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EXPERIMENTAL

The effect of hypoxemic reperfusion on cerebral protection after a severe global ischemic brain insult

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Abstract *Background and purpose*: Reactive oxygen species contribute to membrane lipid peroxidation and neuronal death and have been implicated in anoxic encephalopathy. We tested whether hypoxemic reperfusion (HR) after global cerebral ischemia would improve neurological recovery.
Methods: Two groups of pigs ($n = 11$ in each group) were subjected to a model of a 10-min global cerebral and systemic ischemia to compare the effect of hypoxemic reperfusion (group HR) with the classical hyperoxemic control (group C). A third group not subjected to ischemia served as control to the control group ($n = 6$, group CC), but received hyperoxygenation at the respective period of reperfusion. The outcome was evaluated by means of neurological assessment and the extent of lipid peroxidation measuring the plasma malonaldehyde (MDA) together with hydroxyalkenals (HALK).
Results: Animals of group HR exhibited a significantly superior neurological outcome compared with those of group C at all three consecutive assessments after reperfusion (post-resuscitation $P = 0.006$, at 8 h $P = 0.003$, and at 24 h $P = 0.007$). The levels of MDA and HALK are lower in the HR group than in group C ($P = 0.029$). Additionally, in the CC group these molecules increased significantly early at hyperoxygenation ($P = 0.02$). A faster lactate metabolism in the HR group was observed during reperfusion, though non-significant.
Conclusions: Hypoxemic reperfusion during resuscitation from a severe global ischemic cerebral insult improves the neurological outcome compared with classic hyperoxemic reperfusion. This is additionally confirmed by the decreased production of the molecules of lipid peroxidation. In the absence of preceding ischemia, these molecules may increase by simple over-oxygenation.

Key words Hypoxemic reperfusion · Reactive oxygen species · Overall performance category · Malonaldehyde · Hydroxyalkenals · Postanoxic encephalopathy

Introduction

Postanoxic encephalopathy remains the major problem following successful but prolonged or delayed resuscitation after cardiac arrest [1]. Reactive oxygen species (ROS) produced during reperfusion/reoxygenation have been implicated in several pathways leading to permanent cerebral damage [2, 3]. Agents that either inhibit [4] or scavenge ROS [5] have to be given soon after or preferably before the ischemic insult in order to achieve a meaningful effect and, therefore, their clinical application cannot be recommended.

We conceived the hypothesis that if oxygen supply during reperfusion is reduced, then both the burst of ROS and the degree of their damaging effect could also be reduced based on the following clinical observation: since July 1988, four patients have fully or efficiently recovered neurologically, despite being laboriously resuscitated from a particularly protracted (15–32 min) cardiac arrest. The observation common to all patients was that they were severely hypoxemic with a PaO_2 ranging from 42 to 55 mmHg for various reasons (hemothorax, severe ARDS). Another observation that supports the hypothesis is the low production of superoxide anion by neutrophils when they are exposed to hypoxia [6].

We tested the above hypothesis by applying hypoxemic or hyperoxemic reperfusion to pigs subjected to a model of cerebral global ischemic insult. Comparisons were made by correlating the neurological outcome and quantifying some biochemical markers of lipid peroxidation.

Materials and methods

Twenty-eight male pigs weighing 20–24 kg, fasted overnight with free access to water, were randomly allocated to receive either hypoxemic [group of hypoxemic reperfusion (HR), $n = 11$] or hyperoxemic reperfusion [control group (C), $n = 11$] after a global ischemic cerebral insult. Another group not submitted to ischemic insult, but ventilated as group C at the respective reperfusion period, served as control group for group C (group CC, $n = 6$). After premedication with 10 mg/kg i.m. ketamine, 1 mg/kg i.m. diazepam, and 0.02 mg/kg i.m. atropine, the animals were intubated administering pentobarbital sodium at a dose of 3–5 mg/kg. They received mechanical intermittent positive pressure-controlled ventilation using a Soxitronic Class 1 respirator (Felino, Italy) to maintain a $PaCO_2$ of 42–48 mmHg. A mixture of air and O_2 was administered to keep a PaO_2 level of 95–105 mmHg. Anaesthesia was maintained by continuous i.v. propofol at a dose of 0.035–0.09 mg/min and the central body temperature was kept between 37° and 38 °C with the aid of an electrical blanket. Fentanyl and atracurium were given intermittently as needed, and fluids were given accordingly to maintain a CVP of 3–5 mmHg and blood glucose of 90–120 mg/dl.

Experimental set-up

The right femoral artery was canulated and connected to a transducer (Abbott Laboratories, Ireland) for the continuous monitoring of the blood pressure waveform. In addition, a sensor (Paratrend-7, Biomedical Sensors, Bucks., UK) was introduced for continuous arterial blood gases and body temperature monitoring.

Both carotids were exposed and vessel loops were left in place. The right internal jugular vein was canulated retrograde and a catheter was advanced to the jugular bulb for blood sampling and monitoring of regional blood gases. A specific balloon catheter with a free lumen on the tip, a small balloon for flow direction, and a large balloon 2 cm from the tip was inserted via the external jugular vein into the right atrium. The intra-atrial placement of the large balloon was ensured by withdrawing the catheter to the level where the ventricular waveform disappeared. By injecting normal saline, the large balloon expanded to 30–40 ml, decreasing the right ventricular preload and, consequently, the arterial pressure to the desired level [7].

Finally, 1 g of ceftriaxone was given intravenously for infection prophylaxis, and the animals were left to calm down for 60 min after the end of the preparation in order to achieve a steady state. Subsequently, 2,500 UI heparin and 10 mg atracurium were given intravenously, before the onset of the ischemic insult.

Cerebral global ischemic insult

Cerebral global ischemic insult consisted of the following three manipulations lasting for 10 min: (a) severe systemic hypotension with a mean arterial pressure (MAP) of 15–35 mmHg by filling the atrial balloon with normal saline; (b) bilateral clamping of the carotid arteries; and (c) cessation of respiration by disconnecting the respirator under paralysis. In case of cardiac arrest, the experiment was excluded from further analyses. The procedure was recorded on videotape throughout the 10-min period.

Reperfusion

After completion of the 10-min ischemic insult, balloon emptying, carotids release, and respirator reconnection were applied simultaneously. Return on spontaneous circulation (ROSC) was considered when a MAP of 60 mmHg was restored and the elapsed time was recorded. Epinephrine solution drip was given after 60 s of inefficient ROSC.

FiO_2 at the moment of respirator re-connection was randomly allocated to be either equal to 1 (group C), or equal to 0.12 (group HR). Ten minutes after initiation of reperfusion, FiO_2 was gradually increased or decreased in group HR or group C, respectively, to achieve a PaO_2 of 100 mmHg over the following 50 min, with alterations of about 20% every 10 min.

The animals of the CC group were not subjected to the 10-min period of ischemic insult. However, they underwent exactly the same manipulations, the same blood sampling, and the same oxygenation during the respective period of reperfusion, to solely test the effect of O_2 abundance in the absence of the preceding ischemia.

The animals were extubated, since they had recovered from anaesthesia and had effective ventilation. The neurological assessment was performed on recovery from anaesthesia as well as 8 and 24 h after the insult and was recorded on videotape. Two observers scored each case blindly and independently based on the view of the videotape. A substantial agreement in the scoring was noticed (kappa coefficient = 0.668, 95% confidence interval: 0.564–0.772). In case of disagreement, the corresponding videos were re-evaluated jointly by the two observers so that a consensus could be reached. The neurological evaluation of the pigs was standardised according to the overall performance category score (OPC, modified from [8]). This score evaluates the state of awareness, ambulatory abilities, muscle tone, abnormal movements, presence or absence of hyperventilation and seizures, response to noise, pain, and light, and the ability to feed. It served to categorise each pig as either normal (grade I), or encephalopathic with mild impairment (grade II), moderate impairment (grade III), severe impairment (grade IV), and brain dead (grade V).

The protocol was approved by the National Committee on Use and Care of Animals.

Table 1 The comparative mean ± SD values of mean arterial pressure *(MAP)*, PaO_2, pH, and $PaCO_2$ at the various phases of the experiment

	Base	Ischemic insult (min)			Reperfusion (min)					
Groups		1'	5'	10'	1'	3'	10'	20'	30'	60'
MAP (mmHg)										
HR	103 ± 10	31 ± 9	24 ± 5	16 ± 5	96 ± 44	92 ± 46	86 ± 27	93 ± 24	92 ± 17	92 ± 14
C	102 ± 12	35 ± 5	29 ± 6	21 ± 5	129 ± 31	155 ± 23	120 ± 32	103 ± 20	97 ± 14	94 ± 11
CC	102 ± 13	102 ± 12	101 ± 14	105 ± 15	99 ± 14	103 ± 11	108 ± 14	106 ± 13	106 ± 11	105 ± 5
PaO_2 (mmHg)										
HR	105 ± 5	70 ± 12	45 ± 14	27 ± 7	37 ± 8	37 ± 6	37 ± 5	39 ± 6	50 ± 5	101 ± 9
C	102 ± 4	67 ± 13	39 ± 11	26 ± 8	208 ± 98	357 ± 113	446 ± 110	280 ± 56	174 ± 30	100 ± 2
CC	101 ± 2	100 ± 1	100 ± 2	101 ± 2	462 ± 109	514 ± 104	460 ± 51	375 ± 47	185 ± 36	101 ± 2
pH										
HR	7.45 ± 0.03	7.44 ± 0.9	7.33 ± 0.13	7.14 ± 0.08	7.16 ± 0.13	7.20 ± 0.06	7.25 ± 0.09	7.31 ± 0.1	7.29 ± 0.07	7.35 ± 0.04
C	7.46 ± 0.03	7.39 ± 0.06	7.31 ± 0.05	7.14 ± 0.06	7.15 ± 0.03	7.19 ± 0.04	7.28 ± 0.04	7.33 ± 0.04	7.37 ± 0.02	7.44 ± 0.02
CC	7.46 ± 0.03	7.46 ± 0.02	7.44 ± 0.03	7.45 ± 0.02	7.47 ± 0.02	7.46 ± 0.03	7.43 ± 0.03	7.44 ± 0.03	7.44 ± 0.02	7.45 ± 0.03
$PaCO_2$ (mmHg)										
HR	44 ± 3	44 ± 3	47 ± 13	83 ± 20	67 ± 14	57 ± 10	58 ± 9	46 ± 7	41 ± 3	37 ± 4
C	46 ± 3	50 ± 6	61 ± 9	80 ± 19	72 ± 14	66 ± 7	52 ± 5	45 ± 10	41 ± 1	38 ± 5
CC	45 ± 2	45 ± 1	46 ± 1	45 ± 2	44 ± 3	45 ± 3	47 ± 3	46 ± 2	46 ± 2	45 ± 3

Biochemical determinations

Blood sampling was performed from the right femoral artery and the right jugular bulb at the following periods: the end of the steady state, at minute 5 and at the last minute of the ischemic insult as well as at minutes 3, 10, 20, 30, and 60 of the reperfusion. The samples collected in sterile tubes were centrifuged at 2000 rpm for 10 min at 4 °C. The serum was stored at –75 °C in cryotubes for the following determinations:

1. Lactate measurement: using the REA (radiative energy attenuation) method on the Tdx system (Abbott Laboratories, Ill., USA) based on the application of two coupled enzymatic reactions with lactate dehydrogenase and diaphorase [9].
2. Malonaldehyde (MDA) in combination to hydroxyalkenals (HALK) have been used both as an indicator of lipid peroxidation [10]. These two molecules were measured in combination (Bioxytech LPO-586, OXIS, USA). Briefly, in 200 μl of the sample, 10 μl of 0.5 M bytilated hydroxytoluene (Sigma, St. Louis, Mo., USA) in acetonitrile (BDH, Pool, UK) and 650 μl of diluted 3:1 with methanol 10.3 Mm *N*-methyl-2-phelyndole in acetonitrile were added and mixed gently. One hundred and fifty microliters of 15.4 M methasulfonic acid was then added and mixed well. After 45 min of incubation at 45 °C, samples were centrifuged at 15,000 *g* for 15 min at 4 °C, and the supernatant was transferred to a clear cuvette. Absorbance was measured at 586 nm in a spectrophotometer (Hitachi U-2000, Japan) against a sample and a reagent blank. Results were calculated according to the kit manual.

Statistical analysis

Data are presented as means ± SD for quantitative variables. The median value and range are reported for ROSC and the frequency distribution within each examined group for OPC. For the evaluation of OPC differences among groups, χ^2 for linear trend statistic was used. The exact Wilcoxon rank sum test was used to evaluate ROSC differences between the HR and C groups. Parametric analysis of variance and paired *t*-tests were used for comparisons between and within groups, respectively. For PjO_2, lactate, and MDA, together with HALK concentrations, the method of analysis of variance for repeated measures was performed. The effect of three independent factors (groups, time, jugular/arterial) as well as their interaction was examined. The Tukey-Kramer multiple comparisons test was employed to detect significant differences. A probability of less than 0.05 was considered significant. Finally, in order to evaluate the overall agreement between the two observers who performed the neurological assessment, the kappa coefficient and its 95 % confidence interval were calculated.

Results

The mean ± SD of MAP and PaO_2 values during various stages of the experiment is presented in Table 1. It may be seen that both the C and HR groups were subjected to an ischemic insult of similar intensity. However, in group HR, the drop of MAP is more pronounced, especially at minute 10 ($P < 0.05$). The animals of group CC had not been subjected to ischemic insult, and PaO_2 and MAP levels at the corresponding period are normal. Animals of groups C and CC were subjected to hyperoxemic reperfusion with the PaO_2 levels being significantly ($P < 0.001$) higher than group HR till minute 30 of reperfusion. With reference to the variation of pH and $PaCO_2$, it may be seen that their mean levels are similar throughout the experiment and a difference towards a more acidotic pH is observed at minutes 30 and 60 of reperfusion in group HR than in group C ($P < 0.01$).

In Fig. 1 it may be seen that during the insult period no significant differences were observed in PjO_2 values between groups HR and C. In contrast, groups HR and C differ significantly from group CC ($P < 0.001$) at the

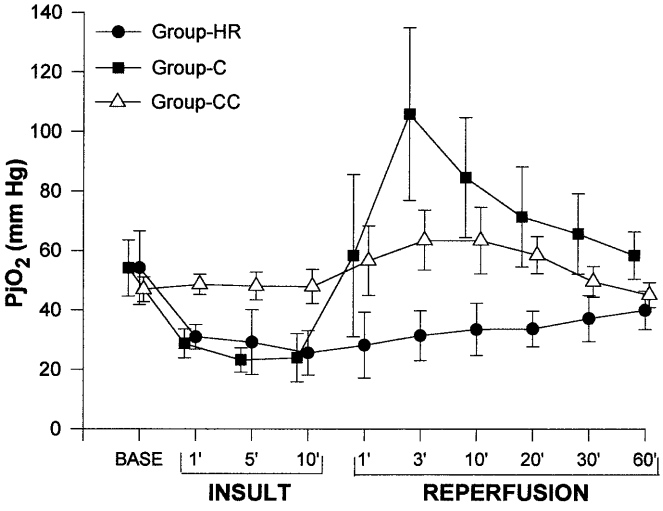

Fig. 1 The mean ± SE levels of PjO_2 in the three groups during the various stages of the experiment. For the statistical evaluation, see text

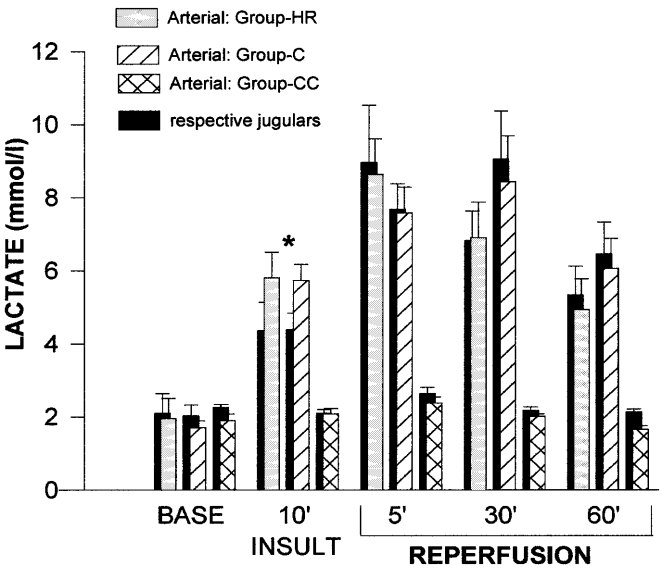

Fig. 2 The mean ± SE levels of lactate in the arterial and jugular blood in the three groups during the various stages of the experiment. *$P = 0.0002$ between arterial and jugular levels in groups C and HR

corresponding time period. Additionally, from minute 1 to 10 of reperfusion, the PjO_2 of group HR is significantly lower than groups C ($P < 0.002$ for each time period) and CC ($P < 0.03$ for each time period). At minute 3 of reperfusion, the PjO_2 of group C is higher than group CC ($P < 0.0001$). At minute 20 to 60 of reperfusion, the values of PjO_2 are similar between group CC and groups HR and C. In contrast, these values are different between the groups HR and C ($P < 0.004$) for each time period of reperfusion with the exception of minute 60 ($P = 0.35$).

The median time of ROSC was 60 s (range, 15–200 s) and 40 s (range, 10–180 s) in groups C and HR, respectively ($P = 0.6$, NS). Six animals in group C and two in group HR received inotropes to restore MAP above 60 mmHg at resuscitation.

The OPC for each group is listed in Table 2. Animals of the HR group exhibited a significantly superior neurological outcome than those of group C. This difference was observed during the three consecutive assessments over time. The pigs of group HR were continuously improving in a similar way to those of group CC, but all the pigs of the latter group recovered completely at 24 h.

The mean ± SE of arterial and jugular lactate levels is presented in Fig. 2. Lactate levels in group C are higher than those of group HR for each time point after the initial 5 min of reperfusion although these differences are not statistically significant at the nominal level. Although jugular levels are similar to the arterial at all times in all groups, they are significantly lower at the end of the ischemic insult in groups HR and C (mean arterial and jugular levels of the two groups jointly 5.76 and 4.38 mmol/l, respectively, $P = 0.0002$).

The mean ± SE of arterial and jugular MDA and HALK levels is presented in Fig. 3. The levels of the molecules are significantly lower at minute 10 to 60 of

Table 2 The overall performance category *(OPC)* variation of the three groups

		OPC					P^a
Assessment	Groups	I	II	III	IV	V	
After resuscitation	HR		4	7			
	C			6	5		0.006
	CC		2	4			0.90
8 h	HR	3	5	2	1		
	C		1	3	7		0.003
	CC	2	3	1			0.56
24 h	HR	6	2	2	1		
	C	1	1	2	6	1	0.007
	CC	5	1				0.20

[a] Significance level from χ^2 test for linear trend in proportions, comparing group HR with groups C and CC

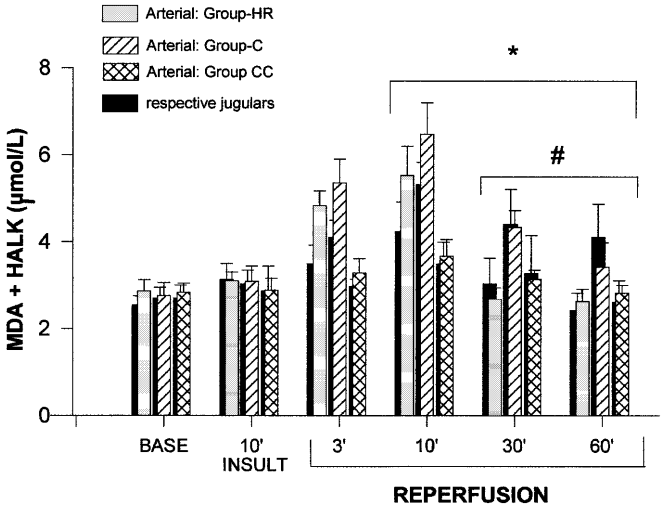

Fig. 3 The mean ± SE levels of malondialdehyde *(MDA)* + hydroxyalkenals *(HALK)* in the arterial and jugular blood in the three groups during the various stages of the experiment. *$P = 0.029$ between group HR and C, #$P < 0.0001$ between the levels at minute 30 and 60 of reperfusion compared to the levels at minute 10

reperfusion in group HR than group C ($P = 0.029$). There is no difference between jugular and arterial concentration of the molecules at all times and in all groups ($P = 0.35$). A significant decrease in the levels of molecules is observed from minute 10 of reperfusion to minute 30 and 60 ($P < 0.0001$) in groups HR and C. In the CC group, the arterial levels of the molecules increase at minute 10 of the over-oxygenation period to a statistically significant degree ($P = 0.02$) compared to the reference levels.

Discussion

In this study, an improvement of neurological outcome was observed by applying hypoxemic instead of classic hyperoxemic reperfusion after a global cerebral ischemic insult. This effect has been additionally verified by the decreased production of the molecules of lipid peroxidation.

The choice of the model of cerebral global ischemic insult appeared to be a major challenge in this study. The pig brain is supplied both by the internal carotid arteries and the vertebral arteries. Additionally, the internal carotid artery forms a rich anastomotic network with the maxillary artery [11] that is unique in the pig. Therefore, by simply ligating the major arteries, complete ischemia of the brain may be impossible. Moreover, since it was desirable to assess the animals clinically, major interventions needing thoracotomy had to be ruled out. The application of a neck tourniquet in combination with a severe decrease of MAP [12] represents an alternative model of cerebral ischemia, adapted perhaps in rats but not in pigs, which present a short and thick neck with massive tissue structures and a system of vertebral arteries that are not occluded by neck compression.

In other studies, global cerebral ischemia was produced by raising intracranial pressure either to 100 mmHg above arterial pressure [13] or equal to MAP [14] with rapid infusion of artificial cerebrospinal fluid into a lateral ventricle. However, mechanical may overshadow ischemic injury of the brain, and the post-resuscitative syndrome is not reliably reproduced since no systemic ischemia occurs.

The model applied in the present study ensures a global ischemic cerebral insult of particular reproducibility, leading to a severe neurological deficit without abrogating respiration. Consequently, the animals are capable of breathing spontaneously after recovery from anaesthesia and their neurological assessment is simplified since there are no major interventions demanding analgesia or the environment of intensive care.

The control group was reperfused with hyperoxemia after the insult, since this is the common clinical practice after ischemic insults or cardiac arrest and, in addition, it is imposed by the current principles of cardiopulmonary resuscitation. The control group of the control (group CC) was added in part to serve as the standard for the neurological assessment, since no insult was occurred, and in part to test solely the effect of over-oxygenation on the markers of lipid peroxidation.

A significant improvement of the acute brain recovery was observed in group HR compared with group C assessed by the clinical scale of OPC. There are at least three studies supporting the notion that ventilating with air atmosphere versus 100 % O_2 during resuscitation after cardiac arrest may be advantageous with respect to neurological outcome. Mickel, for example, [15] showed a threefold decrease in 14-day mortality in gerbils when exposed to an air atmosphere instead of to 100 % O_2 after ischemia. Zwemer et al. [16] similarly showed that hyperoxically resuscitated dogs sustained significantly worse neurological deficit at 12 and 24 h than did both antioxidant-pretreated hyperoxically resuscitated and normoxically resuscitated dogs after cardiac arrest. Similarly, Liu et al. [17] tested normoxic versus hyperoxic reperfusion after cardiac arrest showing a better outcome in the normoxically treated animals together with lower levels of oxidized brain lipids. It seems therefore, that the normal- compared to the high-oxygen mixtures ventilation during reperfusion favours diminished ROS formation and lower cerebral lipid peroxidation. These results may lead to the rationale that further diminishment of elemental tissue O_2 concentration may further minimise the post-arrest cerebral damage. In fact, the hypoxemically treated ani-

mals in the present study achieved an even better neurological outcome, and therefore, a more effective protection of the brain during reperfusion seems to have occurred. No other study has tested the effect of hypoxemic reperfusion after brain ischemia, with the exception that of Ulatowski et al. [13], which discloses the limitations stated above.

The protective role of hypoxemia during reperfusion is further supported by the detection of lower levels of MDA and HALK in the hypoxemic group animals than in the control group, suggesting a lesser degree of lipid peroxidation. In contrast, the administration of hyperbaric oxygen after global cerebral ischemia in rabbits did not appear to promote brain lipid peroxidation [14]. However, in that study, both oxidized glutathione and the ratio of oxidized to reduced glutathione were higher, indicating an increase of free radical generation. This, in fact, implies that the elemental presence of more oxygen during reperfusion after ischemia enhances the production of ROM which, however, did not produce an increase in lipid peroxidation. This may simply be due to lack of detection because MDA was measured too late in the reperfusion i.e., in minute 75. In the present study, the peak of MDA and HALK detection is at minute 10 while by minute 60 their levels return almost to normal. Additionally, these molecules at the end of the insult were found at similar levels to the base condition. These results therefore, contrast with findings supporting that the level of oxidized lipids does not change with increasing periods of reperfusion as well as that lipid peroxidation occurs during ischemia in the absence of reperfusion [17]. The finding of the significant increase of MDA and HALK during reperfusion in the CC group is of similar importance. It seems, therefore, that the use of high O_2 concentration mixtures alone may lead to lipid peroxidation even in the absence of preceding anoxia.

The improvement of brain recovery in the HR group is additionally supported by the fact that the high PjO_2 observed during reperfusion in animals of group C indicates a defective cerebral O_2 utilisation representing a degree of brain damage. In contrast, PjO_2 of HR animals indicates a strong desire for oxygen utilisation.

In contrast to the results of the present study, no improvement of acute brain electrical recovery assessed till minute 120 of reperfusion was observed in the study of Ulatovski et al. [13]. They also tested the effect of transient hypoxic reperfusion for 30 min after 15 min of global cerebral ischemia. Two points of controversy may be cited here: first, that while PaO_2 was maintained at 35–45 mmHg during reperfusion, it was abruptly turned to 80–120 mmHg at min 30. Possibly, at that moment, an oxidative brain injury might have occurred, apparently detected as poor electrical brain recovery. This is further supported by the observation that till minute 30 of reperfusion, hypoxic animals showed a somewhat better recovery in the change of amplitude of somatosensory evoked potentials than the controls. Thereafter, the favourable effect was scattered and both groups showed a similar poor recovery. In the present study, a progressive return to the normal PaO_2 was considered to obviate this eventual delayed effect. Second, in the model of ischemia per se, raising the intracranial pressure to 100 mmHg above arterial pressure seems to be inappropriate for such an investigation, for the reasons cited above.

There is a trend towards a faster metabolism of lactate in the HR group during reperfusion together with better cardio-circulatory stability and the need for fewer inotropes. This may indicate a better preservation of cellular and mitochondrial structure integrity, permitting better cardiac performance and effective cellular metabolism of lactate. The latter is compatible with the study [17] that found higher brain lactate levels in the hyperoxemic group. An additional point of interest is the observation that jugular lactate levels are lower than the arterial levels during the ischemic insult although similar during reperfusion. This implies that during ischemia the brain produces less lactate than the periphery.

Protection against anoxia/reoxygenation injury has been shown to occur in acidotic pH, a phenomenon known as "pH paradox" [18, 19]. In the present study, the mean pH and PCO_2 values were similar in groups HR and C in the various point periods. It is rather doubtful whether a slightly more acidotic pH observed at minutes 30 and 60 of reperfusion exerts any protection.

Given the potential of residual postanoxic encephalopathy, there is evidence from the results of several studies [15, 16, 17], combined with those of the present study, suggesting that the current principles of cardiopulmonary resuscitation might be in question. Because of the innovative character of the idea of hypoxemic reperfusion after ischemic insults, these results have to be confirmed by histopathological studies, and such a study is currently under way. The need for additional experimental work confirming the possible beneficial effect of hypoxemic reperfusion after global cerebral ischemia is of paramount importance before attempting relative clinical trials becomes justified.

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