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Are slaughterhouse-obtained livers suitable for use in *ex vivo* perfusion research?

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Abstract

Objectives: The success of the *ex vivo* machine perfusion of pig livers used for preclinical research depends on organ quality and availability. In this study, we investigated whether livers obtained from slaughterhouses are suitable and equivalent to livers obtained from laboratory pigs.

Methods: Livers were obtained from slaughterhouse pigs stunned by electrocution or CO_2 inhalation and from laboratory pigs. For the latter group, 45 minutes of warm ischemia was mimicked for a subgroup, ensuring a valid comparison with slaughterhouse-derived livers.

Results: Livers from CO_2-stunned pigs showed lower indocyanine green clearance and bile production, higher blood lactate and potassium concentrations, and higher alanine aminotransferase activities than electrically stunned pigs. Furthermore, livers from electrically stunned pigs, and livers from laboratory pigs, subjected or not to warm ischemia, showed similar performance in terms of perfusion and metabolism.

Conclusion: For an *ex vivo* liver model generated using slaughterhouse pigs, electrical stunning is preferable to CO_2 stunning. Livers from electrically stunned slaughterhouse pigs performed similarly to laboratory pig livers. These findings support the use of livers from electrically stunned slaughterhouse pigs, which may therefore provide an alternative to livers obtained from laboratory pigs, consistent with the principle of the 3Rs.

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Introduction

Ex vivo normothermic machine perfusion (NMP) maintains a perfused organ in a viable state, improves its preservation *versus* cold storage, and might even be suitable for the repair of organs rejected for transplantation.[1–4] Thereby, NMP is not only important for transplantation research, but also as model for preclinical research purposes.[5] Reliable *ex vivo* models are of great importance for the bridging of the gap between bench testing and *in vivo* modeling for the assessment of potential novel therapies.[6] The ability to directly control the experimental parameters and monitor effects in real time renders *ex vivo* organ perfusion a valuable preclinical model for use in intervention studies.[7]

Here, the use of porcine livers obtained from the slaughterhouse as an *ex vivo* liver perfusion model for research purposes that is compliant with the 3Rs principle (i.e., replacement, reduction, and refinement) was investigated. The 3Rs principle guides the selection of methods for use in experiments that do not involve the use of animals, minimize their use, or involve less painful procedures.[8] Today, in addition to laboratory animal-derived organs,[9–12] slaughterhouse-derived organs are increasingly used in perfusion research,[5,13–16] but standardized protocols for organ harvesting and the slaughter of the animals have not been established. The fixed processes used in slaughterhouses present a challenging environment for organ sampling, because the infusion of substances such as heparin prior to slaughter is not permitted, which increases the risk of intrahepatic thrombosis. Moreover, organ collection in this context is subject to prolonged periods of warm ischemia, as well as prolonged exposure to a non-sterile environment, which is not the case for laboratory animals. In addition, the stunning technique used might have a substantial impact on the quality of the organ and its subsequent perfusion.

In Europe, the most commonly used stunning techniques are CO_2 stunning and electrical stunning.[17] CO_2 stunning has the major advantage of being suitable for use with groups of animals, but is also known to significantly affect the balance of blood gases and chemicals in pigs.[18] Electrical stunning also affects blood gases, but blood chemicals are affected to a lesser extent.[18,19] Therefore, we aimed to study how these stunning techniques affect liver function during *ex vivo* perfusion, in order to optimize the utility of slaughterhouse-derived livers. The second aim was to compare livers obtained from slaughterhouses with those obtained from laboratory pigs to investigate whether the former represents a suitable alternative to the latter, despite the challenges posed by the slaughterhouse processes. Finally, to ensure that we compared equivalent situations, and to assess the influence on liver function of warm ischemia time (WIT), which is inherent to slaughterhouses, we mimicked a WIT of 45 minutes in livers from laboratory pigs.

Methods

Liver and blood procurement

Livers were harvested from clinically healthy Dutch Landrace Hybrid pigs in

two local slaughterhouses and from Norsvin Landrace laboratory pigs. Permission was not required from an Ethics committee, because the livers were harvested from slaughterhouse pigs that had been exsanguinated for human consumption. The protocols were consistent with EC regulations 1069 and 2009 regarding the use of slaughterhouse material for diagnosis and research, as supervised by the Dutch Government (Dutch Ministry of Agriculture, Nature, and Food Quality) and were approved by the associated legal authorities for animal welfare (Food and Consumer Product Safety Authority). Furthermore, livers were obtained from fresh cadavers of surplus pathogen-free laboratory pigs (surplus material, UMCU, 3Rs policy) that had been used in non-liver related research (approval numbers: AVD1150020209706, AVD11500201 72624, and AVD1150020209965). All the personnel involved were trained according to EU Directive 2010/63/EU regarding the protection of animals used for scientific purposes and had adequate knowledge regarding appropriate hygiene measures for the prevention of zoonosis.

The pigs were 4-to-6 months old and had a mean body mass of 80 to 100 kg. The pigs were stunned with CO_2 (>90% CO_2 for 15 seconds) in one slaughterhouse (SH_gas, n = 6) and head-to-body electrically stunned (1.3 A for 10 seconds) in the other slaughterhouse (SH_elec, n = 6). Dictated by the slaughter process, livers from CO_2-stunned pigs could only be harvested after a WIT of approximately 45 minutes, while livers from electrically stunned pigs were harvested after a WIT of a maximum of 20 minutes. The complete gastrointestinal tract was excised from each of the pigs, and the liver was then isolated. The livers were cannulated and cold-flushed (4°C) with 2 L of heparinized (5,000 U/L, LEO Pharma, Amsterdam, The Netherlands) Custodiol® solution (Dr. Franz Köhler Chemie GmbH, Bensheim, Germany) *via* the portal vein (PV).[14]

Laboratory animals were sedated with ketamine (10 mg/kg), midazolam (0.4 mg/kg), and atropine (0.05 mg/kg), and euthanized with potassium (LB, n = 4). Some livers were obtained from surplus animals to study the effect of a WIT of 45 minutes (WI45, n = 6). The livers were cannulated and cold-flushed *in situ* immediately after euthanasia, then excised. WI45 livers were prepared in the same manner, but only flushed after they had undergone a WIT of 45 minutes at 37°C outside the body.

For all livers obtained during this study, a perfusate of 3 L of blood was collected from electrically stunned donor pigs and supplemented with 5,000 U/L heparin (LEO Pharma) and 50 mg/L gentamicin (Carl Roth, Karlsruhe, Germany). The livers were transported in Custodiol® solution at 4°C. The cold storage time was a maximum of 2 hours.

Reperfusion

The total perfusion volume was 3.5 L, and was composed of 0.7 L priming solution and 2.8 L heparinized blood. The perfusion circuit (Figure 1) consisted of an organ receptacle, which also functioned as the main blood reservoir, a centrifugal pump (Biomedicus 550, Dublin, Ireland), an arterial filter (38 µm, Medtronic, Dublin, Ireland), and an oxygenator with an integrated heat exchanger (Quadrox-ID, Maquet, Getinge Group, Rastatt, Germany). The pressure (Merit Medical Systems, Pressure Transducer, South Jordan, UT, USA) and flow rate (SonoTT™ Clamp-On Transducer, em-tec, Lerchenberg, Germany) were measured at the inlets of the hepatic artery (HA) and PV. The liver was connected as described previously.[16] The gas provided to the oxygenator was composed of a mixture of O_2 (95% O_2, 5% CO_2) and N_2 (95% N_2, 5% CO_2) in a 1:3 ratio. The oxygen saturation of the blood

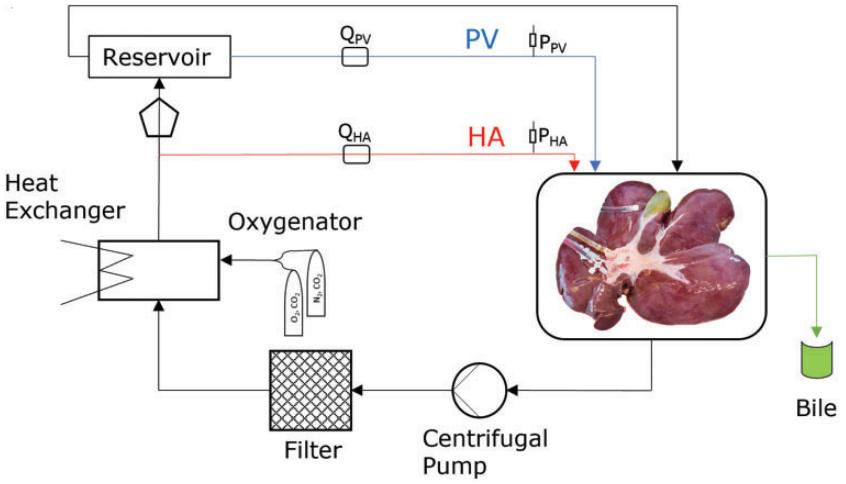

Figure 1. Schematic representation of the perfusion circuit. PV, portal vein; HA, hepatic artery; Q, flow sensor; P, pressure sensor.

was maintained at >95%. At the beginning of the reperfusion, the pressures were set to 30 mmHg and 5 mmHg for the HA and PV, respectively. Over the 30 minutes of rewarming, the pressures were increased gradually until they reached the physiological levels of 80 mmHg and 8 mmHg for the HA and PV, respectively. After these values were reached (T0), the livers were perfused for 5 hours at 39°C.

Analysis

Gas parameters were measured in blood samples obtained after 0, 60, 180, and 300 minutes using a VetScan i-Stat 1 (CG4+, Chem8+, Abaxis, Union City, CA, USA), and used to maintain the pH (7.40 ± 0.4) and ionized calcium (1.05 to 1.3 mmol/L) levels by adding bicarbonate or calcium, respectively. The pressures and flow rates in the HA and PV were recorded continuously. Blood samples were centrifuged (15 minutes, $2{,}300 \times g$) and the plasma samples obtained were stored at −80°C until further analysis. Bile was collected continuously and weighed at the end of the period of perfusion. The albumin concentrations (Albumin assay kit, 124CA02A2, Sigma-Aldrich, St. Louis, MO, USA) and alanine aminotransferase (ALT) activities (700260, Cayman Chemical, Ann Arbor, MI, USA) were measured in plasma samples, according to the manufacturers' instructions. The livers were weighed using a Quintix 5101 balance (Sartorius AG, Göttigen, Germany) (Table S1). To analyze liver functionality, indocyanine green (ICG) 5 mg/L (7695-2, Carl Roth, Karlsruhe, Germany) was added to the perfusate at the −15 and 285-minute time points and the distributions were analyzed as described previously.[16] Tissue samples were obtained using a biopsy punch (8 mm diameter, Megro GmbH & Co. KG, Wesel, Germany) for histologic evaluation following cold storage and 5 hours of perfusion. As a control, tissue samples were also obtained immediately after the harvesting of the livers. The samples were further processed as described previously.[16]

Histologic evaluation was performed by a board-certified veterinary pathologist.

Data analysis

Statistical analysis was performed using GraphPad Prism v.9 (San Diego, CA, USA). Liver functionality was compared using two-way ANOVA, followed by Tukey's *post hoc* test, with specific reference to the effects of the stunning technique, the liver source, and the WIT over time. Bile production was analyzed using a two-tailed unpaired *t*-test. Differences were considered to be statistically significant when $p < 0.05$.

Results

Blood chemistry analysis showed that livers procured after CO_2 stunning had poorer functionality. The plasma lactate concentrations increased significantly from 7.0 ± 3.1 (T0) to 12.01 ± 6.6 mmol/L ($p = 0.0346$) (T300), whereas SH_elec was associated with a low lactate concentration throughout the perfusion (1.2 ± 1.0 mmol/L (T300)) (Figure 2a). The blood glucose concentrations decreased in SH_elec from 17.3 ± 11.3 (T0) to 6.5 ± 3.4 mmol/L (T300), whereas those of SH_gas were initially higher (24.0 ± 9.9 mmol/L (T0)) and only slowly decreased over the 5 hours of perfusion (to 15.8 ± 11.1 mmol/L (T300)) (Figure 2b).

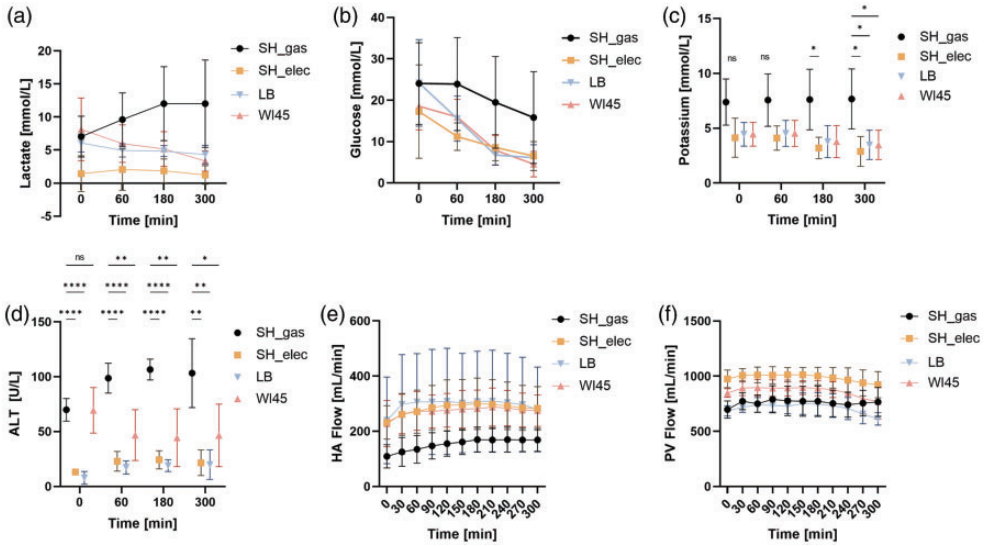

Figure 2. Blood chemistry for *ex vivo* perfused livers procured from CO_2- and electrically stunned pigs and from laboratory pigs. (a) Blood lactate concentrations over the 5 hours of perfusion. These were higher for SH_gas, as were the (b) plasma glucose and (c) potassium concentrations, *versus* SH_elec, LB, and WI45. (d) ALT activities, which were significantly higher for SH_gas from the beginning of the perfusion period. The ALT activities of SH_elec and LB increased from T0 to T300, but stayed within the reference range. (e) HA flow and (f) PV flow during the 5 hours of liver perfusion. Data are reported as mean ± s.d. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$; ns, non-significant. SH_gas, livers from pigs stunned with CO_2 gas; SH_elec, livers from pigs that were electrically stunned; LB, laboratory pig livers; WI45, laboratory pig livers subjected to warm ischemia for 45 minutes; ALT, alanine aminotransferase; HA, hepatic artery; PV, portal vein.

The potassium concentrations in the perfusate were higher in SH_gas than in SH_elec throughout the perfusion (T180, $p = 0.036$; T300, $p = 0.024$) (Figure 2c). Hepatocellular injury was assessed by measuring ALT activity, and in SH_gas, this increased from 69 ± 10.0 (T0) to 103.3 ± 31.0 U/L (T300), which was approximately five times higher than in SH_elec, in which it increased from 13.2 ± 2.0 (T0) to 21.6 ± 12.0 U/L (T300) (Figure 2d). Furthermore, the ALT activities of SH_elec remained within the reference range of 7 to 70 U/L.[20] The flows through the HA in livers from CO_2-stunned pigs were slower (152.5 ± 20 mL/minute) than those in the other groups (SH_elec: 281.6 ± 20.1 mL/minute, LB: 272.6 ± 16.3 mL/minute, WI45: 272.6 ± 15.5 mL/minute), whereas a physiological PV flow was achieved in all the livers (Figure 2e, f). Marginally lower bile production was identified in SH_gas (6.6 ± 3.4 g/hour) than in SH_elec (11.5 ± 7.5 g/hour) (Figure 3a). The half-life of the ICG significantly increased from 9.8 ± 4.4 (T0) to 18.7 ± 5.0 min ($p = 0.0027$) (T300) in SH_gas (Figure 3b), which was consistent with the decrease in functionality.[21] The plasma albumin concentrations were maintained in both groups over the 5 hours of perfusion (Figure 3c).

Tissue integrity was analyzed using hematoxylin and eosin staining. Although biochemical and plasma protein analysis indicated good viability and functionality for livers of the SH_elec group, the histological findings indicated tissue damage, including congestion and mild leukocytosis, in both groups (Figure 4a, b). Most importantly, prominent portal and parenchymal disruption was noted for SH_gas, and prominent portal and mild parenchymal disruption was noted for SH_elec at the end of the perfusion period. Mild sinusoidal leukocytosis was already visible in samples obtained immediately after the harvesting of the livers in both slaughterhouses. In addition, early apoptosis/necrosis was apparent in samples from both groups, identified on the basis of eosinophilia of the cytoplasm and karyopyknosis and karyorrhexis of the hepatocytes on hematoxylin and eosin-stained sections. We did not perform additional testing, such as immunohistochemistry for caspase-3 or a TUNEL assay to differentiate between these two types of cell death.

Livers from SH_elec and LB pigs showed similar viability and functionality. The blood lactate concentrations of SH_elec (1.2 ± 1.4 mmol/L (T300)) were lower than

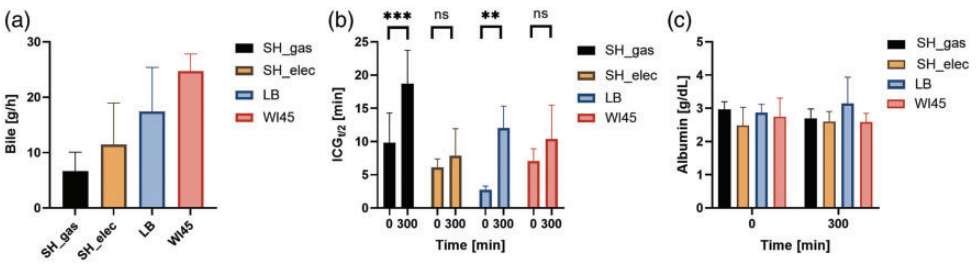

Figure 3. Functionality of livers obtained from slaughterhouse and laboratory pigs. (a) Mean bile production by SH_gas, SH_elec, LB, and WI45 at the end of perfusion. (b) Indocyanine green half-life. Those for SH_gas and LB had significantly increased by T300, but those for SH_elec and WI45 had not and (c) Plasma albumin concentrations were maintained in all the groups over 5 hours. Data reported as mean ± s.d. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$; ns, non-significant. SH_gas, livers from pigs stunned with CO_2 gas; SH_elec, livers from pigs that were electrically stunned; LB, livers from laboratory pigs; WI45, double laboratory pig livers subjected to warm ischemia for 45 minutes.

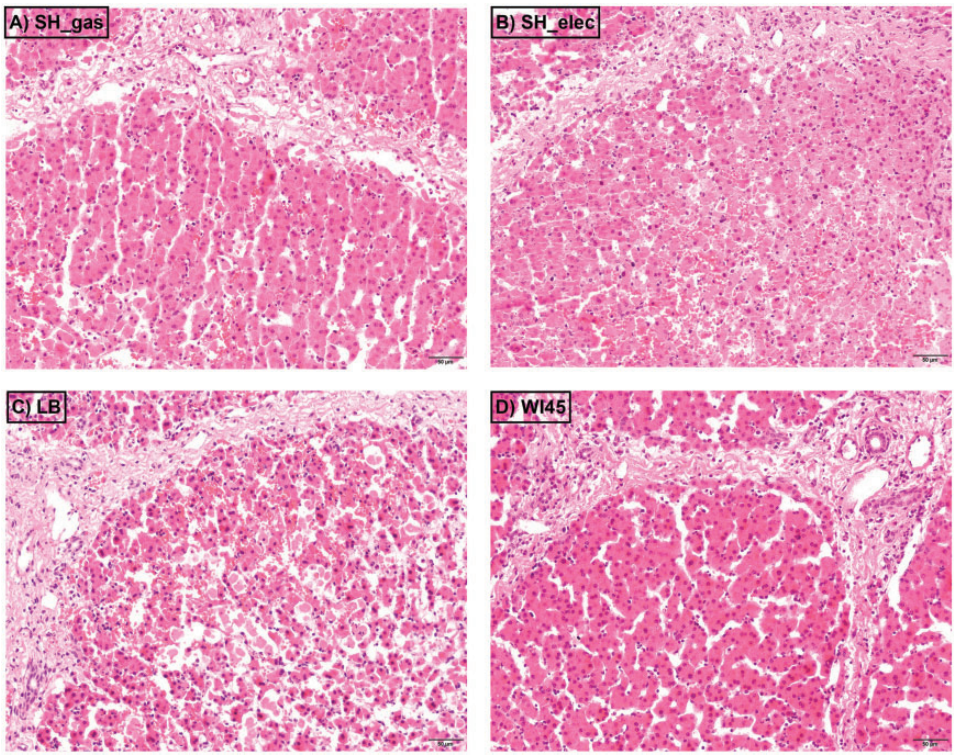

Figure 4. Histology of liver samples after 5 hours of perfusion. Representative images of hematoxylin and eosin-stained sections, showing liver damage in (a) SH_gas, (b) SH_elec, (c) LB, and (d) WI45. Scale bars represent 50 μm. SH_gas, livers from pigs stunned with CO_2 gas; SH_elec, livers from pigs that were electrically stunned; LB, livers from laboratory pigs; WI45, double laboratory pig livers subjected to warm ischemia for 45 minutes.

those of LB at the end of the perfusion period (4.3 ± 1.4 mmol/L (T300)) (Figure 2a). The plasma glucose concentrations of LB decreased to 6.1 ± 3.1 mmol/L (T300), which was comparable to those of SH_elec (6.5 ± 3.5 mmol/L (T300)) (Figure 2b). Bile production during the perfusion was similar (17.4 ± 8.0 vs 11.5 ± 7.5 g/hour in LB and SH_elec, respectively) (Figure 3a). Interestingly, LB showed an increase in ICG half-life from 2.7 ± 0.5 (T0) to 12.0 ± 3.3 minute (T300), which did not occur in SH_elec (Figure 3b). On histologic analysis, LB also showed mild leukocytosis at T0 and T300. In addition, some tissue congestion was present, indicating impaired outflow from the tissue in LB, and disruption of the tissue architecture was visible (Figure 4c).

To determine whether the slaughter procedure or the WIT alone resulted in significant changes in the viability or functionality of the livers, we mimicked a WIT of 45 minutes using surplus livers obtained from laboratory animals, which allowed us to compare the results with those for livers that did not undergo WIT. Overall, blood chemistry analysis showed no significant differences between livers that did or did not undergo WIT (Figure 2a, d, Figure S1A–F). However, the ALT activities were significantly higher at T0 for WI45 (69.4 ± 20.0 U/L) than for LB (7.8 ± 5.0 U/L) ($p = 0.0018$), but

had decreased after 60 minutes of perfusion (Figure 2d). Notably, the data from the WI45 was more highly variable than those from LB (Figure 2a, d). A comparison of the plasma ALT activities of slaughterhouse pigs and WI45 showed that those for WI45 were similar at T0 (69.4 ± 20.7 U/L) to those measured for SH_gas (69.9 ± 10.3 U/L) and were significantly higher than those for SH_elec (13.2 ± 1.5 U/L) ($p = 0.0043$). However, at T300, the ALT activities of WI45 (46.6 ± 28.5 U/L) were lower than those of SH_gas (103.3 ± 31.4 U/L) ($p = 0.0358$), but still higher than those of LB (19.8 ± 13.0 U/L).

WI45 showed a slightly higher mean bile production of 24.7 ± 3.0 g/h than that of LB (17.4 ± 7.9 g/hour) and a significantly higher bile production than that of SH_gas (6.6 ± 3.4 g/hour) ($p < 0.0001$) (Figure 3a). Liver functionality testing showed a 2.5-times higher ICG half-life at T0 for WI45 than for LB (Figure 3b). However, the half-life had not increased significantly by the end of the perfusion period, as for SH_gas and LB. In addition, in WI45, signs of sinusoidal leukocytosis and mild parenchymal disruption were visible before and after the perfusion, as in the other experimental groups; however, there was superior tissue integrity at a histologic level than for all the other samples (Figure 4d).

Discussion

In the present study, we aimed to investigate the utility of slaughterhouse-derived livers for research purposes using an *ex vivo* liver perfusion platform. We showed that the CO_2 stunning of pigs has a negative effect on liver viability and functionality during subsequent *ex vivo* perfusion, compared to electrical stunning. In addition, electrical stunning was associated with superior liver functionality and similar quality to livers obtained from laboratory animals. Furthermore, we found that warm ischemia for 45 minutes did not cause significant liver damage, and therefore concluded that the differences resulted from the stunning technique itself.

CO_2 is known to cause coma and death in humans at concentrations of >10%[22] and induces unconsciousness in pigs after exposure for <1 minute at concentrations >90%.[19] Forslid and Augustinsson identified acidosis and stress hormone release in response to 1 minute of 80% CO_2 inhalation.[23] They showed high plasma potassium concentrations during and 1.5 minutes after CO_2 exposure, as well as a high plasma glucose concentration 5 min following exposure. In the same study, the plasma potassium concentration was found to decrease again after exposure to air.[23] This temporary increase in potassium might explain the potassium and glucose concentrations in SH_gas that were measured in the present study, which were 2.7- and 2.3-fold higher than those of SH_elec (Figure 2). After CO_2 exposure (>90% for 1 minute), the pigs in the present study were immediately exsanguinated, and therefore the high potassium and glucose concentrations might not decrease until the livers are harvested and put in cold storage. CO_2 stunning is known to reduce pH and induce lactic acidosis,[24] and despite adding bicarbonate during the perfusion as a pH buffer, there was a very low base excess (-10 ± 4 mmol/L) for SH_gas until the end of perfusion period (Figure S1D), which together with the lower lactate clearance (Figure 2a) confirms the presence of metabolic acidosis in SH_gas.

Hyperkalemia is a known risk during liver transplantation after cold storage in humans,[25] and Burlage *et al.* also showed this in *ex vivo* normothermically perfused livers after they had been in cold storage, as in the present study.[26] However, there was no hyperkalemia in livers from electrically stunned or laboratory pigs in the present study, despite their exposure to cold storage and the subsequent normothermic perfusion. Therefore, we excluded these factors as the explanation for the high

potassium concentrations in SH_gas. Interestingly, Burlage *et al.* showed in human donor livers that hypothermic machine perfusion prior to normothermic perfusion and after cold storage is associated with significant lower circulating potassium concentrations.[26] Therefore, in future experiments, it might be useful to add a hypothermic perfusion phase prior to the normothermic perfusion to evaluate its effects on livers from CO_2-stunned pigs.

The housing, care, and euthanasia of laboratory animals and farm animals must follow European Commission protocols to ensure animal welfare.[27] However, laboratory animals are subject to more strict procedures with regards to housing, feeding, and transportation time, to minimize inter-individual variation.[28] Furthermore, the livers that were harvested from both slaughterhouses came from a number of farms, which might have meant that there were more differences in their husbandry and transportation. Therefore, using slaughterhouse-derived material may be associated with greater variation between animals and their organs. Parameters for SH_gas showed noticeably higher standard deviations than those for SH_elec. This greater variability may have arisen because of the presence of confounding factors prior to slaughter, but also because of variations in WIT during the procurement of the liver. Employees of slaughterhouses require different lengths of time (30 to 45 minutes) to harvest the intestinal tract, which results in varying periods of warm ischemia during the slaughter procedure. Therefore, strict protocols and close collaboration between slaughterhouse technicians and researchers need to be established to ensure consistency of the harvesting protocol.

Although there were significant differences in the values of markers of functionality between SH_gas and SH_elec, these groups showed similar tissue changes on histologic examination, including mild tissue disruption, at the end of the perfusion period. A prolonged perfusion might also be associated with abnormal function in SH_elec after T300, but this possibility requires further analysis. In summary, SH_elec showed superior functionality and viability to SH_gas during the 5 hours of perfusion. With respect to transplantation, electrocution is not a contraindication for organ donation,[29] and in the present study, electrical stunning had less damaging effects on the liver than CO_2 stunning. Terlouw *et al.* showed that there was less severe acidosis in the muscles of electrically stunned animals than in CO_2-stunned animals, and the less acidic environment of livers from electrically stunned animals may explain the physiological values obtained for SH_elec.[30]

We also compared livers harvested at the slaughterhouse with livers harvested from laboratory animals, and found similar functionality during the 5 hours of perfusion. The ICG half-life for SH_elec was approximately 5 minutes, which is comparable to the reference values in humans.[21] In LB, the ICG half-life increased two-fold between T0 and T300. When considered alongside the results of previous studies, in which tissue damage was assessed by measuring the ICG half-life up to 183 min,[16,31] this increase seemed not to be significant. However, the increase in ALT activity during the perfusion that occurred in LB might indicate initial tissue damage. Because cold storage and reperfusion of the liver cause tissue stress, increases in markers of liver damage were expected. Although LB showed higher values of markers of tissue damage, they were still within the reference ranges. However, longer perfusions should be evaluated to determine if the level of functionality would remain stable or decrease in SH_elec, or recover in the case of LB.

Finally, to better understand the liver injury that occurred in SH_gas, the effect

of a longer WIT during the slaughter process was assessed. Studies of porcine livers described a WIT of <30 minutes[14,15,32] up to 60 minutes.[33,34] Nassar *et al.* described the ability of NMP to permit the recovery of injured organs after an extended WIT of 60 minutes, but stated that 30 minutes is the maximum accepted WIT in most transplantation protocols.[33] In addition, He *et al.* found that a WIT of 45 minutes was the maximum for an animal transplantation model before irreversible injury was observed.[35] In the present study, WI45 showed comparable functionality to LB, but this WIT also resulted in significantly higher initial ALT activities than in LB, indicating that 45 minutes of WIT does have an initial deleterious effect on the liver, with the values of markers of damage decreasing within the first hour.

A limitation of the present study was the lack of a comparison of the use of CO_2 stunning technique with or without a WIT. To assess whether the CO_2 stunning procedure itself is the major cause of the lower hepatic viability and functionality that characterized these pigs, compared to electrocuted and laboratory pigs, additional experiments should be performed. Such experiments could not be performed during the study because of the lack of availability of livers from CO_2-stunned pigs that did not experience warm ischemia. However, we can conclude that CO_2 stunning under the slaughter conditions used does have major effects on the livers used for *ex vivo* perfusion, and that these might depend on the WIT that the livers experience, alongside the acidic environment that is induced by the stunning technique. Hence, livers obtained from CO_2-stunned animals are not recommended for use in *ex vivo* perfusion studies.

Histologic examination revealed mild portal and parenchymal disruption in liver samples obtained at T300 from laboratory pigs, whereas more marked disruption was visible in livers obtained from slaughterhouses. The degree of disruption in SH_gas showed clear inter-individual variation, which typifies the variability of livers obtained from slaughterhouse animals. The noted leukocytosis and the differences in the amount of fibrosis in the portal region (Glisson's capsule) was already visible in samples taken immediately after liver harvesting, and these were therefore interpreted as representing individual variation at baseline, and not as being the result of either the sampling method or the perfusion. The disruption of the hepatic parenchyma was visible only at T300, and was sometimes associated with apoptosis or necrosis of hepatocytes, which might be the result of the perfusion technique, although influences of the method of harvesting and the characteristics of the animals cannot be excluded. To further analyze the architectural consequences of the disruption, additional histological evaluation of the livers, for example using reticulin staining, would be useful. To further assess liver viability, bile chemistry should be assessed, because this has been shown to be an important biomarker of viability.[36,37] Improvement of the perfusion technique is necessary for future studies, especially because architectural changes are already present after 5 hours of reperfusion, whereas robust applicability in research would require that the livers remain viable and functional for longer periods of time. The use of a longer duration of perfusion might also be useful for the evaluation of incipient tissue damage, which is already visible on histologic examination.[31] Tissue damage might occur because of ischemia, which results in reperfusion injury, a feature of every transplantation or *ex vivo* reperfusion.[38] Schlegel *et al.* showed a protective effect of hypothermic machine perfusion prior to normothermic reperfusion against

reperfusion injury, which also resulted in less tissue necrosis.[39] In future research, hypothermic perfusion could be used prior to normothermic reperfusion in an attempt to reduce tissue damage and improve the condition of livers harvested from CO_2-stunned animals.

In conclusion, we have shown the utility of pig livers obtained from a slaughterhouse, but also the important effect of the stunning technique used on this. *Ex vivo* organ perfusion using slaughterhouse tissue represents a more ethical and easily accessible research model for preclinical studies, thereby reducing the use of laboratory animals. The source of the material and the harvesting procedure should be standardized as much as possible to create an attractive research model, or materials for use in tissue response or interventional studies. The results of this study contribute to this area of research by evaluating the effect of the source of porcine livers (slaughterhouse and experimental pigs), and show superior performance of livers obtained from electrically stunned pigs over CO_2-stunned pigs. In conclusion, livers obtained from pigs that were slaughtered following electrical stunning could be used as an alternative to laboratory pigs that are euthanized specifically for the harvest of livers for *ex vivo* studies.

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Author contributions

AR and MS conceived and designed the study. AR, CP, IP, and R-AS performed the experiments and analyzed the data. AR and GCMG analyzed the data. AR wrote the original draft of the manuscript. LK, MS, and BS provided the laboratories and research equipment. AR, LK, BS, and MR finalized the version of the text to be published.

Declaration of conflicting interests

The authors declare that they have no conflict of interest.

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