DIFFERENT SLAUGHTERING TECHNIQUES AND POSSIBLE PHYSIOLOGICAL AND BIOMOLECULAR EFFECTS

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Accepted 10 August 2017, Published online 31 December 2017

ABSTRACT

The aim of the study was to identify possible physiological and biomolecular changes during slaughtering. For slaughtering, before the neck cutting, chickens are immobilized manually or immobilized using shackles. Neck cutting is generally performed using automated knife, often results in decapitation. Both of these immobilization and neck cutting conditions are expected to influence muscle contraction and blood loss. We have investigated the activity and transcription of acetylcholinesterase (AChE) which terminates cholinergic synaptic transmission by hydrolysing the neurotransmitter acetylcholine that is responsible for muscle contraction and relaxation. We have also analysed the residual haemoglobin content of the skeletal muscle as indicator of blood loss. Skeletal muscle sample was collected from the chickens that were slaughtered either by decapitation (C) or by severance of the jugular veins, carotid arteries, oesophagus and trachea only (P); whilst immediately after slaughtering, chickens were either released (R) or manually constrained (T). Differences in the conditions of slaughtering: CR, PR or PT did not affect blood loss as measured by the residual Hb content and the amount of Zn and Fe either in muscle or liver, deducing no significant difference (p>0.05) in blood loss due to different type of slaughtering. No significant differences (p>0.05) were observed in AChE activity in muscles taken from all slaughter groups. However, AChE transcripts were detected in muscles from chickens from PT and CR groups which might be due to the decapitation and/or constrained muscular activity after neck cutting. Hence the results of the current study indicate that, constraining during slaughtering and decapitation might induce stress to the animals.

Key words: Motor neuron, neuromuscular junction, acetylcholine, stress, haemoglobin, central nervous system

INTRODUCTION

Industrial poultry slaughtering is mostly done by neck cutting of immobilized (shackled) birds with or without water bath stunning (Davis, 2009; Shields & Raj 2010; Löhren, 2012). An immobilized bird would struggle to release the external pressure resulting in additional muscle contraction-relaxation. Slaughtering activity increases the muscular activity due to the struggling of the birds during slaughtering resulting in an increased blood flow in skeletal muscle, by compressing vasculature, thus promoting venous return and expelling blood towards the heart (Hussain & Comtois, 2005). At rest, average blood flow rate in skeletal muscle is 10- to 12-fold lesser than in highly metabolic organs such as heart and brain (Korthuis, 2011). This blood flow during slaughtering can be increased due to sudden increase in muscle contraction and relaxation leading to an increased blood loss.

One of the fastest methods to induce brain death, to ensure humane slaughtering, is performed by the neck-cutting using sharp knife. Neck cutting is performed by severing two carotid arteries and jugular veins and wind pipe without damaging the spinal cord to let rapid blood loss (Raj, 2010). However, neck cutting often results in decapitation, i.e., complete separation of the head from the body, reported to be practiced in industrial slaughter plants which raised significant ethical concerns (Shields & Raj, 2010). Notably, decapitation causes the severance of the spinal cord. Failure to cut both
CAROTIDS CAN ELONGATE THE TIME REQUIRED FOR BRAIN DEATH. SEVERANCE OF ONLY ONE JUGULAR VEIN CAN ALSO CAUSE BIRDS TO RETAIN CONSCIOUSNESS WITH SEVERE PAIN (DAVIS, 2009).

TAKEN TOGETHER, IT CAN BE CONSIDERED THAT BLOOD LOSS DURING SLAUGHTERING BY NECK CUTTING CAN BE AFFECTED NOT ONLY BY THE VEINS AND ARTERIES THAT ARE CUT DURING SLAUGHTERING BUT ALSO BY THE IN/VOLUNTARY MUSCLE CONTRACTION BOTH OF WHICH ARE CONTROLLED BY THE NEUROTRANSMITTER, ACETYLCHOLINE (ACh) PRESENT AT NEUROMUSCULAR JUNCTION OF VERTEBRATE. DUE TO THE SHORT HALF-LIFE OF ACh, ITS RELEASE AT NEUROMUSCULAR JUNCTION OF VERTEBRATE IS OFTEN MEASURED INDIRECTLY BY MEASURING THE ACTIVITY AND TRANSCRIPTION OF ACETYLCHOLINESTERASE (AChE) WHICH TERMINATES CHOLINERGIC SYNAPTIC TRANSMISSION BY HYDROLYZING THE NEUROTRANSMITTER ACh THAT IS RESPONSIBLE FOR MUSCLE CONTRACTION AND RELAXATION.

IN THE PRESENT STUDY WE ANALYZED SKELETAL MUSCLE OF THE CHICKENS THAT WERE SLAUGHTERED IN CONDITIONS THAT VARIED IN (I) NECK CUTTING EITHER DECAPITATED OR PARTIALLY NECK CUT AND, (II) AFTER NECK CUTTING MUSCLE MOVEMENT WAS EITHER CONSTRAINED MANUALLY (A REGULAR PRACTICE IN SMALL ABATTOIRS) OR RELEASED. WE HAVE ANALYZED THE RESIDUAL HEMOGLOBIN (Hb), Fe AND Zn CONTENT IN THE SKELETAL MUSCLE AS AN INDICATOR FOR BLOOD LOSS/RETENTION. WE HAVE ALSO ANALYZED THE ACTIVITY OF ACETYLCOLINESTERASE (AChE) EXPRESSION, AS A MARKER OF ACh WHICH IS LINKED TO SKELETAL MUSCLE CONTRACTION.

MATERIALS AND METHODS

Chicken slaughtering and sample preparation

Chickens (Gallus domesticus), 21 days old having 750-850 gm of body weight were slaughtered (n = 28) by manual slaughtering using sharp knife. All experiments were conducted at the research facilities in Faculty of Science, International Islamic University of Malaysia (IIUM) following appropriate institutional ethical approval. The act of slaughtering was performed by decapitation (C) or conventional neck cutting, i.e., without severance of the spinal cord (P); while the legs and wings of the chickens were manually constrained (T) or released (R) after neck cutting. For conventional (partial) neck cutting both jugular veins, both carotid arteries, trachea, and the oesophagus were cut and for complete neck cutting the head was separated from the body.

Muscle and liver haemoglobin (Hb) concentration

Heme protein extraction was performed in a conventional method with modification which was first described by Andersen and Shoemaker in 1965. Briefly, 1 gm of muscle/liver tissue sample was homogenized at 4°C in a solution containing 1 mL of 2N HCl and deionized H2O (total volume 10.2 mL) and centrifuged at 4°C either at 2500 g for 80 minutes (for muscle) or at 2000 g for 45 minutes (for liver). Supernatant fluid was collected to determine Hb content as follows: 7 mL of supernatant was mixed with 2 mL of 1N NaOH; a few crystals of NaCN were added to the solution. One mL of 10% sodium lauryl sulfate was added and allowed to stand at 4°C for 24 hours and the supernatants were centrifuged either at 3000 g for 60 minutes (for muscle) or at 2000 g for 30 minutes (for liver) at 4°C. Absorbance was measured for supernatant solution at 540 nm. Hb content was determined using the following equation, adopted from Kranen et al. (1999).

\[
\text{Hb (mg/gm) = } \frac{\text{A} \times \text{Dilution Factor}}{\text{Mole extinction coefficient x light path (cm)}
\]

Determination of Zn and Fe

Muscle and liver samples were dried in the oven at constant temperature of 62°C until a constant weight was achieved. After that, each portion of sample was divided in triplicate and digested in 10 mL of 65% HNO3 for 5 hours in a water bath at 95°C. The digested samples were then diluted with deionized H2O, filtered and were analyzed using automated atomic absorption spectrophotometric (AAS) machine (Perkin Elmer Analyst 700, Waltham, USA). The standards prepared were Zn (1000 ppm) and Fe (1000 ppm) (Perkin Elmer, Waltham, USA).

Measurement of ACh esterase (AChE) activity

ACh esterase activity in skeletal muscle was measured as previously described (Chavez et al., 2007). Briefly, 1 gm of muscle tissue sample was homogenized in 10 mL of 0.1 M phosphate buffer (PB, pH 8.0) for approximately 1 minute on ice; the homogenate was then centrifuged at 3000 g for 15 minutes at 4°C; the filtered supernatant was used to measure the AChE activity. 131 μL of PB was added to 1μL of substrate (75 mM acetylthiocholine iodide), followed by the addition of 5 μL of 10 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) in a well of a 96-wells microplate. This mixture was incubated at 37°C for 10 minutes and the absorbance at 405 nm was recorded as ‘blank’ reading. 20 μL of brain homogenate was added and the absorbance was recorded every 2 until 32 minutes. For control wells, 20 μL PB was added instead of the muscle homogenate and absorbance was recorded accordingly. Both control and muscle homogenate were in triplicate.
Evaluation of AChE mRNA expression using RT-PCR

Total RNA was extracted from skeletal muscle using RNeasy fibrous Tissue Mini Kit (QiAgen, Hilden, Germany). RNA was eluted in 40μl of RNase free water. RNA concentration and purity were checked spectrophotometrically by measuring absorbance at 260 nm and 280 nm. One-Step RT-PCR was performed using Maxime RT-PCR premix (iNtRON, Boca Raton FL, USA). AChE forward primer 5’-CTC TTA TCG CCC CAT AGC AA-3’ and reverse primer 5’-CGA AAG CGA ACC TAA AGA CG-3’ were designed using Primer3 (v. 0.4.0, http://frodo.wi.mit.edu/primer3) to amplify a sequence of 250 bp. Primers for β-actin, as housekeeping gene to amplify a sequence of 123 bp, (forward: 5’-CTG GCA CCT AGC ACA ATG AA-3’ and reverse: 5’-CTG CTT GCT GAT CCA CAT CT-3’), were used as described earlier (Maddineni et al., 2005). All primers were synthesized and supplied by Research Biolab, Singapore.

The reverse transcription was performed at 45°C for 30 minutes followed by one step for enzyme inactivation at 94°C for 5 minutes. The subsequent PCR (34 cycles) steps were denaturation at 94°C for 1 minute; annealing at 62.8°C (for AChE mRNA) or 55°C (for β-actin mRNA) for 1 minute; and, extension at 72°C for 1 minute; final extension at 72°C for 5 minutes. PCR amplified retro-transcripts were electrophoresed on 2% agarose gel and visualized under UV illumination with ethidium bromide staining.

Statistical Analysis

Effects of slaughtering methods on the possible interaction with AChE enzyme activity, Hb content, Fe and Zn in the meat and liver were tested by one way ANOVA using excel spreadsheet 2007. The means were considered different when $p<0.05$ by ANOVA, unless stated otherwise.

RESULTS

Muscle and liver Hb content were not affected

Hb content was measured in liver and different parts of skeletal muscle (right and left legs and chest muscle). Difference in the mode of neck cutting i.e., partial or complete neck cutting in combination with manual restraining or releasing the chickens after slaughtering did not show any significant effect on Hb content (Table 1).

Zn and Fe content were not affected

Difference in mode of neck severance i.e., partial or decapitation in combination with manually restraining or releasing the chickens after slaughtering did not have any significant effect on Zn (Figure 1A) and Fe (Figure 1B) content in different parts of muscles and liver.

Table 1. Hb content in muscle and liver right after slaughtering

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Average ±SD of Hb content (mg/gm of tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CR</td>
</tr>
<tr>
<td>Leg</td>
<td>0.81±0.21</td>
</tr>
<tr>
<td>Chest</td>
<td>0.09±0.08</td>
</tr>
<tr>
<td>Liver</td>
<td>13.60±1.55</td>
</tr>
</tbody>
</table>

Fig. 1. Zn and Fe content of chicken muscle and liver. Total Zn (A) and Fe (B) content from different parts of skeletal muscle (both legs and both chests) and liver were analyzed using atomic absorption spectrophotometer (AAS). Zn and Fe contents from chickens slaughtered in different ways (i.e., CR, PR, and PT) did not show any significant difference ($p>0.05$).
AChE activity remains similar

Activity of AChE in muscles from chickens slaughtered in different ways did not show any variation. Average AChE activity was found in the range of 4-8 μmol/min/gm of the muscle tissue (Figure 2A).

Decapitation and restrained muscle contraction induces AChE transcription

AChE mRNA was not detected as analyzed by RT-PCR in the muscles from chicken slaughtered by partial neck cutting and released after slaughtering (PR) (Figure 2B); AChE mRNA was detected in muscles of chickens that are slaughtered by complete neck cutting (decapitation) or manually restrained after slaughtering (CR and PT, Figure 2B).

DISCUSSION

One of the fastest methods to induce brain death and to ensure humane slaughtering is by performing neck-cutting i.e., the severance of two carotid arteries and jugular veins (Figure 3). Failure to cut both carotids can elongate the time required for brain death. Severance of only one jugular vein can cause birds to retain consciousness with severe pain (Davis, 2009; Gregory & Wotton, 1986). If not decapitated, i.e., the connectivity of the central and peripheral nervous system remains intact through intact spinal cord, muscle contraction would be less due to the feedback inhibition related to increased serotonin release during high level of motor activity at the axon initial segment of the motor neuron (Cotel et al., 2013). Again, skeletal muscle pump of contractile muscle can increase blood flow (Korthuis, 2011). While muscle contraction during stunning was found associated with bleeding and muscle haemorrhage (Joseph et al., 2013). Taken together, it can be stated that, humane slaughtering through quick blood loss can be ensured by (i) cutting of both carotid arteries and jugular veins and, (ii) optimising muscle contraction.

Since Hb content can be a measure of blood loss during slaughtering, we attempted to determine Hb content in muscle and liver collected from chicken slaughtered in different conditions (Table 1). No significant difference in Hb content was recorded among the three groups: (i) decapitation and releasing chicken after neck cutting (CR), (ii) partial neck cutting and holding chicken tight after neck cutting (PT) and, (iii) partial neck cutting and releasing chicken after neck cutting (PR). The result supports previous findings that concluded, rapid drop of blood pressure due to neck cutting causes insufficient driving force to drain blood from capillary beds adjacent to the muscle; therefore, this eventually causes poor blood removal from breast muscle (Alvarado, 2007). He also showed that blood content of animals slaughtered by cutting of throats were significantly lower than that of the animals which were killed by methods with no bleeding step. Although different methods of slaughtering which had bleeding steps, showed no significant difference in Hb content in breast muscle (Alvarado, 2007). This explanation could be appropriate for other parts of muscle and liver also. However, it is not unlikely that the heme of muscle- and liver- myoglobin might have contributed to the observed indifferences in the current study.
We have also checked Fe content in the same tissues. Since Hb content was not affected, therefore as expected, Fe content of muscle and liver did not show any significant difference among the three groups. In addition to Fe, we have also analyzed Zn content which is known to be the second most abundant metal in animal muscle tissue (Chasapis et al., 2012). Moreover, Zn works as the prosthetic group of carbonic anhydrase one of the most abundant enzymes in blood. Similar to the Fe content, Zn content also did not show any significant differences among the groups (Figure 1B).

Our next attempt was to evaluate the biochemical impact of skeletal muscle contraction that might vary depending on whether the bird is decapitated or how the bird is being restrained manually after the neck cutting. For this we chose to measure the activity of AChE. Earlier it was reported that the activity of AChE is higher in motor neurons than in sensory neurons (Massoulié et al., 1993) while in avian species a substantial amount of AChE nerves is projected to the internal carotid arterial system via the internal ethmoidal artery (Kusaba et al., 2001).

We have found that decapitation or partial neck cutting while manually restraining or releasing the chicken after neck cutting did not affect AChE activity (Figure 2A). To our surprise, AChE mRNA was found differentially expressed. AChE mRNA was detected in muscles of chickens which were (i) released after decapitation (CR) or (ii) restrained manually after partial neck cut (PT). To the contrary, AChE mRNA was not detected in muscles from chickens which were slaughtered by partial neck cut and released after neck cutting (PR). It has been reported earlier that, the neural activity mediated regulation of the expression of contractile proteins in specific muscle is controlled at transcriptional level (Bounanno & Fields, 1999; Musaro & Rosenthal, 2002). Therefore, mRNA expression and enzyme activity often found to be differently expressed.

The observed differences in AChE transcripts raised two fundamental questions: (i) could the resistance to muscle movement and/or decapitation...
induces AChE mRNA expression? And, (ii) what would be the role and/or fate of the inducible AChE mRNA in the skeletal muscle of the chickens that are slaughtered by decapitation and/or restricted muscle movement?

In relation to the current study, manual restraining of the chicken after neck cutting means additional mechanical pressure on the slaughtered chicken – hence the slaughtered chickens might have exerted additional force against the mechanical pressure that in turn might cause additional skeletal muscle contraction (Figure 3B). This additional contraction might contribute to induce AChE transcription. At the same time, less contraction due to inhibition of spontaneous contraction of myotubes and muscle paralysis were shown to reduce activity and expression of AChE (Fernandez-Valle & Rotundo, 1969; Michel et al., 1994). While AChE also found to decrease in exercise induced fatigue (Wen et al., 2009) where muscle movement is comparatively less.

However, the causal relationship between muscle contraction and AChE expression raised further question to explain absence of RT-PCR amplified AChE mRNA in the skeletal muscle of the chickens which were slaughtered by partial neck cutting and immediately after the neck cutting, chickens were released (PR). Using turtle muscle, Perrier et al. (2005) showed that during motor activity, serotonin released in synapses that contact motor neurons can promote muscle contraction. However, during high level of motor activity, the amount of serotonin released increases and a spill-over occurs. Serotonin binds to extra-synaptic receptors located on the axon initial segment of motor neurons with the result that nerve impulse initiated and thereby muscle contractions are inhibited (Cotel et al., 2013). This feedback inhibition of muscle contraction through serotonin spill-over in spinal motor neuron requires CNS-PNS connectivity. Thus less muscle contractions are expected in the skeletal muscle taken from the chickens slaughtered by PR, as they had the intact CNS-PNS connectivity. This also explains why the band intensity of RT-PCR amplified AChE mRNA was less in skeletal muscles taken from PT compared to that taken from CR.

In search for an answer to the second question: the role and/or fate of the inducible AChE mRNA in the chickens that are slaughtered by decapitation and/or experience restricted muscle movement, it might be worth mentioning that the degradation of AChE, at least in cultured muscle cells, follows a first-order decay kinetics with a half-life of about 50 hr (Rotundo & Fambrough, 1980). It indicates that observed indifferent AChE activity (Figure 2A) is related to the AChE that was present in skeletal muscle before slaughtering. Since within a minute after neck cutting, the chickens would die, the induced AChE mRNA might not be translated to active enzyme. Nonetheless, the induced AChE mRNA might reflect more muscle contraction hence more stress related to decapitation and/or restrained muscular activity after neck cutting.

ACKNOWLEDGEMENTS

The authors would like to thank all the fellow friends of IIUM specially Ja’afar Nuhu Ja’afar who helped during slaughtering. Our special thanks to Hamzah Mohd. Salleh, Faculty of Engineering, IIUM, whose technical advice was invaluable for the reported research. This work was supported by the Fundamental Research Grant Scheme (FRGS0106-24), Ministry of Higher Education, Malaysia.

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