of age [28]. Rats at 9 weeks of age were developed hepatic injury associated with severe jaundice at age-dependent manner from 4 to 14 weeks of age [11] and DISCUSSION and exposed to RF radiation for 2 weeks (Fig. 4). The present study showed that RF radiation at 40 kHz induced hepatic injury in LEC rats. This is the first report concerning biological effects of RF radiation at 40 kHz. Although the RF electromagnetic field in the present study included both alternating current at 40 kHz and direct current at 6,000 V (data not shown), exposure of LEC rats to only direct current to for 2 weeks had no effects on the activities of serum ALT and AST (data not shown). Therefore, induction of hepatic injury in LEC rats may be rats (197.6 ± 38.5 ppm). Copper contents in the kidney and serum of LEC rats after exposure to RF radiation for 2 weeks were significantly higher than those in sham-exposed rats. Copper contents in the kidney (46.5 ± 24.8 ppm) and serum (37.3 ± 28.2 ppm) of LEC rats exposed to RF radiation were approximately 4.2-fold and 12.9-fold higher than those in the kidney (11.1 ± 2.2 ppm) and serum (2.9 ± 0.3 ppm) of sham-exposed rats, respectively. In the case of WKAH rats, no significant differences were observed in copper contents in the liver, kidney and serum of rats that were sham-exposed and exposed to RF radiation for 2 weeks (Fig. 3).

Relative scavenging activities of superoxide radical (O2\(^{-}\)). Relative O2\(^{-}\)-scavenging activities in the S-100 fraction of the liver were assessed by the spin trapping method (Fig. 4). Relative O2\(^{-}\)-scavenging activities in the S-100 fraction of the liver of LEC rats after exposure to RF radiation for 2 weeks were significantly higher (1.6-fold) than those in sham-exposed rats. These results suggested that RF radiation for 2 weeks induced hepatic injury in LEC rats. Copper contents in the kidney and serum of LEC rats after exposure to RF radiation for 2 weeks were significantly higher than those in sham-exposed rats. These results also suggested that hepatic injury occurred in the 2-week-exposed LEC rats, since it is thought that copper that has accumulated in the liver is released from severely damaged hepatocytes into the blood and deposited in the kidney [13, 27]. It is well known that copper can efficiently produce reactive oxygen species (ROS) such as superoxide (O2\(^{•-}\)), hydroxyl radical and hydrogen peroxide, and that ROS induce tissue and DNA damage [17, 31, 32, 36]. We and other investigators have shown that DNA damage is induced in hepatic and renal cells of LEC rats by copper accumulation [11–13, 35]. Superoxide dismutase (SOD) that scavenges O2\(^{•-}\) is a major antioxidant enzyme and regulation of SOD genes plays a pivotal role in balancing the concentration of ROS. The compartmentalization and control of SODs at both the gene expression and the activity contribute to the level of SOD and consequent localized ROS level [8, 24]. It has been reported that relative O2\(^{•-}\)-scavenging activities in the S-100 fraction of the liver of LEC rats change from 4 to 14 weeks of age [12], although whether the change of activities affects the occurrence of hepatic injury remains unclear. The relative O2\(^{-}\)-scavenging activities in the S-100 fraction of the liver of exposed LEC rats to RF radiation for 2 weeks were significantly higher than those of sham-exposed rats. These results showed that O2\(^{-}\)-scavenging activities in the liver of LEC rats were induced after exposure to RF radiation. However, since it has been reported that small molecules such as bilirubin have scavenging activities of O2\(^{•-}\) [30], it remains unclear whether the higher activities in LEC rat liver after exposure to RF radiation are due to an induction of O2\(^{-}\)-scavenging enzymes such as SOD or due to an increase in bilirubin concentration by hepatic injury.

LEC rat shows accumulation of copper in the liver in an age-dependent manner from 4 to 14 weeks of age [11] and develops hepatic injury associated with severe jaundice at about 16 weeks of age [28]. Rats at 9 weeks of age were exposed to RF radiation at 40 kHz for 1 week and 2 weeks. Copper contents in the livers of sham-exposed LEC rats were approximately 200-fold higher than those in the liver of sham-exposed WKAH rats at 11 weeks of age (Fig. 3, [11, 12]). Although ALT and AST activities in the serum of sham-exposed LEC rats were higher than those in the serum of WKAH rats at 10 and 11 weeks of age, clinical symptoms of hepatic injury such as jaundice were not observed in sham-exposed LEC rats (data not shown). Furthermore, no significant differences were observed in AST and ALT activities in the serum of sham-exposed LEC rats at 10 and 11 weeks of age (Fig. 2a and b). Activities of ALT and AST in LEC rats exposed for 2 weeks were significantly higher than those in sham-exposed rats. These results suggested that RF radiation for 2 weeks induced hepatic injury in LEC rats. Copper contents in the kidney and serum of LEC rats after exposure to RF radiation for 2 weeks were significantly higher than those in sham-exposed rats. No significant changes were observed in relative O2\(^{•-}\)-scavenging activities in the S-100 fraction of the liver of LEC rats that were sham-exposed and exposed to RF radiation for 2 weeks (Fig. 4).

**Fig. 4.** Effects of RF radiation on activities of relative O2\(^{•-}\)-scavenging activity in rats. Relative O2\(^{•-}\)-scavenging activities in the S-100 fraction of sham-exposed (S) and exposed (E) LEC and WKAH rats for 2 weeks. Each bar represents the average from four separate experiments with standard deviations (SDs) of mean values. * represents significant differences at \(p<0.05\) when compared with sham-exposed and exposed rats.
mainly due to RF radiation of alternating current at 40 kHz. The mechanisms by which RF radiation interacts with metals or ions in cells and tissues remain unknown. Although it has been reported that RF radiation changed calcium efflux rates in culture cells [7, 19], whether RF radiation affected calcium efflux or not was still contradictory [6]. The present results showed that exposure to RF radiation at 40 kHz induced hepatic injury in LEC rats, although adverse effects were not found in normal control rats (WKAH rats) after exposure to RF radiation. Therefore, it may be necessary to establish a different safety level of RF radiation for patients with abnormal metal metabolism such as human Wilson disease from that for the general public.

ACKNOWLEDGMENTS. The authors thank Dr. Akihiro Amaya, Dr. Naoto Nagaoka and Dr. Nobutaka Mori, Department of Engineering and Technology, Doshisha University for scientific discussion for the present study.

REFERENCES


