ARTICLES

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Central Nervous System Tissue Contamination of the Circulatory System Following Humane Cattle Stunning Procedures

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SUMMARY

Two studies were conducted to assess the risk of central nervous system (CNS) material dissemination to edible tissues via blood circulation, following stunning of cattle with non-air injecting penetrating captive bolt (PCB) devices. In one study, an electric shock was applied with a heart defibrillator (HD), after rendering cattle insensible by use of a cartridge-fired PCB gun, to stop heart activity and subsequently blood circulation. In a second study, baseline levels of CNS tissue-marker Glial Fibrillary Acidic Protein (GFAP) were established in blood from cattle following pneumatic-PCB stunning and Kosher slaughter (without stunning) in twelve and one commercial beef packing plants, respectively. Electric shock after stunning produced heart fibrillation, which reduced heart rate and therefore blood circulation between stunning and sticking. The marker GFAP was not detected in the blood of cattle before or after stunning with or without HD. GFAP was detected in the blood of 1 (.28%) and 0 carcasses out of 360 (pneumatic-PCB) and 30 (Kosher) carcasses, respectively. Post-stunning mitigation practices to reduce the likelihood of CNS tissue dissemination in blood would not be necessary, as the risk of CNS tissue being present is low when non-air injecting PCB stunning protocols are employed.

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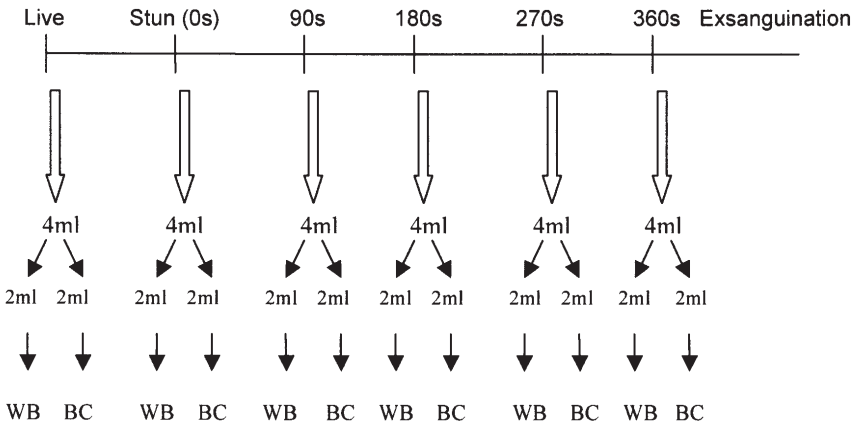
FIGURE 1. Experimental blood sampling protocol (WB: Whole Blood; BC: Buffy Coat)



INTRODUCTION

Contamination of edible carcass portions with infectious Bovine Spongiform Encephalopathy (BSE) prions (PrP^{Sc}) is suspected to increase the risk of human infection with new variant Creutzfeldt-Jakob disease (vCJD) *(11)*. Although the removal of Specified Risk Materials (SRMs) such as brain and spinal cord, which have been shown to transmit BSE, serves as the single most important food safety risk-mitigation intervention *(12)*, there are other possible sources of infectious prions that may reach the human food supply. Brown et al. *(3)* affirmed that cerebral vascular emboli, created by use of cranial stunning instruments to immobilize cattle before killing by exsanguination, could result in PrP^{Sc} dissemination. These stunning methods may cause clots in blood vessels, that if they remain fixed, are known as thrombi; however, if the clot becomes dislodged and floats freely in the bloodstream, it is known as an embolus *(2)*.

Most cattle within the United States (US) are stunned with pneumatic non-air injection penetrating captive bolt stunning devices before exsanguination, and these devices may damage intracranial blood vessels and dislodge central nervous system (CNS) tissue *(1)*. It has been reported that air-injection penetrating captive bolt stunning results in CNS tissue entering the blood *(1)*, passing through the right side of the heart *(17)*, and lodging in the lungs *(7)*, potentially entering the arterial circulation *(4)* even though dissemination throughout the carcass has not been reported. For that reason, the USDA-Food Safety and Inspection Service (FSIS) prohibited the use of penetrating captive bolt (PCB) devices that deliberately inject air into the cranial cavity of cattle *(19)*. Nonetheless, there is international concern about the continual use of non-air injection PCB stunning of cattle based on the evidence that such devices also can result in CNS tissue dissemination in the blood *(5)*.

A reliable analytical test for CNS tissue is essential to ensure consumer confidence of beef and reduce consumer fears of BSE in meat products *(17)*. One of the ways to detect and measure presence and concentration of CNS materials following stunning is by quantifying markers for CNS tissue in the blood of animals. Schmidt et al. *(18)* developed a simple, safe, sensitive, and specific assay for the detection of CNS tissue in blood and meat products with a Fluorescent-ELISA test based upon the immunological detection of Glial Fibrillary Acidic Protein (GFAP). Glial Fibrillary Acidic Protein is an antigen that is highly, but not completely, restricted to astrocytes in the CNS *(18)*. It thus provides an excellent marker for the presence of CNS tissue in blood and meat products. The objectives of this study were: (1) to determine the necessity for BSE risk mitigation practices associated with stunning or immobilization of slaughter cattle by quantifying the concentration of GFAP in blood from living animals and from animals exsanguinated following non-air injection stunning before exsanguination or from animal slaughter using ritual practices (Kosher), and (2) to evaluate heart fibrillation as a potential post PCB-stunning intervention to prevent CNS dissemination.

MATERIALS AND METHODS

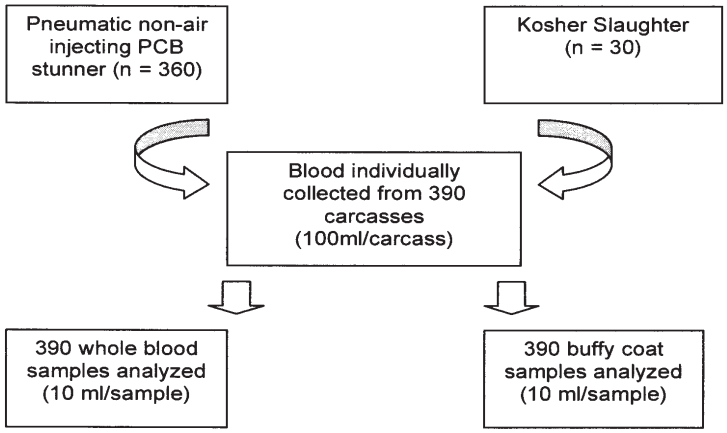
Evaluation of heart fibrillation as an intervention to prevent CNS dissemination

Intravenous catheters were inserted into the jugular veins of 10 market-ready heifers (average weight 505 kg) at the Colorado State University Agricultural Research Development and Educational Center (ARDEC, Fort Collins, CO) (ACUC Protocol Number 05-049A-01). Following a 48-hour withdrawal period for Lidocaine, two defibrillator and three electrocardiogram (ECG) pads were firmly affixed to each heifer externally on the brisket and thoracic wall (2 on the left side and 1 on the right side), respectively. Cattle were then transported and harvested at the Colorado State University Meat Laboratory. Cattle were stunned using a cartridge-fired, non-air injection, penetrating captive bolt (PCB) stunning device (Schermer Model ME) and all were rendered insensible following a single shot. Animals were considered insensible when the head was completely limp, the tongue was fully extended, and the eyes had a blank stare *(9)*. Five of the cattle were immediately shackled, hoisted, and exsanguinated (Treatment 1). The remaining five cattle were shackled and hoisted, after which an electrical shock generated by a commercial hands-free heart defibrillator (Hewlett Packard Code Master XL+) charged to 360 Joules was administered (HD) (Treatment 2). Electrocardiograms of animals were recorded pre- and post- – stunning by use of three-wire electrodes. The electrodes were firmly applied such that two electrodes were on the left thoracic wall (black and red leads) and one was positioned on the right thoracic wall (white lead). Amperage (amount of electrical current that reaches the heart) and Impedance (body resistance to the flow of electrical current) were recorded by the defibrillator for each shock. Voltage, which is required to push the electrical current from the defibrillator to the animal, and duration of the shock were calculated based on the following equations:

$$\text{Voltage} = \text{Amperage (Amps)} * \text{Impedance (Ohms)}$$

$$\text{Duration of the shock (milliseconds)} = \frac{\text{Energy (Joules)}}{\text{Amperage (Amps)} * \text{Voltage (Volts)}}$$

FIGURE 2. Commercial blood sampling protocol



Six blood samples were collected from the jugular catheters of each animal (n = 60) to determine if CNS tissue was present in circulatory blood following stunning with and without heart defibrillation. The blood sampling protocol is summarized in Fig. 1. The first blood sample was collected before PCB stunning, and five samples were collected immediately following stunning, at approximately 90-second intervals, during the 6 minutes following stunning. In one instance, all samples were collected during exsanguination because the jugular cannula was damaged during handling and stunning.

At each sampling interval, 4 ml of blood were collected and divided into 2 Vacutainer™ tubes, one containing K_2EDTA anticoagulant and the other containing Sodium Heparin anticoagulant. Samples were immediately refrigerated at 2°C. Heparinized tubes were centrifuged at 800 × g for 30 minutes at 4°C to separate the sample into serum, white blood cells (buffy coat) and red blood cell fractions. Buffy coat (cellular fraction) was removed using Pasteur pipettes and transferred to 5 ml capped tubes (BD Falcon). These fractions were collected and analyzed in order to increase the sensitivity of the test, as cells of the CNS will tend to pellet together with the same density of cells in the buffy coat fraction. Both buffy coat (n = 60) and whole blood (n = 60) samples were kept refrigerated and transported the following day, in an insulated box with ice packs, to Warren Analytical Laboratories Inc. (Greeley, Colorado) for F-GFAP analysis.

A capture Fluorescent – Enzyme Linked Immunosorbent Assay for Glial Fibrillary Acidic Protein (F-ELISA GFAP) was used to detect CNS tissue contamination in whole blood and buffy coat. The protocol followed was previously described in detail by Schmidt et al. *(18).* A standard curve was developed by use of serially diluted commercial Bovine GFAP. Standard curves were utilized to quantify the concentration of GFAP in whole blood and buffy coat samples. An aliquot of each blood sample, before (whole blood) and after (buffy coat) centrifugation, were analyzed at Warren Analytical Laboratories (Greeley, CO) to detect presence of CNS tissue. Two antibodies were used to detect the presence of GFAP (antigen). The first (polyclonal anti-GFAP) was used to coat the wells and to capture GFAP. The second (monoclonal anti-GFAP) was coupled to a peroxidase enzyme to detect GFAP. Finally, the reaction was detected by the addition of a peroxidase substrate that produced fluorescence upon reaction with the enzyme. The detection limit for this assay was 0.3 ng/well or 0.006 ng/mg for whole blood and buffy coat, as each well contained 50 microliters (ul) of sample. A result of < 0.006 ng/mg denoted a non-detectable level of GFAP in whole blood or buffy coat. Inter and intra-assay coefficients of variation were 3.9% (five different assay dates) and 3.3% (12 wells in one assay date), respectively, indicating that this test is repeatable both within and across sample tests.

Commercial survey of GFAP in circulating blood of cattle stunned in the United States

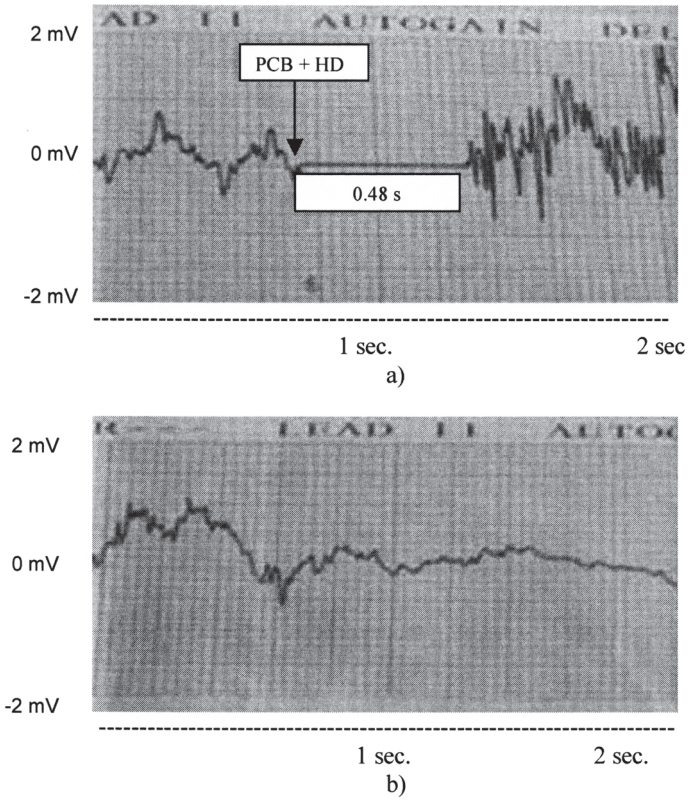
Between July and October 2005, blood samples (N = 390) from random cattle in thirteen commercial beef processing facilities were collected as soon as possible following exsanguination. Twelve of the plants utilized a pneumatic non-air injection PCB device. The remaining plant employed ritual (Kosher) slaughter techniques, immediately followed by pneumatic non-air injection PCB stunning. When possible, cattle that required more than one shot to be rendered unconscious were omitted from the study. However, in two plants, blood samples were collected in a location from which the stunning restrainer was not visible, and we were not assured that samples from these facilities were from single-shot stunned cattle. Blood was aseptically collected in large disposable cups (150 ml) and then transferred to two Vacutainer™ tubes, one containing K_2EDTA (10 ml) and the other containing Sodium Heparin (10 ml) anticoagulant. This resulted in blood samples being collected from 360 pneumatic-PCB stunned cattle and 30 Kosher slaughtered cattle (Fig. 2).

After collection, all samples were refrigerated, placed in coolers with ice packs, and shipped to Warren Analytical Laboratories, Inc. (Greeley, CO) for F-GFAP analysis. After arriving at the Laboratory, heparinized tubes were immediately centrifuged at 800 × g for 30 minutes at 4° C and the buffy coat fraction was collected for analysis. Whole blood and buffy coat samples were analyzed by use of the same F-GFAP ELISA test previously described, again with a detection limit of 0.006 ng/mg.

Statistical analysis

Independent two-sample Student's *t*-test was used for comparisons of heart rates between treatments, as samples had been collected independently of one another. For each treatment, a paired Student's *t*-test was performed to determine the significance of difference in heart rate before and after each stunning protocol, as measurements had been taken from the same animal (correlated samples). The prevalence of GFAP was analyzed statisti-

FIGURE 3. ECG from cattle (a) immediately (1–2 s) following stunning with heart defibrillation (HD), and (b) immediately (1–2 s) following stunning without HD



cally considering a binomial distribution (GFAP detected or not detected in blood circulation). The two blood fractions, whole blood and buffy coat, were analyzed independently. A 95% one-side upper exact binomial confidence limit for GFAP presence was established.

RESULTS AND DISCUSSION

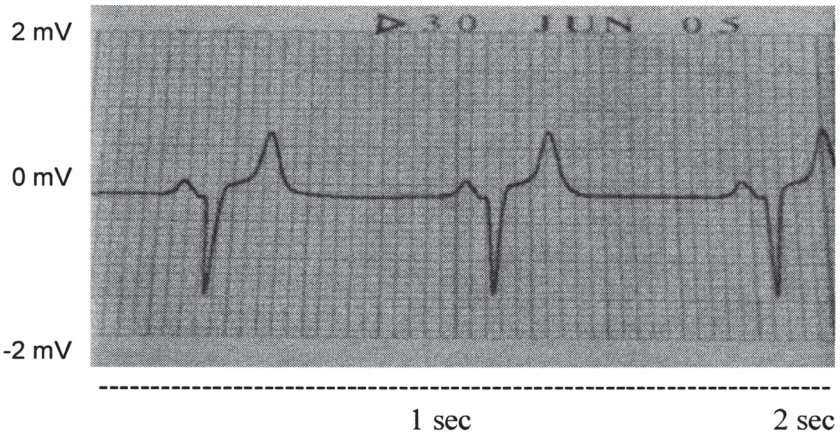
Evaluation of heart fibrillation as an intervention to prevent CNS dissemination

Results of the ELISA test showed that GFAP was not detectable (< 0.006 ng/mg) in whole blood and buffy coat samples collected before and after PCB stunning with or without HD. Absence of detectable levels of GFAP in the whole blood and buffy coat of animals before stunning confirms that GFAP is a protein highly restricted to the CNS (spinal cord and brain) and not found in the normal blood circulation of live animals. For that reason, GFAP is an appropriate protein marker for CNS tissue dissemination/presence in blood. Tests based on protein markers generally are more sensitive than gross tissue examination or microscopic analysis, because stunning may cause leakage of neural tissue across the blood-brain barrier without actual embolization of intact tissue fragments *(14)*.

Electrocardiogram recordings were successfully obtained from 9 of 10 animals before stunning. Mean heart rate of cattle before stunning was 126 (SD = 32) beats per minute (bpm), ranging between 89 and 188 bpm. Gay and Radostits *(8)* reported that the mean heart rate for adult cattle ranges between 60 and 80 bpm and that it is not uncommon for resting heart rate to be accelerated because of acute stress or unfamiliar surroundings. Electric shock delivered using the heart defibrillator charged to 360 Joules following insensibility, created by PCB stunning, delivered an average of 32 Amps (SD = 4) and 3,833 Volts (SD = 219) for 3 milliseconds (SD = 0.2). Animal resistance to the flow of current (Impedance) was 120 ohms (SD = 23). Heart defibrillation (HD) did not permanently render the heart electrically silent (Fig. 3a), yet it resulted in a short electrically silent period (0.48 s) with average heart rate following HD returning to 23 bpm (SD = 8), ranging from 16 to 35 bpm (Fig. 3b), which was lower than the heart rate before PCB stunning ($P < 0.05$). Even though the heart was still beating, cattle were completely insensible because of the PCB stunning applied before HD. Conversely, after PCB stunning without HD (Fig. 3b), animals showed a chaotic heart rhythm (as shown immediately following PCB and HD in Fig. 3a) followed by a tendency to recover and return to a normal heart rate and rhythm. Immediately following PCB stunning, the mean heart rate was 165 bpm (SD = 23); this was higher than the heart rate before PCB stunning ($P < 0.05$), although the heart rate from 2 animals was not recorded because of a very abnormal ECG output. Heart rate immediately following HD was lower than heart rate following PCB stunning without HD ($P < 0.05$). Heart rate of one animal measured three minutes after PCB stunning showed a normal heart activity with 85 bpm (Fig. 4), which reflected normal resting heart rate.

Wotton et al. *(20)* successfully induced ventricular fibrillation and cardiac arrest in adult cattle when >1.51 A sinusoidal AC at 50 Hz was applied for five seconds between the nose and brisket electrodes. According to the description of the defibrillation shock in our study (32 Amps and 3, 833 Volts for 3 milliseconds), the limiting factor that did not allow electrical stoppage of the heart with only one discharge was the short duration of the shock. Increasing the duration of the shock will decrease the animal impedance; therefore, more electrical current will reach the heart, increasing the efficiency of the electric discharge. Although heart fibrillation following PCB stunning did not result in permanent electrical silence, heart rate was reduced and heart rhythm was altered, potentially reducing the blood circulation between stunning and sticking (although blood volume flow was not measured). When there is such incoordinate twitching of the heart, the diastolic period is so short that filling of the ventricles is limited, the blood pressure falls precipitously, and the animal dies within a minute or two of onset as the result of failure of blood perfusion into tissues *(15)*. Conversely, heart rate tended to be normal after PCB stunning without heart fibrillation. Thus, if CNS contamination of the blood were to occur during PCB stunning, the interval be-

FIGURE 4. ECG from an animal 3 minutes after PCB stunning (before sticking)



tween stunning and sticking would result in potential CNS dissemination through the circulatory system if CNS tissue is not trapped in the lungs or heart. Although animal unconsciousness may last up to 10 minutes after PCB stunning *(6)*, and CNS contamination of blood occurs at very low frequencies and at extremely low levels, a best practice would be to complete exsanguination as quickly as possible to reduce any potential organ exposure.

Commercial survey of GFAP in circulating blood of cattle stunned in the United States

Of the 360 samples collected from commercial processing facilities, one sample contained detectable levels of GFAP following pneumatic-PCB stunning. Glial Fibrillary Acidic Protein was detected in both whole blood and buffy coat fractions, with a concentration of 0.010 and 0.015 ng/mg of GFAP, respectively. These values were equivalent to 5.8 and 17 ng of spinal cord tissue per mg of whole blood and buffy coat, respectively, and to 8.7 and 26 ng of brain tissue per mg of whole blood and buffy coat, respectively, considering the concentration of GFAP in CNS as reported by Schmidt et al. *(18)* on a wet weight basis as determined by use of a Fluorescent-GFAP ELISA test. These CNS tissue concentrations were very low, especially when compared to the oral infective dose (150 g of BSE-infected CNS) reported by Lasmezas et al. *(13)*. According to these results, prevalence of CNS tissue in circulating blood was 0.28% of the cattle stunned with pneumatic-PCB protocols. In addition, there was a 95% confidence level that prevalence of CNS tissue in the blood of cattle after pneumatic-PCB is less than 1.31% based on an exact binomial confidence limit. Coore et al. *(5)* reported elevated levels of GFAP in venous blood samples (collected with balloon-catheters) from 4% (95% Confidence Interval: 1.6 to 9.8%) of anesthetized cattle stunned with a cartridge-fired PCB (Cow Puncher™). Limitations of that study included use of anesthetized cattle and balloon catheters, which are inflated to assist in collection of blood, thus blocking venous blood circulation and altering the intracranial pressure. These conditions are not found under commercial stunning protocols and results of that study do not agree with the low GFAP prevalence found in commercial processing facilities in the US in our investigation.

Although all of the plants evaluated in this study used a similar PCB stunning device (Jarvis pneumatic stunner) and processed steers and heifers (variable related to thickness of the skull and bolt penetration), potential differences between plants were observed, such as operator, chain speed (from 150 to 400 animals/hour), interval between stunning and sticking, and interval between stunning and the collection of blood samples. In the plant in which the single positive GFAP result was obtained, blood samples were collected farther from the point of stunning (after electrical stimulation) than in other facilities. Consequently, it was not possible to assure that sampled animals were rendered unconscious on the first stun. However, Grandin *(10)* reported that, during a 4-year period, 97.2% (SD = 6.21) of the cattle slaughtered in the US were correctly stunned on the first attempt. Thus, further research is needed to determine if re-stunning animals, instead of single-shot stunning animals could result in CNS dissemination.

We did not detect GFAP or CNS tissue in the blood of animals following Kosher slaughter protocols (without stunning prior to sticking). In the one plant that we visited, animals were driven to a restraining device that was equipped with a head-catch, and then a shochet (rabbi performing the ritual slaughter) made an incision in the front of the neck of the live animal with a chalaf (knife employed during kosher slaughter). After the shochet had cut the neck of the animal, animals were stunned by use of a pneumatic-PCB device to ensure insensibility of the animals before dressing. Blood samples were collected after pneumatic-PCB stunning, at which time the blood already was flowing because of the previous cut. Although religious slaughter may cause congestion and some microscopic hemorrhages, brain injury is extremely unlikely and of lower risk compared to the PCB-stunning techniques *(16)*, and the circulatory system between the brain and heart are severed before cranial penetration.

CONCLUSIONS

This study indicates that the heart activity and function of cattle after PCB stunning is normal. For that reason, the interval between stunning and sticking is the period of highest risk for organ contamination, as blood circulation remains normal. Even though heart activity of the animals was not permanently stopped by applying an electric shock to the heart with a human heart defibrillator, heart activity was reduced between stunning and sticking as a result of heart fibrillation, which reduced the heart rate of the animals and therefore, presumably, blood circulation. We found a very low prevalence of GFAP in the blood of animals after pneumatic non-air inject PCB stunning in 12 commercial beef slaughter plants in the US, and we did not detect brain tissue in the blood of animals after Kosher protocol in one beef slaughter plant.

Results affirmed the safety of non-air inject PCB stunning protocols used in the United States. For that reason, post-stunning mitigation practices to reduce the likelihood of CNS tissue dissemination would not be necessary when penetrating captive bolt protocols are employed. However, further research is needed to quantify the impact of repeat PCB stunning on CNS tissue dissemination.

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