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● *Original Contribution*

DETRIMENTAL EFFECTS OF 60 KHZ SONOTHROMBOLYSIS IN RATS WITH MIDDLE CEREBRAL ARTERY OCCLUSION

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Abstract—Recent studies have raised concerns about the safety of low frequency ultrasound in transcranial therapeutic application in cerebral ischemia. This study was designed to evaluate safety aspects and potential deleterious effects of low frequency, 60 kHz ultrasound in treatment of experimental middle cerebral artery occlusion (MCAO) in rats. Forty-five male Wistar rats were submitted to either temporary (90 min; groups I and II) or permanent MCAO (groups III and IV) using the suture technique. All animals received recombinant tissue plasminogen activator (rt-PA) starting 90 min after the beginning of occlusion. Groups I and III were additionally treated with 60 kHz ultrasound (time average acoustic intensity 0.14 W/cm^2, duty cycle 50%). Outcome assessment consisted of magnetic resonance imaging (MRI) and clinical evaluation after 5 and 24 h, and histology (perfusion fixation after 24 h). Overall mortality was higher in animals treated with ultrasound (43% versus 29% in controls). Most animals died during the insonation period (25% in group I, 36% in group III, no animals in the corresponding control groups; $p < 0.05$). Histology revealed disseminated microscopic intracerebral bleeding and subarachnoid hemorrhage as one possible cause of death. After temporary occlusion, the hemispheric ischemic lesion volume was more than doubled in animals treated with ultrasound (20.3% ± 14.1% versus 8.6% ± 5.1% in controls; $p < 0.05$). No difference in lesion volume was seen after permanent MCAO. Neurological assessment showed impairment of hearing as an additional specific side effect in ultrasound treated animals (65%, no impairment in controls). Although the results are not directly transferable to the human setting, this study clearly demonstrates the potential limitations of low frequency therapeutic ultrasound and the importance of pre-clinical safety assessment. (E-mail: max.nedelmann@neuro.med.uni-giessen.de) **© 2008 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Ultrasound, Thrombolysis, Stroke, Safety, Efficacy.**

INTRODUCTION

Transcranial application of therapeutic ultrasound is an interesting, yet still experimental option to increase the efficacy of recombinant tissue plasminogen activator (rt-PA) treatment of acute cerebral vessel occlusion. Clinical studies have reported a significantly increased recanalisation rate and a potentially improved outcome in patients receiving transcranial Doppler (Alexandrov et al. 2004; Molina et al. 2006) and duplex insonation (Eggers et al. 2003, 2005) during rt-PA treatment. The largest clinical study to date, the combined lysis of thrombus in brain ischemia using transcranial ultrasound and systemic t-PA trial (CLOTBUST)-trial, noted a recanalisation rate that was more than doubled (46% versus 18%), when continuous 2-h insonation with transcranial 2-MHz Doppler was added to standard rt-PA treatment (Alexandrov et al. 2004).

Experimental results suggest that ultrasound parameters different from the commercially available diagnostic tools may lead to improved treatment efficacy. In particular, the choice of the applied ultrasound frequency may be of importance, with a more pronounced treatment

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effect at lower frequencies (Behrens et al. 2001; Ishibashi et al. 2002; Suchkova et al. 2002; Nedelmann et al. 2005). However, there is also evidence, that incautious choice of ultrasound different from the diagnostic parameter setting may cause severe side effects on brain tissue. A recent clinical multicenter study on transcranial therapeutic application of 300 kHz ultrasound demonstrated an increased rate of cerebral hemorrhage (transcranial low-frequency ultrasound-mediated thrombolysis in brain ischemia (TRUMBI) trial: Daffertshofer et al. 2005). Ultrasound induced blood brain barrier disruption was suggested as one possible cause (Reinhard et al. 2006).

In a previous study on transcranial continuous wave 20 kHz ultrasound (insonation time 20 min), we have found evidence of vasogenic and cytotoxic edema formation and even necrosis in healthy rat brain tissue (Schneider et al. 2006). These effects were dose dependent and were found at intensities ranging from 0.5 to 2.6 W/cm^2. Intensities below this threshold caused no pathological findings on magnetic resonance imaging scans and histology specimens. Application of low intensity 20 kHz ultrasound (0.2 W/cm^2) that had previously not shown side effects in healthy rat brain resulted in an increased death rate of animals subjected to embolic middle cerebral artery occlusion (MCAO) (Wilhelm-Schwenkmezger et al. 2007). As histological evaluation had revealed excessive hemispheric infarction in some of the deceased animals, a potential adverse effect of ultrasound on the ischemic tissue had been postulated.

This study was designed to further evaluate the previously noted deleterious effects of transcranially applied low intensity low frequency ultrasound on ischemic tissue and to gain more insight into the nature of occurring side effects. To extend the knowledge on bioeffects of ultrasound at the low frequency range, a slightly higher (60 kHz) frequency was chosen. An experimental model of MCAO in rats was used. Evaluation was based on MRI, histology and functional testing.

MATERIALS AND METHODS

Animal preparation

Forty-five male Wistar rats weighing 318 ± 32 g were used in the present study (Harlan Winkelmann, Borchen, Germany). One animal that died during the initial operation procedure was excluded from evaluation. Thus, 44 rats were included in the study.

All experiments were performed in accordance with the German animal protection legislation and were approved by the regional ethics committee (Az 1.5 177-07/051-43).

The animals were anesthetized with 5% isoflurane inhalation for 2 min. Anesthesia was maintained with 2% to 3% isoflurane inhalation at 0.5 L/min during surgery. Body temperature was continuously monitored with a rectal probe and maintained at 36.5°C to 37.0°C. Rats were subjected to MCAO by use of the filament model as previously described (Schmid-Elsaesser et al. 1998). In brief, the right external carotid artery (ECA) and the right common carotid artery (CCA) were ligated permanently, while another transient ligation of the internal carotid artery (ICA) was performed right after the bifurcation. A 4-0 silicone-coated nylon suture was introduced through a small arteriotomy between the two ligations of the CCA. The occluder was advanced into the ICA 20 to 25 mm beyond the carotid bifurcation until mild resistance indicated that the tip was lodged in the anterior cerebral artery and, thus, blocked blood flow to the MCA. In temporary occlusion, reperfusion was induced by removing the suture 90 min after MCAO. For permanent occlusion, the suture was left in place.

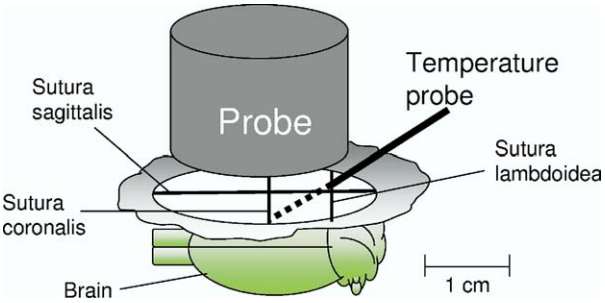

Fig. 1. Experimental set-up: Positioning of the ultrasound probe in relation to the skull and the rat brain. Distance of the probe surface to the skull was 5 mm. The temperature probe for evaluation of intracranial temperature during sonication was placed in the ischemic area in a separate set of experiments.

For transcranial ultrasound application, the animals were prepared as previously described (Schneider et al. 2006). In short, the scalp was mobilized from the skull by longitudinal incision. The skin flaps were attached to a ring (diameter 40 mm, 10 mm above the skull) to form a basin, that was then filled with water. The ultrasound probe (BANDELIN electronic, Berlin, Germany) was placed 5 mm above the skull and into the basin, in order to ascertain full transmission of sound to the skull. The transducer had a plane circular surface with an area of 3.5 cm^2, thus, being similar to the 20 kHz probes that were used in our previous studies (Schneider et al. 2006; Wilhelm-Schwenkmezger et al. 2007; Fig. 1). Twenty-four hours after surgery, the animals were deeply anaesthetized and submitted to transcardial perfusion fixation with 4% paraformaldehyde.

Experimental set-up

The animals were randomly subjected to experimental groups I to IV. Groups I and II received tempo-