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Experimental paper

Hypoxic-ischemic brain injury in pig after cardiac arrest – A new histopathological scoring system for non-specialists

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ABSTRACT

Introduction: After cardiac arrest and successful resuscitation patients often present with hypoxic-ischemic brain injury, which is a major cause of death due to poor neurological outcome. The development of a robust histopathological scoring system for the reliable and easy identification and quantification of hypoxic-ischemic brain injury could lead to a standardization in the evaluation of brain damage. We wanted to establish an easy-to-use neuropathological scoring system to identify and quantify hypoxic-ischemic brain injury.

Methods: The criteria for regular neurons, hypoxic-ischemic brain injury neurons and neurons with ischemic neuronal change (ischemic change neurons) were established in collaboration with specialized neuropathologists. Nine non-specialist examiners performed cell counting using the mentioned criteria in brain tissue samples from a porcine cardiac arrest model. The statistical analyses were performed using the interclass correlation coefficient for counting data and reliability testing.

Results: The inter-rater reliability for regular neurons (ICC 0.68 (0.42 – 0.84; $p < 0.001$) and hypoxic-ischemic brain injury neurons (ICC 0.87 (0.81 – 0.92; $p < 0.001$) showed moderate to excellent correlation while ischemic change neurons showed poor reliability. Excellent results were seen for intra-rater reliability for regular neurons (ICC 0.9 (0.68 – 0.97; $p < 0.001$) and hypoxic-ischemic brain injury neurons (ICC 0.99 (0.83 – 1; $p < 0.001$).

Conclusion: The scoring system provides a reliable method for the discrimination between regular neurons and neurons affected by hypoxic/ischemic injury. This scoring system allows an easy and reliable identification and quantification of hypoxic-ischemic brain injury for non-specialists and offers a standardization to evaluate hypoxic-ischemic brain injury after cardiac arrest.

Introduction

Cardiac arrest, independent of its causes, has a high mortality rate.[1] Despite improved survival rates, the probability for a return of spontaneous circulation (ROSC) after cardiac arrest remains low, with a global rate of about one-third for out-of-hospital cardiac arrest (OHCA).[2] When ROSC is achieved, hypoxic-ischemic brain injury (HIBI) emerges as the primary cause of death[3]. This is often attributed to the withdrawal of care due to an expected poor neurological outcome.[3] It accounts for more than two thirds (73 %) of deaths following OHCA and ROSC.[4]

HIBI is a multifactorial process and the topic of ongoing research. In 2017, Sekhon et al.[5] proposed a "two-hit" model with a primary injury mechanism, also called ischemic injury, which occurs during the cardiac arrest phase. It is caused by the immediate cessation of cerebral blood

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flow due to cardiac arrest.[3,5] The secondary injury, also called reperfusion injury, occurs mainly in the period following cardiac arrest[5]. This phase encompasses the pathophysiological reperfusion processes including the disruptions in calcium homeostasis, immune system activation and tissue inflammation[3,5]. The causal therapy of HIBI is difficult. There are emerging therapeutic approaches, such as targeting metabolic factors and pharmacologic interventions.[6–7] For neuroprognostic purposes, biomarkers[8] and various neuromonitoring instruments[9] can be used in patients with HIBI after cardiac arrest. To evaluate the neuronal damage in an experimental setting, the histopathological evaluation of neurons is a common approach. To especially evaluate HIBI, previous data indicate that cell counting is a valid method.[10]

Traditionally, the death of neurons is classified into passive oncosis and programmed apoptosis. Pyroptosis is a newly described type induced by the inflammasome. Acute ischemia is mainly leading to oncosis (swelling of cells and organelles) and the subsequent necrotic cell death of neurons which is both in contrast to the programmed neuronal cell death such as apoptosis and pyroptosis. Ischemia disrupts homeostasis resulting in cytotoxic oedema, breakdown of the neuronal energy metabolism. Necrotic neurons show cytoplasmic shrinkage, intense eosinophilia and a shrinkage and basophilia of the nucleus. As necrotic neurons are easy to identify in their early stages, HIBI can be exactly quantified in brain sections. As there is a poor neurological outcome and death due to HIBI as well as only limited therapeutic options, we aimed to create and validate a histopathological scoring system that would allow to identify and quantify HIBI after cardiac arrest. Moreover, the scoring system aimed to provide an easily applicable method for non-specialists to be used independently of experienced neuropathologists. This would potentially expand the opportunities for analyses in research groups beyond the neuropathological profession and would refine and standardize the distinct evaluation. For the creation of the scoring system, we collaborated with specialized neuropathologists.

Methods

HIBI-score

In order to be able to quantify regular neurons and neurons which are damaged due to hypoxia (HIBI), we set up a score of precise rating criteria. These criteria should enable an examiner to identify and count regular neurons and HIBI neurons in haematoxylin–eosin-stained brain tissue samples with high accuracy. The criteria were created with the help of experienced neuropathologists (Institute of Neuropathology, University Medical Center Johannes Gutenberg University Mainz, Germany) and are shown in Table 1. Regular neurons are unaffected neurons which do not show any morphological signs of damage. Those regular neurons demonstrate a clearly delimitable round to oval nucleus with a sharp nucleolus and a visibly separated cytoplasm from the surrounding tissue by a smooth membrane (Fig. 1). We employed criteria proposed by Hoque et al.[10] and Stummer et al.[11] with slight modifications to define regular neurons (Table 1). HIBI neurons are neurons which got damaged by hypoxia and reperfusion as suggested by the aforementioned two-hit model.[5] Their nucleolus and nucleus are not clearly delimitable. Furthermore, the nucleus is hyperchromatic (darker than the cytoplasm) and shrunken (pyknotic). The cytoplasm and cell boundary of a HIBI neuron is hypereosinophilic and deformed. Those cells typically become triangular and may show signs of cell homogenization and a shrinkage border (Fig. 1). As we put up the criteria for regular neurons and HIBI neurons, we realized that there is a third category of neurons. These were neurons which in part met both the criteria of regular neurons and of HIBI neurons. They exhibited HIBI criteria, yet the nucleolus remained clearly delimitable (Fig. 1). Consequently, no clear assignment to one of the two categories was possible. Hendrickx et al.[12] described neurons in an intermediate stage and defined them as “ischemic neuronal change (INC) neurons” which are characterized by increased nuclear and cytoplasmic density. We classified those ischemic neuronal change (INC) neurons as ischemic change neurons which made up a third category (Table 1).

Table 1
Criteria for regular neurons, ischemic neuronal change neurons (INC) and hypoxic ischemic brain injury neurons (HIBI).

Regular neurons	1) Nucleolus clearly delimitable 2) Nucleus clearly delimitable, round/oval **and/or** cytoplasm visible and delimitable from the surrounding tissue by a smooth membrane
Ischemic change neurons (neurons with signs of ischemic neuronal change (INC))	Criteria met partly by regular neurons and partly by HIBI neurons, so that no clear assignment to a category is possible. The neurons often show HIBI criteria, but the nucleolus can still be clearly defined.
Neurons with signs of hypoxic ischemic brain injury (HIBI)	1) Nucleolus: not clearly delimitable 2) Nucleus not clearly delimitable **and/or** hyperchromatic (darker than cytoplasm), pyknotic (shrunk) 3) Cytoplasma/cell boundary is deformed: unrounded or triangular **and/or** hypereosinophilic **and/or** signs of cell homogenization, shrinkage border

Sample collection

After setting up the criteria we progressed to validate them. Consequently, we used the brain tissue samples of pigs who underwent cardiac arrest and cardiopulmonary resuscitation (CPR). The trial was approved by the State and Institutional Animal Care Committee Rhineland Palatinate, no. G16-1–042[13]. The trial protocol has been described in detail before.[13–14] In short, piglets, aged between 12 - 16 weeks and weighing between 29 - 34 kg, were administered an anxiolytic medication at a local farm prior to their transfer to our Large Animal Research Facility. Subsequently, they underwent anaesthesia, and standard monitoring was implemented. The airway was secured with an endotracheal tube. They were instrumented with arterial and venous sheaths and catheters. Afterwards, ventricular fibrillation was induced. The no-flow time was 4 or 8 min, respectively, followed by 8 min of basic life support which then was followed by advanced life support (low-flow time). We used different ventilation strategies during CPR. The no– and low-flow times as well as different ventilation strategies lead to variable oxygenation.[13] If a ROSC was reached, the animals were monitored for 22 h and then euthanized using high doses of propofol and potassium chloride. After euthanization, the brain was removed immediately and fixed in a 4 % formaldehyde solution. The brain was sectioned using a pig brain atlas[15] to access the regions of interest: the cornu ammonis (CA) 2 and 3 and the dentate gyrus (DG) of the hippocampus as well as a cortical area next to the corpus callosum (cingulate cortex). The paraffin-embedded tissue was cut into 2–5-µm-thick sections on a microtome, mounted on glass slides, followed by routine haematoxylin–eosin staining (Fig. 2).

Validation

We used nine non-specialists in the field of neuropathology as examiners after a short training course for the histopathological analysis (Table 1 supplemental), who performed cell counting of HE-stained sample images using the mentioned criteria (Table 1). As to our knowledge there was no data available on which we could have based our assumptions for a formal examiner number calculation, we searched the literature for similar experiments. García-Cabezas et al. who conducted a comparable study which included 8 raters was used as a guideline.[16] The sample images were generated by photographing the brain tissue samples using a light microscope (CX43RF, Olympus

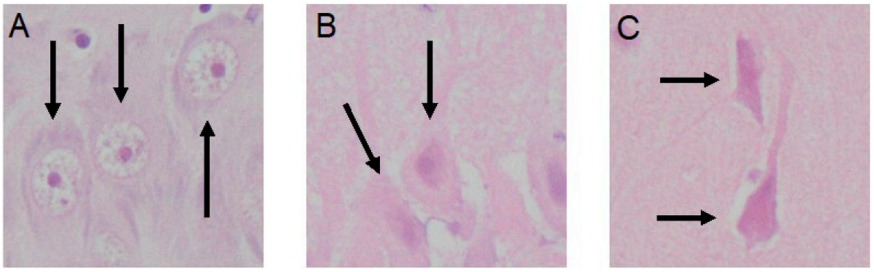

Fig. 1. Haematoxylin-Eosin (HE) staining. Neurons are labeled with an arrow. A: Three regular neurons. B: Two ischemic change neurons. C: Two neurons with hypoxic-ischemic brain injury (HIBI).

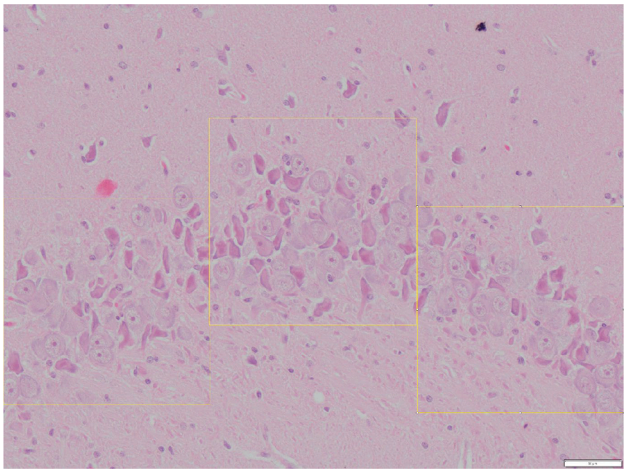

Fig. 2. Sample image within the hippocampus as a region of interest. Neurons are seen in the stratum pyramidale of the cornu ammonis (CA) of the hippocampus (marked with yellow boxes). Haematoxylin-Eosin (HE) staining. Scale bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Cooperation, Tokyo, Japan) and the CellSens Software (CellSens Entry. lnk, creation date 3 December 2018). Lighting conditions were kept constant for all images. The sample images were taken in the three mentioned regions of interest. Each examiner received a set of 8 sample images for each region of interest (CA, DG, cortex), totaling 24 sample images (Fig. 2). Afterwards, cell counting was conducted by each examiner and the inter-rater reliability was assessed. Prior to the cell counting, all nine examiners received an approximately 30-minute introduction explaining the criteria and aiding decisions using standardized images for regular neurons, ischemic change neurons and HIBI neurons as examples. To evaluate the intra-rater reliability, five examiners (randomly chosen from the initial nine) performed a second round of cell counting after a three-month interval. The criteria and the set of 24 sample images remained the same without repeated introduction.

Statistics

The cell-counting results were statistically evaluated via an interclass-correlation coefficient (ICC) for the estimation of reliability, adapted to count data.[17] The ICC serves as a reliability index in the analyses of test–retest, intra-rater and inter-rater reliability[18]. It is suggested that ICC values less than 0.5 indicate poor reliability, values between 0.5 and 0.75 indicate moderate reliability, values between 0.75 and 0.9 indicate good reliability, and values greater than 0.90 indicate excellent reliability.[18]

The ICC (adapted to count data) used the variance components of a generalized mixed model to estimate the ICC. The images were then entered as random effects into the model to account for the repeated assessments. The neuron counting results were assumed to be Poisson distributed; p-values were obtained by a z-approximation using the standard errors of the ICC. The analysis was conducted using R (version 4.2.1,[19]and the R package iccCount[20]. Additionally, the ICC (2,1) (two-way mixed-effect, single rater type, absolute agreement) was based on the proportion of HIBI neurons and compared to the number of regular neurons plus HIBI neurons.[21]

Results

In the first cell counting round we had nine examiners who counted neurons according to the criteria. As a mean, 627.6 regular neurons and 475.2 HIBI neurons (across all images and regions) were counted (Table 2). There were only 40.7 ischemic change neurons identified, which are 3.6 % of all neurons counted. Similar numbers were seen in the second counting round (Table 2).

Our analyses revealed a varying reliability across different neuron types and brain regions during the cell counting assessments. The “overall” category indicates that all three regions of interest are being considered simultaneously.

In the first round of cell counting, inter-rater reliability for regular neurons (overall) was moderate, with an overall ICC of 0.68 (CI 0.42 - 0.84), $p < 0.001$ (Table 3). HIBI neurons (overall) demonstrated good reliability, showing an ICC of 0.87 (CI 0.81 - 0.92), $p < 0.001$ (Table 3). Conversely, ischemic change neurons (overall) exhibited poor reliability, with an ICC of 0.12 (CI −0.02 - 0.24), $p = 0.082$ (Table 3). Notably, the CA region displayed the highest ICC: regular neurons with 0.88 (CI 0.63 - 0.96), $p < 0.001$, and HIBI neurons with 0.93 (CI 0.87 - 0.97), $p < 0.001$ (Table 3) indicating good or even excellent reliability.

In the second round of cell counting, inter-rater reliability for regular neurons (overall) remained at 0.68 (CI 0.41 - 0.84), $p < 0.001$, with HIBI neurons (overall) also showing good reliability with 0.84 (CI 0.74 - 0.91), $p < 0.001$ (Table 3). Regular neurons merely showed a lower ICC in the DG region of 0.59 (CI 0.34 - 0.76), $p < 0.001$ (Table 3), still indicating moderate reliability. Ischemic change neurons continued to demonstrate poor reliability with an ICC of 0.12 (CI 0.05 - 0.29), $p = 0.158$ (Table 3). Once again, the CA region exhibited the highest ICC: regular neurons with 0.98 (CI 0.78 - 1), $p < 0.001$, and HIBI neurons with 0.89 (CI 0.78 - 0.95), $p < 0.001$ (Table 3).

Intra-rater reliability assessments indicated good to excellent reliability for regular neurons and HIBI neurons. The lowest ICC for regular neurons was observed in the Cortex of 0.92 (CI 0.68 - 0.98), $p < 0.001$ (Table 3). Regular neurons (overall) had an ICC of 0.9 (CI 0.68 - 0.97), $p < 0.001$, and HIBI neurons (overall) had an ICC of 0.99 (CI 0.83 - 1), $p < 0.001$ (table 3). Ischemic change neurons consistently showed poor reliability with an ICC (overall) of 0.3 (CI −0.01 - 0.56), $p = 0.045$ and the highest ICC in the CA region with 0.57 (CI −0.09 - 0.88), $p = 0.024$ (Table 3).

Regarding the percentage of HIBI in the total neuron count (regular neurons + HIBI neurons) we excluded ischemic change neurons due to

Table 2
Observations of regular neurons, ischemic change neurons and neurons with hypoxic-ischemic brain injury (HIBI), as well as the % of HIBI versus regular neurons + HIBI, across all images and regions (absolute and relative frequencies) by examination.

Examiner	Regular neurons		Ischemic change neurons		Hypoxic ischemic brain injury neurons (HIBI)		
	n	%	n	%	n	%	% of HIBI vs. regular neurons + HIBI
First examination							
Examiner 1	574	57.8 %	17	1.7 %	402	40.5 %	41.2 %
Examiner 2	873	58.7 %	40	2.7 %	573	38.6 %	39.6 %
Examiner 3	1031	60.5 %	25	1.5 %	647	38.0 %	38.6 %
Examiner 4	598	44.8 %	9	0.7 %	728	54.5 %	54.9 %
Examiner 5	445	58.3 %	27	3.5 %	291	38.1 %	39.5 %
Examiner 6	677	52.1 %	42	3.2 %	581	44.7 %	46.2 %
Examiner 7	567	59.5 %	95	10.0 %	291	30.5 %	33.9 %
Examiner 8	500	52.3 %	69	7.2 %	387	40.5 %	43.6 %
Examiner 9	383	47.8 %	42	5.2 %	377	47.0 %	49.6 %
Mean	627.6	54.9 %	40.7	3.6 %	475.2	41.6 %	43.1 %
Second examination							
Examiner 1	547	56.2 %	17	1.7 %	409	42.0 %	42.8 %
Examiner 4	542	53.1 %	59	5.8 %	419	41.1 %	43.6 %
Examiner 6	650	50.3 %	34	2.6 %	608	47.1 %	48.3 %
Examiner 7	845	53.2 %	16	1.0 %	727	45.8 %	46.2 %
Examiner 8	363	54.0 %	21	3.1 %	288	42.9 %	44.2 %
Mean	589.4	53.1 %	29.4	2.7 %	490.2	44.2 %	45.4 %

Table 3
Inter-rater and Intra-rater reliability by examination and region. Abbreviations: neurons with hypoxic-ischemic brain injury (HIBI), cornu ammonis (CA), dentate gyrus (DG), interclass correlation coefficient (ICC).

	Regular neurons		Ischemic change neurons		Hypoxic ischemic brain injury neurons (HIBI)	
Region	ICC	p-value	ICC	p-value	ICC	p-value
Inter-rater reliability 1st examination (9 examiners)						
CA	0.88 (0.63 - 0.96)	< 0.001	0.2 (−0.08 - 0.45)	0.156	0.93 (0.87 - 0.97)	< 0.001
Cortex	0.66 (0.36 - 0.84)	< 0.001	0.1 (−0.03 - 0.23)	0.141	0.82 (0.67 - 0.91)	< 0.001
DG	0.61 (0.37 - 0.77)	< 0.001	0.12 (−0.07 - 0.31)	0.208	0.84 (0.8 - 0.88)	< 0.001
Overall	0.68 (0.42 - 0.84)	< 0.001	0.12 (−0.02 - 0.24)	0.082	0.87 (0.81 - 0.92)	< 0.001
Inter-rater reliability 2nd examination (5 examiners)						
CA	0.98 (0.78 - 1)	< 0.001	0.17 (−0.1 - 0.41)	0.207	0.79 (0.7 - 0.86)	< 0.001
Cortex	0.81 (0.51 - 0.93)	< 0.001	0.19 (−0.11 - 0.46)	0.197	0.89 (0.78 - 0.95)	< 0.001
DG	0.59 (0.34 - 0.76)	< 0.001	0.2 (−0.14 - 0.5)	0.233	0.83 (0.75 - 0.89)	< 0.001
Overall	0.68 (0.41 - 0.84)	< 0.001	0.12 (−0.05 - 0.29)	0.158	0.84 (0.74 - 0.91)	< 0.001
Intra-rater reliability (5 examiners)						
CA	0.98 (0.77 - 1)	< 0.001	0.57 (−0.09 - 0.88)	0.024	>0.99 (0.69 - 1)	< 0.001
Cortex	0.92 (0.68 - 0.98)	< 0.001	0.28 (−0.05 - 0.55)	0.075	0.99 (0.79 - 1)	< 0.001
DG	0.96 (0.82 - 0.99)	< 0.001	0.37 (−0.08 - 0.69)	0.076	>0.99 (0.87 - 1)	< 0.001
Overall	0.9 (0.68 - 0.97)	< 0.001	0.3 (−0.01 - 0.56)	0.045	0.99 (0.83 - 1)	< 0.001

the low absolute number of ischemic change neurons (Table 2). A good and mainly excellent reliability for regular neurons and HIBI neurons (overall) was found with an ICC (overall) of 0.87 (CI 0.79 - 0.93), $p < 0.001$ (Table 4) in the first round and an ICC (overall) of 0.92 (CI 0.86 - 0.96), $p < 0.001$ (Table 4) in the second round. Intra-rater reliability assessments showed an ICC (overall) of 0.95 (CI 0.93 - 0.96), $p < 0.001$ (Table 4).

Table 4
Inter-rater and Intra-rater reliability of the percentage of HIBI neurons versus RN+HIBI neuron counts i.e., exclusion of ischemic change neurons. Abbreviations: cornu ammonis (CA), dentate gyrus (DG), interclass correlation coefficient (ICC).

Analysis	Region	ICC (95 %-CI)	p
Inter-rater reliability	CA	0.87 (0.72 - 0.97)	< 0.001
1st examination	Cortex	0.82 (0.63 - 0.95)	< 0.001
	DG	0.94 (0.85 - 0.98)	< 0.001
	Overall	0.87 (0.79 - 0.93)	< 0.001
Inter-rater reliability	CA	0.86 (0.68 - 0.96)	< 0.001
2nd examination	Cortex	0.95 (0.88 - 0.99)	< 0.001
	DG	0.96 (0.9 - 0.99)	< 0.001
	Overall	0.92 (0.86 - 0.96)	< 0.001
Intra-rater reliability	CA	0.92 (0.85 - 0.95)	< 0.001
	Cortex	0.98 (0.96 - 0.99)	< 0.001
	DG	0.96 (0.93 - 0.98)	< 0.001
	Overall	0.95 (0.93 - 0.96)	< 0.001

Discussion

To assess the poor neurological outcome and high mortality following HIBI after cardiac arrest and CPR with ROSC,[2–4] we developed a robust scoring system for the histopathological identification and quantification of HIBI neurons and regular neurons by non-specialists. The criteria were established in collaboration with neuropathologists (Table 1), and validation involved nine examiners performing cell counting on haematoxylin–eosin-stained porcine brain tissue samples. The statistical analysis showed moderate/good to excellent reliability for regular neurons and HIBI neurons.

When defining our criteria for regular neurons and HIBI neurons, we leaned on the definition of Hoque et al.[10] and introduced slight adaptations (Table 1). Hoque et al. suggested a quantitative scoring system to evaluate regional and global brain injury in a perinatal and global hypoxia–ischemia pig model.[10] To achieve this, they used a score adapted from Thoresen et al. in which the extent of the regional damage (from individual necrotic neuron to total disintegration of a region of interest) was graded with 0.5-intervals on a 9-step scale[22]. They then correlated the counted number of viable neurons with the

neuropathology score.[10] Our scoring system aims to simply identify regular neurons, ischemic change neurons, and HIBI neurons, to serve investigators with a limited neuropathology expertise. It is designed for any examiner conducting cell counting to identify and quantify HIBI neurons based on our criteria (Table 1). Moreover, no previous experience in microscopy is required.

While our statistical analyses showed a moderate/good to excellent reliability in the category of regular neurons and HIBI neurons, the category of ischemic change neurons only showed a poor reliability (Table 3). Ischemic change neurons or INC neurons were first described by Hendrickx et al.. To our knowledge, the reversibility of ischemic change neurons in this stage is unknown.[12] Hendrickx et al. investigated a rat model of cerebral insult due to asphyxia and CPR. Following several hours of reperfusion lasting up to 24–28 h, an intermediate stage of neurons emerged, characterized by increased density of the nucleus and the cytoplasm.[12] In our scoring system, ischemic change neurons were defined as not meeting the criteria for either regular neurons or HIBI neurons (Table 1). During the validation, both the inter- and intra-rater reliability for ischemic change neurons were found to be poor (Table 3), likely due to the challenge in an accurate identification. Throughout our post-ROSC monitoring period, regular neurons and HIBI neurons were predominant, while ischemic change neurons were fewer. In the first round, one rater counted 9 ischemic change neurons (overall), while another counted 95 (Table 2). This large difference inadvertently lowered the ICC. Given their infrequent occurrence and small proportion of total neurons (Table 2), alongside the aforementioned criteria to identify them (Table 1), it is expected that the reliability was lower (Table 3). However, their clinical relevance still remains unclear according to the available research.

For our scoring system, we validated the neuron criteria in three different regions of interest: CA (CA2 and CA3), DG and cingulate cortex (near the corpus callosum). We selected these regions of interest according to their known and increased sensitivity to hypoxia[3,5,23–24]. Studies using a pig cardiac arrest-model have shown significant HIBI-like changes in the hippocampus, specifically in the CA1, cortex, thalamus and striatum[10,25–26] mirroring the regions of interest in human brains. Following cardiac arrest, HIBI occurs in similar brain regions of pigs and humans.[3,5,24–26] This similarity highlights the potential usefulness of the model for translational studies. The brain of pigs is gyrencephalic and identification of cortical and subcortical structures is enabled.[27–28] Therefore, those post-cardiac arrest models have a very high translational potential.

Högler et al. evaluated 22 brain regions reported to be vulnerable to global ischemia in a pig cardiac arrest-model[25]. They established a semi-quantitative histopathological scoring system: the regions of interest were examined and assigned weighting factor, for example edema (weighting factor 1), eosinophilic neuronal necrosis (weighting factor 2) or infarction (weighting factor 4), and rated on a 5-point scale. The final score for each region of interest was calculated by multiplying the WF for each lesion type with the corresponding WF for extent, considering both hemispheres. They mainly detected neuronal necrosis and edema.[25] To use this score, the examiner must understand complex categories like edema and eosinophilic neuronal necrosis, as a precise identification is essential. In contrast, our score is much easier to use and, as stated before, also usable for any non-specialist. Högler et al. correlated the histological damage with neurological deficits 72 h post-ROSC. Using neurological deficit scores and overall performance categories, they detected a highly significant correlation.[25] While Högler et al. primarily observed neuronal necrosis, which closely resembled the HIBI neurons defined in our scoring system and established a strong correlation between those histopathological findings and neurological deficit scores, it is plausible to infer that our results may also be applicable to the neurological outcomes of our experimental animals. Unfortunately, in our setting, clinical testing of neurological outcome was not possible due to local regulations and limited observation capabilities. Högler et al. examined their animals 72 h post-ROSC.[25] It is important to consider that our post-ROSC monitoring period was only 22 h. The correlation between histopathology and clinical testing was not only examined by Högler et al.. Another porcine cardiac arrest-model also demonstrated, that histopathological HIBI-like changes are associated with pathological neurocognitive testing[26]. Moreover, in humans who died due to cardiac arrest a correlation between the histopathological evaluation of hypoxic-ischemic encephalopathy (evaluated according to the selective eosinophilic neuronal death classification) and current methods for assessing neurological outcomes (such as brain CT imaging or serum Neuron-specific enolase levels) has also been observed.[29]

When developing such an evaluation system, the question naturally arises as to the context in which it could be applied. Our histopathological scoring system could be utilized in experimental settings using animal models or for the post-mortem assessment of human patients to identify and quantify HIBI following cardiac arrest. Our scoring system could also serve as a suitable alternative to neurological assessments, especially in experimental settings where such assessments may not be feasible It provides valuable information about the extent of HIBI neurons and could offer insights into the anticipated neurological outcome. Our score has the great potential to simplify the evaluation of HIBI in experimental settings in critical care research without the requirement for specialized neuropathological expertise if access to those specialties is limited.

Conclusion

We successfully compiled precise criteria for the identification of regular neurons, HIBI neurons and neurons with ischemic neuronal change into an easy-to-use score for non-professional examiners. To detect HIBI neurons our score showed good to excellent reliability and could be used in experimental settings to identify and quantify HIBI neurons while assessing novel therapeutic options for the treatment of HIBI. This scoring approach has the potential to streamline the analysis of HIBI in various critical care or resuscitation research projects, especially when access to neuropathologic expertise is restricted or limited by local availability.

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CRediT authorship contribution statement

Miriam Renz: Writing – original draft, Resources, Investigation, Formal analysis, Data curation, Conceptualization. **Pascal Siegert:** Investigation, Funding acquisition, Data curation, Conceptualization. **Roman Paul:** Writing – review & editing, Visualization, Formal analysis, Data curation. **Adina Lepadatu:** Investigation, Data curation. **Petra Leukel:** Investigation, Data curation. **Katrin Frauenknecht:** Writing – review & editing, Supervision, Methodology, Investigation, Data curation. **Andrea Urmann:** Investigation, Data curation. **Johanna Hain:** Investigation, Data curation. **Katja Mohnke:** Investigation, Data curation. **Alexander Ziebart:** Supervision, Resources, Project administration. **Anja Harder:** Writing – review & editing, Supervision, Methodology, Investigation, Data curation, Conceptualization. **Robert Ruemmler:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

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Parts of this study will be used in the doctoral thesis of Pascal Siegert.

Artificial intelligence was partly used for an optimized translation from German to the English language but was not used to substantially alter the content of this manuscript.

Ethical statement:

The trial was approved by the State and Institutional Animal Care Committee of Rhineland Palatinate, Germany, approval no. G16-1-042. All procedures performed in the described studies involving animals were in accordance with the local ethical standards and the ARRIVE guidelines (supplementary material).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.resplu.2024.100779.

References

1. Gräsner JT, W.J., Herlitz J, Perkins GD, Lefering R, Tjelmeland I, Koster RW, Masterson S, Rossell-Ortiz F, Maurer H, Böttiger BW, Moertl M, Mols P, Alihodžić H, Hadžibegović I, Ioannides M, Truhlář A, Wissenberg M, Salo A, Escutnaire J, Nikolaou N, Nagy E, Jonsson BS, Wright P, Semeraro F, Clarens C, Beesems S, Cebula G, Correia VH, Cimpoesu D, Raffay V, Trenkler S, Markota A, Strömsöe A, Burkart R, Booth S, Bossaert L. Survival after out-of-hospital cardiac arrest in Europe - Results of the EuReCa TWO study. *Resuscitation*. 2020;148:218–226.
2. Yan S, G.Y., Jiang N, Wang R, Chen Y, Luo Z, Zong Q, Chen S, Lv C. The global survival rate among adult out-of-hospital cardiac arrest patients who received cardiopulmonary resuscitation: a systematic review and meta-analysis. *Crit Care*. 2020;24(1):61.
3. Sandroni C, C.T., Sekhon M. Brain injury after cardiac arrest: Pathophysiology, treatment, and prognosis. *Intensive Care Med*. 2021;47(12):1393–1414.
4. Witten L, G.R., Holmberg MJ, Wiberg S, Moskowitz A, Mehta S, Grossestreuer AV, Yankama T, Donnino MW, Berg KM. Reasons for death in patients successfully resuscitated from out-of-hospital and in-hospital cardiac arrest. *Resuscitation*. 2019;136:93–99.
5. Sekhon MS, A.P., Griesdale DE. Clinical pathophysiology of hypoxic ischemic brain injury after cardiac arrest: a "two-hit" model. *Crit Care*. 2017;21(1):90.
6. Hosseini M, W.R., Crouzet C, Amirhekmat A, Wei KS, Akbari Y. Resuscitating the Globally Ischemic Brain: TTM and Beyond. *Neurotherapeutics*. 2020;17(2):539–562.
7. Sekhon MS, G.d.. Individualized perfusion targets in hypoxic ischemic brain injury after cardiac arrest. *Crit Care*. 2017;21(1):259.
8. Hoiland RL, R.K., Thiara S, Fordyce C, Kramer AH, Skrifvars MB, Wellington CL, Griesdale DE, Fergusson NA, Sekhon MS. Neurologic Prognostication After Cardiac Arrest Using Brain Biomarkers: A Systematic Review and Meta-analysis. *JAMA Neurol*. 2022;79(4):390–398.
9. Sandroni C, S.M., Taccone FS. Brain monitoring after cardiac arrest. *Curr Opin Crit Care*. 2023;29(2):68–74.
10. Hoque N, S.H., Maes E, Bishop S, Thoresen M. Validation of a neuropathology score using quantitative methods to evaluate brain injury in a pig model of hypoxia ischaemia. *J Neurosci Methods*. 2014;230:30–36.
11. Stummer W, W.K., Tranmer B, Baethmann A, Kempski O. Reduced mortality and brain damage after locomotor activity in gerbil forebrain ischemia. *Stroke*. 1994;25(9):1862–1869.
12. Hendrickx HH, R.G., Safar P, Gisvold SE. Asphyxia, cardiac arrest and resuscitation in rats. I. Short term recovery. *Resuscitation*. 1984;12(2):97–116.
13. Ruemmler R, Ziebart A, Moellmann C, et al. Ultra-low tidal volume ventilation—A novel and effective ventilation strategy during experimental cardiopulmonary resuscitation. *Resuscitation*. 2018;132:56–62. https://doi.org/10.1016/j.resuscitation.2018.08.031.
14. Mohnke K, Conzelmann P, Renz M, et al. Ultra-low tidal volume ventilation during cardiopulmonary resuscitation shows no mitigating effect on pulmonary end-organ damage compared to standard ventilation: insights from a porcine model. *Intensive Care Med Exp*. 2023;11(1):81. https://doi.org/10.1186/s40635-023-00568-6.
15. Félix B, Léger ME, Albe-Fessard D, Marcilloux JC, Rampin O, Laplace JP. Stereotaxic atlas of the pig brain. *Brain Res Bull*. 1999;49(1–2):1–137. https://doi.org/10.1016/s0361-9230(99)00012-x. PMID: 10466025.
16. García-Cabezas MÁ, J.Y., Barbas H, Zikopoulos B. Distinction of Neurons, Glia and Endothelial Cells in the Cerebral Cortex: An Algorithm Based on Cytological Features. *Front Neuroanat*. 2016;10:107. https://doi.org/10.3389/fnana.2016.00107. PMID: 27847469; PMCID: PMC5088408.
17. Carrasco J. A generalized concordance correlation coefficient based on the variance components generalized linear mixed models for overdispersed count data. *Biometrics*. 2010;66(3):897–904.
18. Koo TK, L.m.. A Guideline of Selecting and Reporting Intraclass Correlation Coefficients for Reliability Research. *J Chiropr Med*. 2016;15(2):155–163.
19. R Core Team, *R: A language and environment for statistical computing. R Foundation for Statistical Computing*.-. Vienna, Austria., 2022. https://www.R-project.org/.
20. Carrasco JL, iccCounts. An R Package to Estimate the Intraclass Correlation Coefficient for Assessing Agreement with Count Data. *The R Journal*.; 2022. https://journal.r-project.org/articles/RJ-2022-034/.
21. Shrout PE, F.j.. Intraclass correlations: uses in assessing rater reliability. *Psychol Bull*. 1979;86(2):420–428.
22. Thoresen M, H.K., Løberg EM, Whitelaw A, Apricena F, Hankø E, Steen PA. A piglet survival model of posthypoxic encephalopathy. *Pediatr Res*. 1996;40(5):738–748.
23. Björklund E, L.E., Rundgren M, Cronberg T, Friberg H, Englund E. Ischaemic brain damage after cardiac arrest and induced hypothermia–a systematic description of selective eosinophilic neuronal death. A neuropathologic study of 23 patients. *Resuscitation*. 2014;85(4):527–532.
24. Nolan JP, N.R., Adrie C, Aibiki M, Berg RA, Böttiger BW, Callaway C, Clark RS, Geocadin RG, Jauch EC, Kern KB, Laurent I, Longstreth WT, Merchant RM, Morley P, Morrison LJ, Nadkarni V, Peberdy MA, Rivers EP, Rodriguez-Nunez A, Sellke FW, Spaulding C, Sunde K, Hoek TV. , *Post-cardiac arrest syndrome: epidemiology, pathophysiology, treatment, and prognostication. A Scientific Statement from the International Liaison Committee on Resuscitation; the American Heart Association Emergency Cardiovascular Care Committee; the Council on Cardiovascular Surgery and Anesthesia; the Council on Cardiopulmonary, Perioperative, and Critical Care; the Council on Clinical Cardiology; the Council on Stroke.* Resuscitation, 2008. **79(3):350-79**.
25. Högler S, Sterz F, Sipos W, et al. Distribution of neuropathological lesions in pig brains after different durations of cardiac arrest. *Resuscitation*. 2010;81(11):1577–1583. https://doi.org/10.1016/j.resuscitation.2010.07.005. Epub 2010 Aug 19.
26. Fries M, N.K., Demir F, Kottmann K, Timper A, Coburn M, Weis J, Rossaint R. Neurocognitive performance after cardiopulmonary resuscitation in pigs. *Crit Care Med*. 2008;36(3):842–847.
27. Lind NM, M.A., Jelsing J, Vajta G, Cumming P, Hansen AK. The use of pigs in neuroscience: modeling brain disorders. *Neurosci Biobehav Rev*. 2007;31(5):728–751. https://doi.org/10.1016/j.neubiorev.2007.02.003. Epub 2007 Mar 4. PMID: 17445892.
28. Swindle MM, M.A., Herron AJ, Clubb FJ Jr, Frazier KS. , *Swine as models in biomedical research and toxicology testing.* Vet Pathol., 2021. **49(2):344-56. doi: 10.1177/0300985811402846. Epub 2011 Mar 25. Erratum in: Vet Pathol. 2012 Jul;49(4):738. PMID: 21441112.**
29. Endisch C, W.E., Kenda M, Streitberger KJ, Kirkegaard H, Stenzel W, Storm C, Ploner CJ, Cronberg T, Friberg H, Englund E, Leithner C. Hypoxic-Ischemic Encephalopathy Evaluated by Brain Autopsy and Neuroprognostication After Cardiac Arrest. *JAMA Neurol*. 2020;77(11):1430–1439.