Study of the effects of electroacupuncture in a rodent model of cerebral ischaemia

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Summary. - The effects of electroacupuncture (EA) has been studied in a model of global cerebral ischaemia performed in gerbils through the bilateral carotid artery occlusion (BCAO). Animals, under isofluorane anaesthesia, underwent 5 min of BCAO and were killed after 7 days. The effects of EA were evaluated both on functional (with electrophysiological recordings of synaptic potentials in hippocampal slices) and morphological parameters (by counting the number of survived neurons in CA1 area of the hippocampus). The results demonstrated that the treatment of animals with EA (5 min before, during and 20 min after BCAO and 30 min per day in the following 5 days) did not modify either the ischaemia-induced reduction of synaptic potentials amplitude, either ischaemia-induced neuronal loss in the hippocampus. We conclude that, at least in this animal model of cerebral ischaemia, EA does not exert a neuroprotective effect.

Key words: electroacupuncture, cerebral ischaemia, hippocampus, gerbils.

Riassunto (Studio degli effetti della elettroagopuntura in un modello animale di ischemia cerebrale). - L’effetto dell’elettroagopuntura (EA) è stato studiato in un modello in vivo di ischemia cerebrale transitoria globale da occlusione bilaterale delle carotidi comuni (BCAO) nei gerbili. Gli animali, in anestesia da isoflurano, erano sottoposti a 5 min di BCAO e sacrificati dopo 7 giorni dall’intervento. L’effetto della EA era valutato sia su parametri funzionali (con registrazioni di potenziali sinaptici da fettine di ippocampo ottenute dal cervello isolato) che su parametri morfologici (attraverso la conta dei neuroni nell’area CA1 dell’ippocampo). I risultati hanno dimostrato che il trattamento degli animali con EA (5 min prima, durante e 20 min dopo l’occlusione delle carotidi e per 30 min al giorno nei successivi 5 giorni) non modificava né la riduzione dell’ampiezza dei potenziali sinaptici indotta dall’ischemia né la perdita di neuroni a livello ippocampale. In conclusione possiamo affermare che, almeno in questo modello di ischemia, l’EA non appare esercitare un effetto neuroprotettivo.

Parole chiave: elettroagopuntura, ischemia cerebrale, ippocampo, gerbili.
ischaemia, epilepsy and spasticity has been proposed [4-6]. However, the scientific reports on the efficacy of acupuncture in the treatment of the above diseases are poor and not well documented. It is thus essential to set up specific studies to establish the real effectiveness of acupuncture. For obvious reasons, this should be done first in experimental models of disorders for which, at the moment, there is a lack of undoubtfully effective therapies.

In this paper we studied the effects of electroacupuncture (EA) in a rodent model of forebrain ischaemia using Mongolian gerbils. The Mongolian gerbil is a suitable model for studying cerebral ischaemia. In this species, forebrain ischaemia can be indeed easily produced by clamping both common carotid arteries, since the circle of Willis connecting the carotid and the vertebrobasilar circulations is incomplete [7]. The reasons for which this specific experimental model of human disease was chosen to study the effects of acupuncture can be summarized as follows: i) as pointed out above, to date there are no effective treatments for patients victims of stroke; ii) acupuncture was suggested to have a potential beneficial effect in preventing cerebral damage associated with brain ischaemia; and iii) the animal model easily reproduces the human pathology [8].

We studied the effect of EA in the CA1 area of the hippocampus, a brain area particularly vulnerable to ischaemic insults. Both histological (count of surviving neurons) and functional (recording of neuronal electric activity) parameters have been evaluated, in order to have a more complete spectrum of a possible neuroprotective activity [9].

Materials and methods

Animals

Male Mongolian gerbils (Charles River, Italy) weighing 60-80 g, fed ad libitum and kept under standardized temperature, humidity and lighting conditions, were used. Animal care and use followed the directives of the Council of the European Communities (86/609/EEC).

Forebrain ischaemia

Gerbils were subjected to a 5-min period of bilateral carotid artery occlusion (BCAO) under anaesthesia using a mixture of 3% isofluorane, 30% O₂ and 70% NO₂. They were fixed in the supine position on a temperature-controlled operating pad. A 2 cm anterior midline incision was made and both common carotid arteries were carefully dissected free of accompanying tissues. Bilateral carotid arterial occlusion was produced by clamping the vessels with microaneurysm clips. During operation, anaesthesia was maintained with 1% isofluorane in the same gas mixture.

Electroacupuncture

Animals were treated with EA before, during and after BCAO. Under general anaesthesia, the common carotid arteries were made free from the accompanying tissue and the needles were inserted to the points 8 Du-Jinsuo e 16 Du-Fengfu. In order to verify the correct position of the needles, a stimulus was delivered to evoke the reaction to the puncture (deqi). Such reaction were judged as positive when the needle was held by muscles and not easily extractable. The EA was then delivered using a specific apparatus (Model G-6805, Shanghai Medical Electronic Apparatus Co., China), 5 min before and during the 5 min of BCAO. The parameters of stimulations were 7 Hz of frequency and 5-6 mA of current intensity. EA continued during the suture of the wound and thereafter for a total of 30 min. In order to deliver EA after the end of BCAO procedure, i.e. in awake animals, gerbils had to be restrained in a special apparatus which allowed to hold needles in place. Of course, this apparatus did not induce pain to the animals. The choice of EA points, the parameters of stimulation and the delivery of EA were performed by Luo Mingfu. EA was repeated 30 min a day for the following 5 days in awake (and therefore restrained) animals.

Experimental groups

Animals were divided in 4 groups:
1) ischaemic group (ISCH): animals underwent BCAO;
2) sham-operated group (SHAM): animals underwent the same experimental procedures of the ISCH except for the artery occlusion;
3) restrained group (RESTR): animals were treated as the ISCH but, in addition, they were restrained for 30 min a day over 5 days;
4) acupuncture group (ACU): animals underwent BCAO, restraint and EA.

For electrophysiological experiments, 8-10 animals per group were used. For histological analysis, brains obtained from 4-6 animals per group were analysed.

Hippocampal slice preparation and electrophysiological recordings

Animals were allowed to survive for 7 days following BCAO. Then they were killed by decapitation under light ether anaesthesia. The skull was opened and the hippocampus rapidly removed.
Slices of hippocampus (450 µm thick) were cut with a tissue chopper (McIlwain) and immediately placed in the recording chamber, where they were constantly perfused (at a rate of 2-3 ml/min) with an artificial cerebral spinal fluid (ACSF), saturated with 95% O₂ and 5% CO₂. The composition of ACSF was the following (mM): 126 NaCl, 3.5 KCl, 1.2 NaH₂PO₄, 1.3 MgCl₂, 2 CaCl₂, 25 NaHCO₃, 11 glucose (pH 7.3). The temperature of the perfusion chamber was maintained at 33 ± 1 °C. An interval of 60-90 min was allowed between the time the slices were cut and the start of the recording session.

Extracellular field potentials (FPs) were recorded through 2 M NaCl-filled glass microelectrodes (1-5 Megaohms) in the CA1 area of the hippocampus after electrical stimulation of the stratum radiatum (0.1 Hz, 70 µs, 100-200 µA). FPs were amplified, averaged and analyzed on line by an ad hoc software package. For each experimental group, the average amplitude of the FPs recorded in the CA1 area of different slices has been considered. Moreover, the percentage of failures to record a FP was considered in each group. Differences among groups were evaluated by the Kruskal-Wallis non parametric analysis of variance. For multiple comparisons, the Mann-Whitney U test with Bonferroni’s correction was used.

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**Histology**

Brains were immersed in a solution of formaldehyde buffered with 1% PBS, where they were maintained until histological processing. Paraffin sections (7 µm) of the hippocampus were then cut by a microtome, stained with Cresyl violet and evaluated by a light microscope. For quantitative analysis, the number of CA1 pyramidal cells was counted in 2 consecutive microscopic fields of 0.12 mm² each. The rostrocaudal plane of the sections analyzed was similar among all the groups. Results have been analysed with the Kruskal-Wallis non parametric analysis of variance. For multiple comparisons, the Mann-Whitney U test with Bonferroni’s correction has been used.

**Results**

**Electrophysiology**

Failures in FPs recording occurred in about 44% of slices from ISCH, 12 of slices from ACU and 9% of slices from RESTR groups. On the contrary, in slices obtained from SHAM group, FPs were recorded in the 100% of slices. In line with previous observations [10], the mean FP amplitude recorded in slices from ISCH animals was significantly lower than in the SHAM group (p < 0.05; Fig. 1). In slices from the ACU group, the mean FP amplitude was not significantly different from SHAM animals. However, also the FP amplitude recorded in slices from RESTR group did not differ versus SHAM or ACU groups (Fig. 1).

**Fig. 1.** - Effect of EA on FP amplitude recorded in the CA1 area of the hippocampus.

Experimental groups: SHAM = animals were treated as the ISCH group except for the occlusion of the arteries; ISCH = animals underwent 5-min bilateral common carotids occlusion; RESTR = animals were treated as the ISCH group plus the restraint procedures (30 min a day over 5 days); ACU = animals were treated as the ISCH group plus the electroacupuncture and obviously the restraint, (30 min a day over 5 days). Bars show a significant reduction in the FP amplitude in the ISCH group with respect to the SHAM. Such a reduction was not seen in the ACU group (Fig. 3). Even though an increase in the number of neurons was seen in the ACU with respect to
RESTR group, such a difference was not statistically significant. In addition, the number of neurons in the RESTR group was not statistically different from SHAM, suggesting that restraint alone can exert a positive effect in reducing histological damage.

**Discussion**

Animal models represent often an effective tool to reproduce human pathologies, allowing the study of specific parameters than can be useful to predict a potential therapeutic activity. The Mongolian gerbil is a suitable model for studying cerebral ischaemia because of the lack of a complete circle of Willis. Therefore, cerebral ischaemia is easily induced and testing the effect of a treatment on ischaemia-induced neuronal death is relatively easy in this species. It has been shown that discrete areas in the brain and certain types of neurons such as the CA1 pyramidal cells in the hippocampus, are selectively vulnerable to ischaemic insults and, following a brief episode of ischaemia, they undergo alterations in synaptic transmission and delayed neuronal death that increase over time reaching a plateau one week after the ischaemic episode [11].

While in oriental medicine, and in particular in Chinese traditional medicine, acupuncture is widely used for the treatment of several pathological conditions, in Western countries its use is limited to the treatment of neuromuscular symptoms (mainly pain). The release of endorphines (endogenous agonists of opioid receptors) following needle stimulation of specific points of the body seems to be at the basis of its therapeutic effect [4, 12, 13]. However, acupuncture has been proposed also for the treatment of pathological states for which an involvement of the opioid system has not been yet demonstrated. In the last years, several studies reported a beneficial effect of acupuncture in the post-ischaemic treatment of experimental stroke [14-16]. In line with these observations, the results of our study demonstrated a significant improvement of cellular functions (measured as synaptic activity of CA1 neurons) in ischaemic animals treated with acupuncture. However, this improvement was not different from that observed in the restrained group. It appears clear, therefore, that most of the neuroprotective effect observed in ischaemic animals treated with EA, should be ascribed to the restraint procedure. In fact, it has been demonstrated that the stress of being restrained, by itself, could reduce ischaemic damage [17]. Restraint stress can activate a plethora of different mechanisms, such as an increase of anti-inflammatory hormones and interleukines [18] as well as an increased expression of heat shock proteins [19] or immediate early genes [20], and all of that could be at the basis of the protection observed. However, the investigation of stress-induced neuroprotection was not the aim of this study. In our experimental conditions, the restraint procedure was necessary to deliver EA in awake animals. Another observation that arises from our result is that the effect of EA plus restraint is apparently more effective in terms of neuronal survival than of functional activity. In fact, even though we had found a statistically significant increase in the number of surviving neurons in the ACU versus RESTR group, no beneficial effects of EA were found in terms of cellular electric activity, confirming the observation that even surviving neurons may be altered under the functional point of view [10].

The main result of our study (i.e. no difference between the groups ACU and RESTR) is apparently in contrast with the neuroprotective effects of EA reported by others [14, 16, 21, 22]. In these studies, however, it was often quite difficult to understand the real experimental conditions. In most cases the experimental protocols were not clear, and appropriate control groups were often lacking. On the other hand, doubts on the actual efficacy of acupuncture in the treatment of post ischaemic patients have been raised in recent studies. Specifically, after a careful analysis of some clinical studies showing beneficial effects of acupuncture in the treatment of post-ischaemic disabilities, some authors
have recently underlined the poor quality of such studies and criticized the criteria chosen to interpretate and evaluate the results [23, 24].

In conclusion, in our experimental conditions we could not demonstrate an effect of acupuncture significantly higher than that exerted by restraint alone. Even though the possibility that EA may exert neuroprotective effects cannot be definitely ruled out, our data confirm the importance of considering the appropriate control groups and of choosing the right parameters for the evaluation of the results.

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