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Research report

Anesthesia specific differences in a cardio-pulmonary resuscitation rat model; halothane versus sevoflurane

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ABSTRACT

Objective: Our asphyxia cardiac arrest (ACA) rat model is well established. The original model was designed in the 1990th using halothane and nitrous oxide for pre-insult anesthesia. Because of its hepato-toxicity and its potential to induce severe liver failures, halothane is no longer used in clinical anesthesia for several years. In order to minimize the health risk for our laboratory staff as well as to keep the experimental settings of our model on a clinically oriented basis we decided to replace halothane by sevoflurane. In this study we intended to determine if the change of the narcotic gas regiment causes changes in the neurological damage and how far our model had to be adjusted.

Methods: Adult rats were subjected to 5 min of ACA followed by resuscitation. There were four treatment groups: ACA - halothane, ACA - sevoflurane and with halothane or sevoflurane sham operated animals. Vital and blood parameters were monitored during the 45 min post-resuscitation intensive care phase. After a survival time of 7 days histological evaluation of the hippocampus was performed.

Results: We observed that resuscitated rats anesthetized prior by sevoflurane (i) have had a lower heart rate and a higher MAP compared to halothane anesthetized animals; (ii) The neurological damaged were significantly reduced in the hippocampal CA1 region in sevoflurane treated rats.

Conclusion: Using sevoflurane instead of halothane for anesthesia requires some physiological and experimental changes. However the model keeps its validity. Sevoflurane caused less pronounced neurodegeneration in the CA1 region of the hippocampus. This had to be considered in further resuscitation-studies containing sevoflurane as anesthetic.

Institutional protocol number for animal studies: 42502-2-2–947 Uni MD.

1. Introduction

The rat model of asphyxia cardiac arrest (ACA) (Ebmeyer et al., 2002, 2014; Keilhoff et al., 2010, 2011, 2013) is well established in our lab. Animal models for resuscitation research are accepted and unalterable. For ethical reasons new therapeutic approaches can be transferred into clinical practice only after profound animal studies. In animal models it is possible to reduced interfering factors like pre-existing illnesses of the cardiovascular system or the CNS, variety of environmental factors and other individual conditions, to a minimum. However, an animal model can simulate the situation in human patients only to a certain degree. Most laboratory animals are young adults, whereas most patients are elderly. There are no comorbidities in animals compared to elderly patients who often have cardiac or other illnesses. Often human patients have different preexisting medical therapies (Hausenloy et al., 2010).

The original model was designed in the 1990th using halothane and nitrous oxide for pre-insult anesthesia. Because of its hepato-toxicity and its potential to induce severe liver failure (Kumar et al., 2005; Qureshi et al., 2007), halothane is no longer used in clinical anesthesia for several years. In order to minimize the health risk for our laboratory staff as well as to keep the experimental settings of our model on a clinically oriented basis we decided to replace halothane by sevoflurane. Sevoflurane is a widely used volatile anesthetic. Because of its lower blood solubility the anesthetic effects of sevoflurane are significantly faster as compared to halothane. This effect is similar during induction as well as for the wake up. Like halothane sevoflurane has a very good hypnotic potential, but shows only poor analgesic and muscle relaxant effects. Similar to other volatile anesthetics sevoflurane acts negative inotropic, leads to bronchodilatation, and increases cerebral blood flow. It is known that in the presence of particularly dry carbon dioxide absorbents containing sodium or barium hydroxide sevoflurane

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may build nephrotoxic halo alkenes which may lead to renal failure in rats. Therefore we decided to use a semi-open anesthetic system without carbon dioxide absorbents.

The potential neuroprotective effect of sevoflurane is well established (Adamczyk et al., 2010; McAuliffe et al., 2009; Payne et al., 2005; Wang et al., 2010, 2008). The replacement of the anesthetic agent leads to more modifications of our model. Because of sevoflurane specific shortened wake up we were forced to reduce the so called washout period immediately prior the insult from 5 to 4 min. Otherwise there would be a risk that animals could awake from anesthesia before cardiac arrest is established. On the others side a minimal duration of the washout period is mandatory to reduce potential side effects caused by the pre asphyxiation applied anesthetics. In addition the new anesthesia regiment caused a reduction of the required post resuscitation intensive care period from 60 to 45 min. Preliminary tests have shown that animals that went through an only 5 min cardiac arrest period regained faster a normal metabolic status and sufficiently stable vital parameters. Asphyxiation periods of less than 5 min could, however, appears to be too short to generated sufficiently detectable as well as reproducible neurological damages.

In this study we wanted to determine all necessary model adaptations caused by the change from halothane to sevoflurane. Our aim was to optimize resource utilization and to alleviate stress for the animals. In addition, we would demonstrate potential differences on the neurological damages and the preconditioning potential of the both volatile anesthetics in our ACA rat model.

2. Results

2.1. Vital parameters

The average preparation time was 25.05 ± 6.5 min without group differences. By the end of the preparation phase, all baseline parameters were within physiological ranges. An overview of the vital parameters is shown in Table 1. After asphyxia was started, animals went into CA within 172.6 ± 20.6 s without group differences. After five minutes of asphyxiation, resuscitation was initiated and ROSC was achieved within an average of 29.8 ± 9.9 s. Three animals were excluded. One animal could not be resuscitated within the required 2 min of CPR time. Two animals failed to survive the prior asphyxiation via randomization predetermined survival time because of pulmonary edema. Immediately after resuscitation, the MAP temporarily increased in all animals above 200 mmHg (initial hypertensive bout). Overall the trend from the sham groups was as expected. Animals treated with sevoflurane developed a higher heart rate (Fig. 1**A**) and a higher MAP (Fig. 1**B**) during the intensive care period in the ACA and sham groups. Hypotonic blood pressure was not measured at any moment in one of the ACA or sham groups. During the 45 min

Table 1
Vital parameters overview. t rec (rectal temperature); t tym (tympanal temperature); HR (heart rate); MAP (mean arterial blood pressure); pH (pH-value); pCO_2 (partial pressure of carbon dioxide); pO_2 (oxygen partial pressure); Bicarb.(bicarbonate); BE (base excess); Glucose (blood glucose).

		BL	5 min	15 min	30 min	45 min
sham halothane	t rec	36.1 ± 0.1	36.6 ± 0.2	36.7 ± 0.3	36.9 ± 0.1	36.8 ± 0.2
	t tym	37.3 ± 0.3	37.2 ± 0.4	37.1 ± 0.3	37.1 ± 0.1	37.1 ± 0.1
	HR	255 ± 30	335 ± 30	320 ± 28.3	330 ± 42.4	330 ± 35.5
	MAP	64.8 ± 6.9	78.0 ± 21.2	79.5 ± 15.7	85.5 ± 27.6	86.8 ± 29.7
	pH	7.4 ± 0.03	7.4 ± 0.05	7.4 ± 0.03	7.4 ± 0.02	7.4 ± 0.03
	pCO_2	35.3 ± 3.3	38.0 ± 4.1	36.3 ± 3.0	37.0 ± 2.8	36.5 ± 2.9
	pO_2	164.5 ± 25.8	174.5 ± 16.9	178 ± 27.6	197.5 ± 31.8	204.4 ± 22.4
	Bicarb.	26.1 ± 1.7	23.3 ± 2.1	24.2 ± 2.1	22.9 ± 0.3	23.2 ± 1.2
	BE	1.6 ± 2.1	−1.3 ± 2.3	0.1 ± 2.4	−1.0 ± 0.7	−0.6 ± 1.0
	Glucose	9.2 ± 0.2	11.8 ± 1.4	10.6 ± 1.3	9.6 ± 1.3	8.5 ± 1.2
sham sevoflurane	t rec	35.8 ± 0.5	35.8 ± 0.5	35.9 ± 0.5	35.9 ± 0.5	36.1 ± 0.4
	t tym	36.5 ± 0.4	36.2 ± 0.3	36.4 ± 0.2	36.5 ± 0.2	36.5 ± 0.2
	HR	368.1 ± 43.4	357.5 ± 29	360.6 ± 29	370.0 ± 34.6	377.5 ± 45.2
	MAP	89 ± 12.2	94.8 ± 9.4	91.4 ± 9.7	92.6 ± 8.9	97.1 ± 9
	pH	7.4 ± 0.04	7.4 ± 0.02	7.4 ± 0.02	7.4 ± 0.02	7.4 ± 0.02
	pCO_2	40.4 ± 2.6	40.4 ± 4.5	40.6 ± 2.6	40.4 ± 1.4	40.6 ± 0.8
	pO_2	172 ± 29.6	172.4 ± 26.2	174.6 ± 23.4	177.4 ± 14.7	177.8 ± 15.2
	Bicarb.	24.98 ± 2.3	24.7 ± 2.0	24.1 ± 1.7	23.8 ± 1.6	23.8 ± 1.4
	BE	0.7 ± 2.8	0.3 ± 2.4	−0.3 ± 1.9	−0.7 ± 1.8	−0.7 ± 1.5
	Glucose	11.0 ± 4.3	11.6 ± 4.9	11.7 ± 4.2	11.1 ± 4.1	11.1 ± 3.5
ACA halothane	t rec	35.9 ± 0.4	36.5 ± 0.7	37.2 ± 0.6	37.5 ± 0.5	37.7 ± 0.3
	t tym	37.3 ± 0.5	36.8 ± 0.6	37.1 ± 0.4	37.5 ± 0.4	37.3 ± 0.2
	HR	256 ± 47.2	418.9 ± 13.7	341.1 ± 37.8	315.6 ± 31	306.7 ± 9.4
	MAP	69 ± 13.9	137.6 ± 16	89.8 ± 26.7	82.9 ± 22.6	96.3 ± 25.1
	pH	7.4 ± 0.04	7.1 ± 0.05	7.3 ± 0.04	7.3 ± 0.05	7.3 ± 0.07
	pCO_2	38.4 ± 9.4	63.3 ± 5.6	50.0 ± 6.6	42.1 ± 2.9	43.7 ± 1.2
	pO_2	129.2 ± 27.6	136.1 ± 39.4	126.3 ± 42.3	211.8 ± 60.4	235.3 ± 55.3
	Bicarb.	23.8 ± 2.3	15.9 ± 1.9	19.9 ± 1.8	22.2 ± 2.2	21.9 ± 3.4
	BE	−0.9 ± 2.7	−10.4 ± 2.3	−5.5 ± 2.1	−2.8 ± 2.6	−3.2 ± 4.1
	Glucose	10.7 ± 1.6	15.0 ± 2.3	13.0 ± 1.7	10.1 ± 1.2	8.6 ± 1.3
ACA sevoflurane	t rec	36.1 ± 0.7	36.4 ± 0.5	36.8 ± 0.7	36.9 ± 0.5	36.8 ± 0.4
	t tym	36.3 ± 0.3	36.3 ± 0.5	36.7 ± 0.5	36.6 ± 0.3	36.7 ± 0.4
	HR	340.5 ± 37	450.5 ± 29.2	381 ± 27.7	358 ± 31.6	332 ± 33.4
	MAP	91.1 ± 14	169.2 ± 15	99.9 ± 18.1	87.1 ± 19.3	87.3 ± 12.7
	pH	7.4 ± 0.05	7.1 ± 0.03	7.2 ± 0.05	7.3 ± 0.03	7.3 ± 0.06
	pCO_2	42.8 ± 8.8	74.2 ± 9.9	68.2 ± 11	56.2 ± 6.8	55.8 ± 12.4
	pO_2	145.6 ± 36.5	98.2 ± 33.2	85.2 ± 21.4	182.6 ± 56.8	154.4 ± 24.3
	Bicarb.	26.7 ± 2.9	20.7 ± 1.5	21.8 ± 1.8	24.1 ± 1.5	24.9 ± 0.8
	BE	2.96 ± 2.2	−6.3 ± 1.1	−3.5 ± 0.5	0.3 ± 1.4	1.1 ± 0.9
	Glucose	11 ± 2.7	16.9 ± 5.2	17.0 ± 3.5	12.96 ± 3.1	10.6 ± 2.9

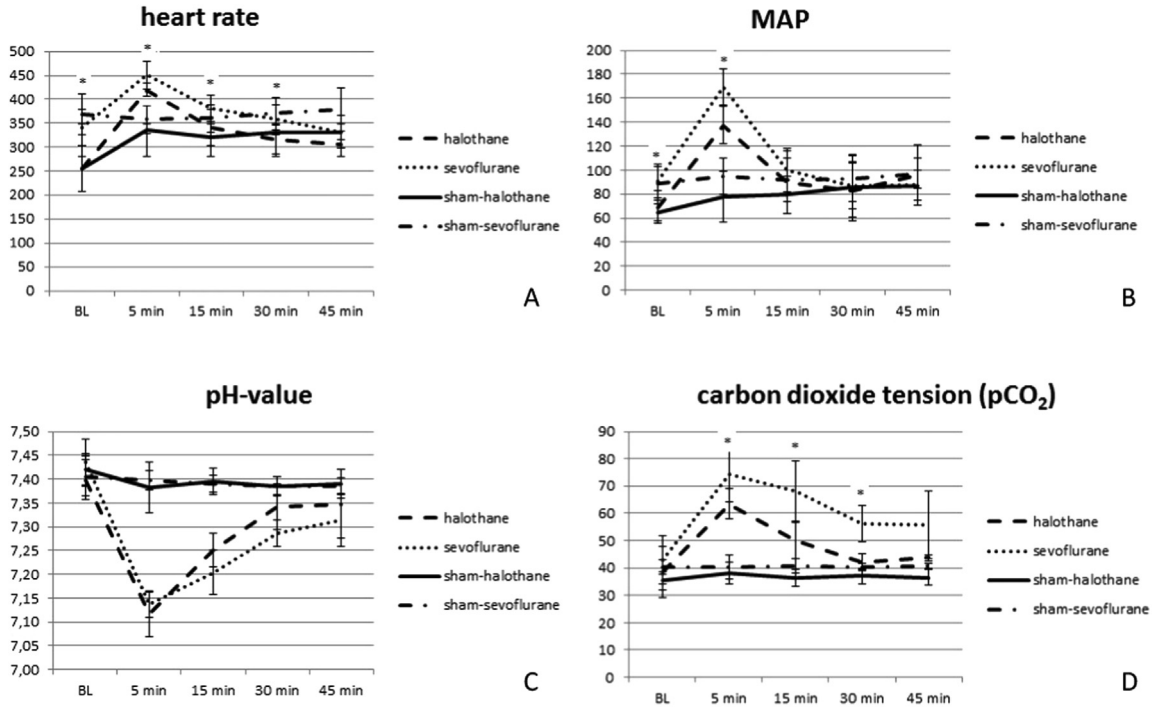

Fig. 1. A Overview heart rat; B Overview mean arterial blood pressure; C Overview pH-value; D Overview carbon dioxide tension, (significances between ACA groups indicated by * $p < 0.05$).

intensive care period, the MAP decreased to 95.8% of the baseline level in ACA-sevoflurane group and increased at the same time to 139% in ACA-halothane group. In all ACA-sevoflurane and ACA-halothane animals, the MAP was from a clinical standpoint sufficiently stable during the intensive care period without any additional interventions.

Heart rates of all animals were elevated for the first minutes post-resuscitation to about twice the baseline values but returned to physiological ranges within 45 min. Animals of the sham-sevoflurane group impressed generally with more stable vital parameters then the rats of the sham-halothane group. Fig. 1**A** show an overview of the first 45 min of the intensive care period. It becomes apparent that the heart rate is lower in the sham-halothane compared to the sham-sevoflurane group. As shown in Fig. 1**B** the MAP was also lower in the sham-halothane group.

As expected, all asphyxiated rats went initially into acidosis (Fig. 1**C**) but returned to baseline values within 45 min. Likewise arterial carbon dioxide tension (pCO_2) was highest immediately after resuscitation, with the values observed in the ACA-sevoflurane animals always slightly above those of the ACA-halothane animals (Fig. 1**D**). All animals could be weaned and extubated on time. During preparation, insult, and intensive care rectal and tympanal temperatures were measured permanently. Artificial hypothermia could be excluded in all groups. After a brief recovery period all resuscitated animals were housed during the following night in an incubator (34 °C). If needed, animals were fed and nursed. Neurological and behavioral performance improved rapidly in all resuscitated animals without group differences; all sham operated animals were at all times without any neurological impairment.

2.2. Hippocampal degeneration

Sham-operated animals were independent on the used anesthetic free of neurodegeneration in any region of the hippocampus as demonstrated by both NeuN (Fig. 2**A-B′**) and MAP2 (Fig. 2**E–F′**) immunostainings. The GFAP-positive astroglia pattern was regular (Fig. 2**A–B′, E–F′, I–J′**), and IBA1-positive microglia was detectable but not predominant (Fig. 2**I–J′**).

ACA caused a selective degeneration of CA1 pyramidal cells. The quantitative analysis of neuron-specific MAP2 immunostaining revealed significant ($p < 0.0001$) differences between ACA and sham operated animals in both anesthetic groups (Fig. 3**A vs. B**). The level of degeneration, however, clearly depended on the used anesthetic and was significantly more prominent in the ACA-halothane group (Fig. 3**B**). The reduction of CA1 neuronal cell loss in the ACA-sevoflurane group could be seen by NeuN (Fig. 2**C,C′**) as well as MAP2 (Fig. 2**G,G′**) immunostaining. NeuN and still more MAP2 staining indicated that a distinct population of CA1 neurons survived. In halothane animals, ACA induced a dramatic loss of the respective neuronal cell bodies (NeuN staining, Fig. 2**D,D′**) and a complete loss of CA1 neuronal fibers (MAP2 staining, Fig. 2**H,H′**). The differences between MAP2 and NeuN staining verified the methodological sensitivity of our model. There is evidence that many neurodegenerative diseases exhibit a so called “dying-back” pattern of neurodegeneration; a progressive and substantial loss of (MAP2-expressing) axons precedes loss of (NeuN-positive) neuronal cell bodies (Saxena and Caroni, 2007).

In accordance with the anesthetic induced differences of the neurodegenerative pattern the subsequent astrogliosis was more pronounced in the ACA-halothane group compared to the ACA-sevoflurane animals (Fig. 2**C,C′ vs. D,D′; G,G′ vs. H,H′; K,K′ vs. L,L′**; Fig. 3B).

Neurodegeneration induced microglia activation, demonstrated by IBA1 immunostaining, acted up to the same principle and was more intensive in ACA-halothane animals in comparison to the ACA-sevoflurane group (Fig. 2**K,K′ vs. L,L′**; Fig. 3**B)**. This phenomenon proofed the more massive ACA-induced neurodegeneration in the halothane animals. Moreover, it indicated that these neurodegenerative processes persisted longer.

3. Discussion

Using sevoflurane instead of halothane for anesthesia caused some physiological and experimental changes in our asphyxia cardiac arrest rat model whereby criteria defining a successful cardio-pulmonary

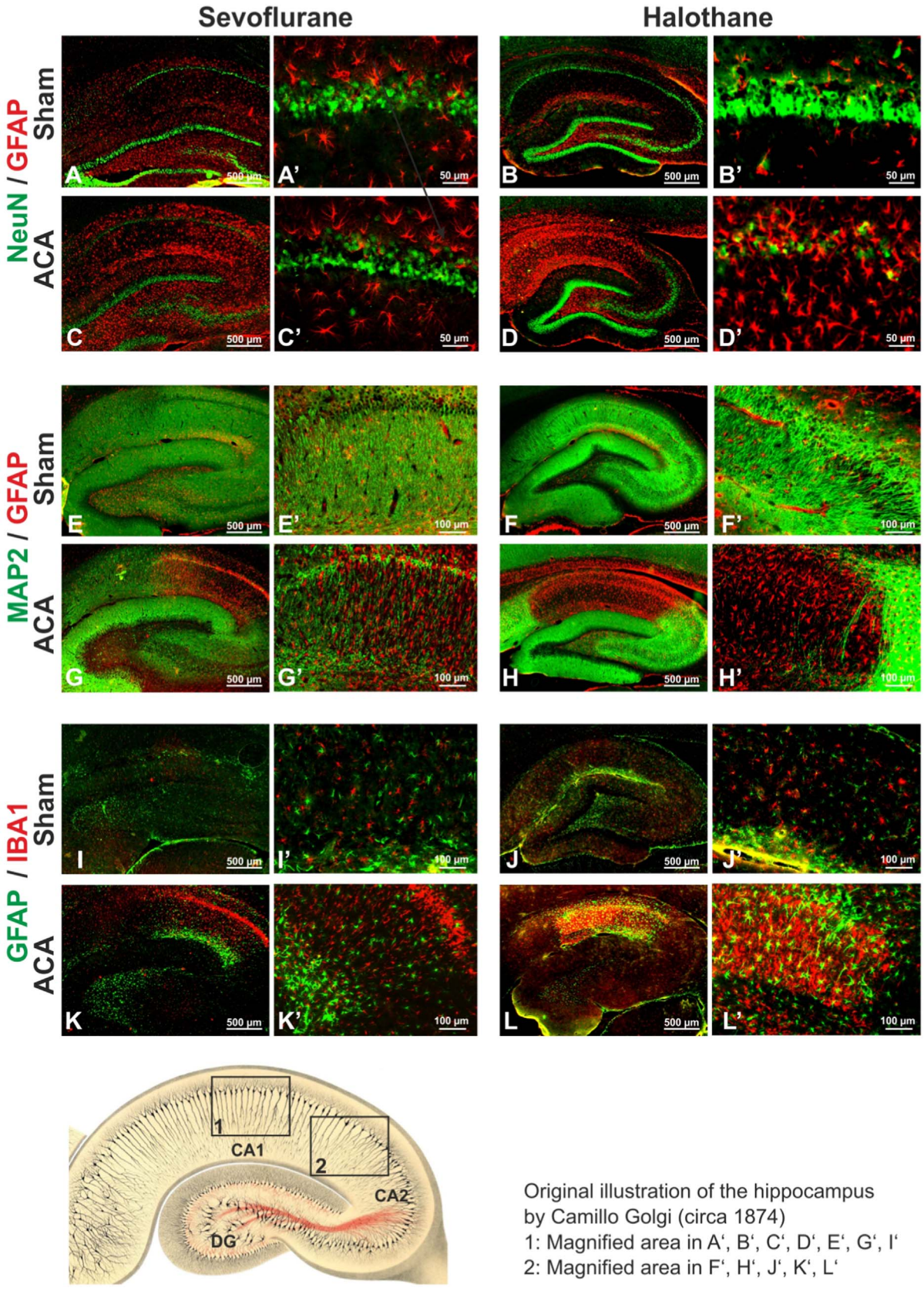

Fig. 2. Representative fluorescence images of hippocampus 7 days post-intervention. Sham-operated animals show a normal pattern of NeuN (green; A, A′, B, B′) and MAP2 (green; E, E′, F, F′) immunostaining. ACA induces a massive degeneration of NeuN-positive CA1 pyramidal cells (green; C, C′, D, D′). Additionally, MAP2 immunostaining reveals the ACA-induced loss of the respective nerve fibers (green; G, G′, H, H′). Note the more intense neurodegenerative signs under halothane (C, C′ vs. D, D′; G, G′ vs. H, H′). Consistently, under halothane a more massive GFAP-expressing astroglial scar is seen (red; C, C′ vs. D, D′; G, G′ vs. H, H′). ACA-induced microglia activation, indicated by IBA1 staining (red), is also more evident under halothane (K, K′ vs. L, L′). Sham-operated animals express regular distribution pattern of GFAP-positive astroglia (red; A, A′, B, B′) and IBA1-positive microglia (red; I, I′, J, J′).

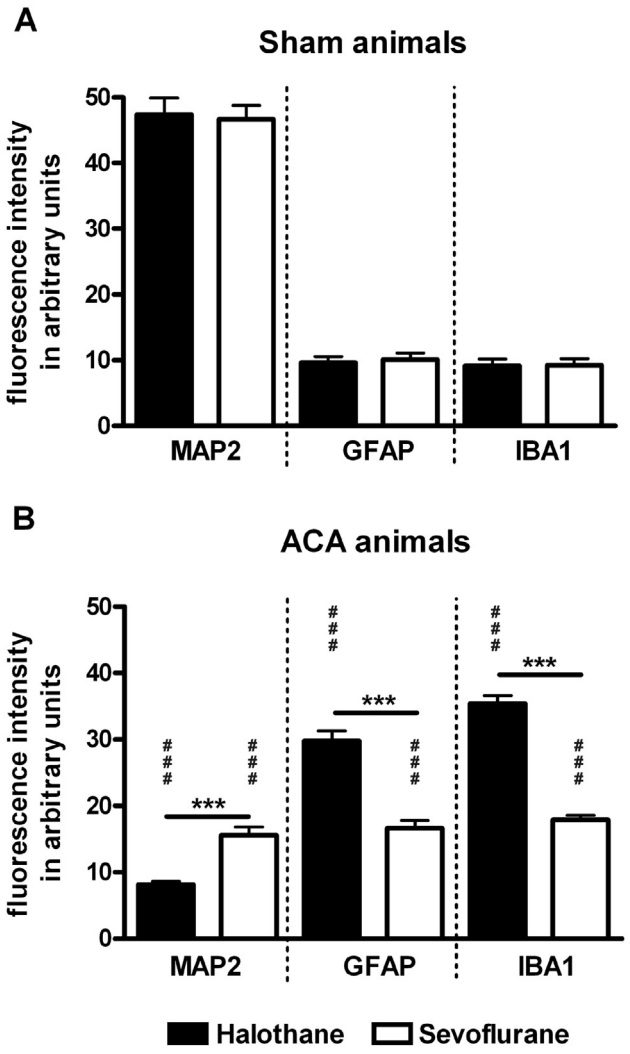

Fig. 3. Quantitative analysis of immunohistochemistry; corresponding stainings of sham and ACA animals (significances indicated by $^{###}p < 0.001$) and of halothane and sevoflurane treated animals (significances indicated by $^{***}p < 0.001$) were compared directly using a paired Student's *t*-test. Data are mean ± SD; n=3 animals/group; for each animal, a mean of 3 slices/staining was entered into the calculation.

resuscitation rat model (high rate of ROSC, distinct/appraisable neuronal damage and a solid reproducibility) were furthermore fulfilled. Because of the lower blood solubility of sevoflurane as compared to halothane the time for washout had to be reduced to maintain sufficient anesthesia once asphyxia is induced. Shorter washout periods lead to increased asphyxia tolerance. Therefor ACA-sevoflurane animals went into cardiac arrest tendendially but not significantly later. This effect is expected to be caused by a higher arterial oxygen partial pressure at the beginning of the asphyxiation period. The reason for the elevated arterial oxygen partial pressure lies in the necessity to reduce the washout for the sevoflurane treated animals by one minute and therefore by a for one minute shortened ventilation time with room air.

The averaged time to establish ROSC was shorter in the ACA-sevoflurane group without significant difference between the groups. The use of sevoflurane causes therefore a slightly delayed onset of cardiac arrest and discreetly reduces resuscitated time. Both effects together should sufficiently explain the partially reduced neurological damage seen in the sevoflurane animals. As seen in the neuron-specific MAP2 and NeuN immunostainings, sevoflurane was able to reduce the ACA-mediated CA1 neuronal cell loss significantly. This lower degree of neurodegeneration was accompanied by a reduced astrogliosis demonstrated by lower GFAP immunostaining intensity as well as by a reduced microglia induction demonstrated by lower IBA1 immunostaining intensity.

Nevertheless the model keeps its validity, because the impairment in the CA1-region of the hippocampus is still detectable. Indeed we want to postulate that marginal neurodegenerative damage extends the range of application and meaningfulness of our model relating to neuroprotective interventions. It can be speculated that a moderate degeneration requires only a moderate intervention. This, however, should be more clinical relevant as a massive intervention which is often characterized by massive side effects.

Another reason for the differences in brain damage between the ACA-groups is the more stable vital parameters in the sevoflurane anesthetized animals. It is well known that halothane has pronounced negative inotropic and chronotropic effects. We have seen these effects in the lower heart rates and lower MAP's in all animals anesthetized with halothane. This anesthesia-mediated differences of the vital parameters were observed in other studies too (Warner et al., 1993). A further negative characteristic of halothane is its ability to influence the cerebral autoregulation (Bode et al., 1994). Accompanied with a decrease in MAP it is possible that the cerebral perfusion pressure might be reduced (Bode et al., 1994).

Another factor is the ischemic preconditioning effect caused by volatile anesthetics. In a study by Warner et al. (1993) it was shown that both halothane and sevoflurane reduced neuronal damage in a focal ischemia model. According to their data halothane was in opposition to our data even more neuroprotective (Warner et al., 1993) as sevoflurane. Maybe these different observations are caused by the different ischemia model (focal (Warner et al., 1993) vs. global ischemia). Moreover, the tetrazolium salt method used by the Warner group is quite rough. In another global ischemia model Payne et al. (2005) demonstrate that preconditioning with sevoflurane can reduce the neuronal damage in the CA1 region of the hippocampus (Payne et al., 2005). The animals were preconditioned for 30 min with sevoflurane once or on 4 consecutive days and were submitted to 7 min of cardiac arrest. In our model the preparation time under anesthesia almost equals this thirty minutes of preconditioning time by Payne. Therefore we consider a similar effect in our model. However it is also to be regarded that early ischemic preconditioning appears to be more effective than late preconditioning.

The positive effect of ischemic preconditioning is well documented. It is also widely accepted that this protective effect is not unique to brain tissue. In a review by Tapuria et al. (2008) it was summarized that ischemic preconditioning is not only effective in the brain but also in renal, heart, intestinal, and other tissues.

The mechanisms of volatile anesthetics mediated ischemic preconditioning is currently under investigation. Related to the brain there are some explanatory approaches. Kehl et al. (2004) have shown in an in-vitro experiment that mitochondrial K_{ATP}-channels play an important role by sevoflurane mediated ischemic preconditioning. By the use of a channel blocking agent the protective effects of sevoflurane was reversed (Kehl et al., 2004). In a review Wang et al. (2008) revealed additional mechanisms beside the K_{ATP}-channels. An important part is the activation of Akt (serine-threonine kinase). This activation can control the balance between survival and death signaling in neuronal cells. Another protective mechanism is related to NO, respectively to the inducible NO-synthase. Some studies showed a reduction in neuronal damage that maybe is related to NO (Hashiguchi et al., 2004; Huang, 2004). In another trial Ye et al. (2012) have demonstrated, that the neuroprotective potential by sevoflurane is referable to inhibition of a mitochondrial permeability transition pore. An inhibiting effect on apoptosis is exerted to be the underlying mechanism. By opening the channel via atractyloside the positive effects of preconditioning with sevoflurane disappeared.

In a model of focal brain ischemia Yang et al. (2012) have shown the neuroprotective effect of sevoflurane. It is indicated that a possible

mechanism for sevoflurane ischemic preconditioning is the Notch signaling pathway.

In our trial we showed that neurological damage followed cardiac arrest was reduced in animals treated with sevoflurane compared to animals treated with halothane. A study by Haelewyn et al. (2003) showed a similar result compared desflurane and halothane. Both volatile anesthetics were neuroprotective, but desflurane causes lesser neuronal damages (Haelewyn et al., 2003). However, the mentioned positive effects of sevoflurane should not tempt into careless handling of this volatile anesthetic. There are reports that sevoflurane is neurotoxic to the developing brain and cause neurobehavioral abnormalities like agitation and delirium (Wang et al., 2013; Xiao et al., 2016; Sun and Guo, 2014). Moreover, there were controversy reports concerning a renal toxicity potential of sevoflurane (Gentz and Malan, 2001; Gallego et al., 2015). Although the harmlessness of sevoflurane in respect to renal and as well as hepatic toxicity is increasingly accepted, sevoflurane should be carefully used in humans with known limited renal function whereby again children bear the highest risk (Jang and Kim, 2005). The particularity of sevoflurane in children (human development) is why sevoflurane should be avoided during the first trimester of pregnancy (Preckel and Bolten, 2005).

4. Conclusion

The transformation of our cardiac arrest model from a halothane to a sevoflurane anesthesia based model was successful. However a few modifications had to be made. Especially the different physiological effects of both substances and the resulting need to adapt particularly the pre-insult phase of this model have to be considered prior further use of this model. The fact that equal asphyxiation periods cause in sevoflurane treated animals a slightly less pronounced neurological damage requires consideration for further research projects. However less pronounced neurological damages in an asphyxia cardiac arrest rat model opens the chance to detect treatment effects that may be destroyed in more severely damaged brains. This opens new opportunities for clinically oriented treatment approaches.

5. Methods and materials

5.1. Asphyxial cardiac arrest model

Ethical approval for this study was granted according to the requirements of the German Animal Welfare Act on the Use of Experimental Animals and the Animal Care and Use Committees of Saxony-Anhalt (permit number 42502-2-2-947 Uni MD). Animals were housed under controlled laboratory conditions (20 ± 2 °C, relative air humidity 55–60%, LD 12:12), with free access to standard diet (Altromin 1326) and tap water. Every effort was made to minimize the amount of suffering and the number of animals used in the experiments.

The ACA rat model was described previously in detail (Adamczyk et al., 2010). Briefly, 34 male Wistar rats (Wistar, inbred, Harlan-Winkelmann; Borchen, Germany) weighing 400–500 g were randomized into four treatment groups: (1) ACA – halothane, 10 animals; (2) ACA – sevoflurane, 10 animals (3) sham operated – halothane (sham-halothane), 7 animals; (4) sham operated – sevoflurane (sham-sevoflurane), 7 animals.

Animals were anesthetized with either 3% halothane or 5% sevoflurane in an oxygen/nitrous oxide mixture (40:60). After endotracheal intubation was established mechanical ventilation was performed with intermittent positive pressure ventilation (IPPV). Both left femoral vessels were cannulated used for drug administration, continuous blood pressure monitoring and blood sampling. Muscular relaxation was achieved with vecuronium bromide (1 mg/kg).

ACA was induced by an end-expiratory interruption of IPPV for 5 min. Cardiac arrest (CA) was established within approximately 3 min. Resuscitation was initiated by the administration of epinephrine (5 µg/kg) and sodium bicarbonate (1 mEq/kg), restarting mechanical ventilation with 100% oxygen, and manual external chest compression (200/min). Return of spontaneous circulation (ROSC) was defined as a pulsatile mean arterial pressure (MAP) above 40 mmHg. Animals without ROSC within 2 min were excluded. During the 45 min post-resuscitation intensive care phase ECG, blood pressure, and temperature were monitored continuously. Arterial blood samples were collected at specific time points and immediately evaluated for blood gases and glucose concentration. Respirator settings were adjusted according to the blood gas values as needed. At the end of the intensive care period the catheters were removed, skin incisions were closed, and the endotracheal tubes were removed after sufficient spontaneous respiration was verified.

5.2. Assessment of hippocampal degeneration

To evaluate hippocampal degeneration, animals were re-anaesthetized and sacrificed by transcardial perfusion (4% 0.1 M phosphate-buffered paraformaldehyde (PFA), Merck, Darmstadt, Germany, pH 7.4). Brains were quickly removed from the cranium, post-fixed in the same fixative at 4 °C overnight, cryoprotected in 30% sucrose in 0.4% PFA (pH 7.4) for 2 days, and rapidly frozen at −20 °C. Serial sagittal sections (20 µm) were cut on a cryostat (Jung Frigocut 2800 E, Leica, Bensheim, Germany) and stained free-floating. Slices were washed 3 times with PBS and nonspecific binding sites were blocked with 10% FCS and 0.3% Triton-X in PBS for 1 h. Afterwards, slices were incubated overnight with the primary antibodies diluted in 10% FCS and 0.3% Triton PBS: polyclonal rabbit anti-GFAP (glial fibrillary acidic protein, astroglia marker, Progen, Heidelberg, Germany; 1:500), monoclonal rabbit anti-MAP2 (neuronal marker, microtubule-associated protein 2, Chemicon, Billerica, USA; 1:2.500); mouse monoclonal anti-NeuN (neuronal nuclei antibody, neuronal marker, Chemicon; 1:100) and rabbit polyclonal anti-IBA1 (ionized calcium binding adaptor molecule 1, microglia marker, Wako Pure Chemicals Industries, Osaka, Japan; 1:1000). This incubation was followed by PBS washing and secondary antibody incubation for 3 h with anti-mouse Alexa 488 (green) and anti-rabbit Alexa 546 (red, Invitrogen, Carlsbad, USA; 1:250) also diluted in 10% FCS and 0.3% Triton in PBS. After washing again with PBS, the slices were embedded with Immu-Mount (Thermo Scientific, Waltham, USA) and inspected by fluorescence microscope AxioImager. M1 (Zeiss, Jena, Germany). Negative controls were performed by substitution of the primary antibody by buffer.

5.3. Statistical analysis

All vital parameters are presented as the mean ± SD. After verifying that the results were normally distributed using a Smirnov test, a student *t*-test was performed to compare group effects. A p value≤0.05 was considered statistically significant.

The stainings were computer-assisted semi-quantitative analyzed using ImageJ software (http:/rsbweb.nih.gov/ij/). Therefore, 3 alternating slices per animal and staining (MAP2, GFAP, IBA1) were scanned in with a Plan-Neofluar objective (×5.0/0.16) getting an image (1388×1040 pixels) representing the complete hippocampal CA1 region, being the most sensitive to/damaged by ischemia brain area, with its adjacent parts. The microscopic settings and the exposure time of the fluorescence channels were set on the basis of control slices and kept equal for the corresponding preparation. The immunostaining intensities were measured in a standard evaluation window (1380×540 pixels) which was placed manually and included the complete CA1 region with all strata. Data are given as mean ± SD. The 3 slices per animal were analyzed individually. The mean was calculated and used as one value for the statistical analyses. Per staining, corresponding sham and ACA animals were compared directly using a paired

Student's *t*-test (GraphPad Software, La Jolla, USA). A p value ≤0.05 was considered statistically significant.

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