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Effect of pre-slaughter stunning on the death of the poultry and myofiber apoptosis

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Abstract

The effectiveness of poultry stunning in producing swift slaughtering was analysed in response to the time needed for the chickens to become insensible upon neck cutting (T_d) and the induction of myofiber apoptosis. In total, 49 chicken broilers (BW of 2.17 ± .24 kg) were sacrificed with pre-slaughter stunning, using a constant voltage stunner where the electric current varied between 7.2 to 124.3 mA, and without stunning. The electric current applied during stunning was found to have no effect on T_d. Number of apoptotic myonuclei did not vary among stunned and unstunned meat. Apoptosis inducing factor (AIF) and caspase 3 expressions were also not detected in the meat samples of both stunned and unstunned groups at 1 d postmortem. Since the slaughtering process and stunning are associated with stress, the expression of 70 kDa-heat shock protein (Hsp70) was investigated. Moreover Hsp70 is also an inhibitor of apoptosis, by preventing the activation of AIF and apoptosome which stimulates caspase 3 activation. However, expression of Hsp70 was not induced in both stunned groups and unstunned groups. Together, this study found that poultry stunning does not affect T_d and myofiber apoptosis.

Introduction

Water bath electrical stunning (WES) is adopted as part of a slaughtering method for large-scale poultry meat processing. Stunning may result in various physiological mechanisms which lead to the induction of death in chickens. Cardiac arrest induced stun-to-kill method in chickens, was reported to be the fastest method to cause brain failure, within 90 s after stunning (Kettlewell and Hallworth, 1990). While decapitation required 136 s, and pre-slaughter stunning prior to cutting carotid arteries required 163 s. The slowest method (332 s) was found to be pre-slaughter stunning followed by cutting only the two jugular veins (Kettlewell and Hallworth, 1990). Stunning chickens followed by cutting only one carotid artery and one jugular vein results in brain failure within 302 s (Kettlewell and Hallworth, 1990). This study will determine whether WES is consistent with compassionate slaughter and swift killing of the chickens by investigating the application of various voltage/current during stunning on the induction of death after neck cutting.

Generally, WES results in immobilisation of physical activity of the chickens as compared to unstunned chickens. Physical activity such as wing flapping, moving the body upwards whilst being shackled and induction of muscle spasms after neck cutting may induce apoptosis as these activities resembles exercise that is known to induce apoptosis (Podhorska-Okolow *et al*., 1998; Favier, Benoit and Freyssenet, 2008; Shenkman, Turtikova, Nemirovskaya and Grigoriev, 2010). During stunning, electrical current flows from head to legs through skin, bones and muscles (i.e. myofibers). Hence lack of physical activity due to stunning induced immobilisation is expected to have an impact on myofiber apoptosis (cell death). Therefore, this paper will also provide data on the impact of stunning at different voltage on myofiber apoptosis.

Materials and Methods

Animal sacrifice

Ross broilers (n=49), which are 42 days old and weighed between 1.6 to 2.6 kg, were purchased from a commercial slaughter plant in Kuantan, Malaysia. Broilers were delivered in the morning, and slaughtered within 4 h of delivery. All slaughtering and sample collection were performed at the outdoor (atmospheric temperature between 30 to 32°C) research facilities of the Faculty of Science, International Islamic University Malaysia (IIUM), with the approval from the IIUM Research Ethics Committee and IIUM Animal Ethics Policy Committee.

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Stunning

A water bath stunner was built in the Biomedical Engineering Laboratory, National University of Malaysia by connecting relevant parts of electrical equipment (Contact Voltage Regulator, OET, Beijing and multimeter, Pro'sKit® MT-125, Taipei) which were purchased from local electrical shops. The stunner was designed to provide a square alternating current wave with a voltage range of 0 to 250 V to allow for the adjustment of voltage as used by the different slaughter plants. In this study, the stunner was preset at 30, 100, 125, 100 and 200 V, and chickens were individually stunned for 5 s, according to the method described by Hindle *et al.* (2010).

Sacrifice by neck cutting

After stunning, chickens were observed for 10 s lapse for signs of life as suggested by Farm Animal Welfare Council (1982), such as presence of breathing, constricted pupils and positive response to comb pinching. Once the chicken was confirmed to be alive, neck cutting on the ventral part of the broiler's upper neck was performed manually using a sharp knife, and severing major blood vessels whilst keeping the spinal cord intact.

Determination of time between neck cutting and chickens becoming insensibile (T_d)

Authors have used voluntary movements such as wing flapping and heartbeat as well as the absence of response from comb pinching to describe the signs of live chickens and to monitor T_d. T_d was recorded using the stopwatch as the time between neck cutting and the absence of wing flapping, pupil reflex, heartbeat, as well as the absence of response from comb pinching. The stopwatch started immediately at neck cutting and stopped when the chicken failed to exhibit all signs of life including pupil reflex and heartbeat, and was completely unresponsive to comb pinching.

Sample preparation for histology

Muscle samples from unstunned (0 V) and stunned at 30 V and 100 V were collected and fixed within 30 min postmortem. Immediately after slaughtering, samples were harvested from pectoralis major, and fixed in 10% buffered formalin in PBS overnight, dehydrated, cleared, infiltrated and embedded in paraffin.

TUNEL assay

Transverse sections (7 µm) were placed on poly-L-lysine treated slides, deparaffinised, rehydrated and processed using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) assay kit (Calbiochem, Darmstadt, Germany; catalog: QIA39). The TUNEL kit was performed according to the manufacturer's instructions. Briefly, muscle cross sections were permeabilised with Proteinase K for 20 min at room temperature, incubated with TdT buffer for 1 h at room temperature, followed by addition of TdT labelling mix. The slides were incubated at 37°C for 1.5 h, rinsed and mounted using Calbiochem® mountant. The total cell population was visualized using ultraviolet filter for DAPI, 330 – 380 nm. TUNEL-positive nuclei were analysed using a standard fluorescein filter, 465 – 495 nm and images were captured using inverted research microscope (Nikon Eclipse Ti, Tokyo, Japan).

Evaluation of caspase 3, AIF and Hsp70 proteins

The caspase 3, AIF and Hsp70 proteins were assessed using immunohistochemistry. Antigens expressed in the tissue sections were detected using rabbit polyclonal caspase 3 (catalog: Ab90437), AIF (catalog: Ab1998) and Hsp70 (catalog: Ab31010) antibodies, in 1:100 dilution for 90 min at room temperature, followed by goat polyclonal secondary anti-rabbit streptavidin-HRP conjugated enzyme (1:100 dilution; 30 min; room temperature; catalog: Ab97051). All antibodies were purchased from Abcam®, England, United Kingdom.

Statistical analysis

The effect of stunning voltage and electric current with BW, resistance and T_d; were analysed with ANOVA (post hoc with equal variances assumed using Tukey test) and Spearman's correlation. The significant differences of the stunning current, body resistance, BW and T_d between genders were tested using independent t-test. All calculations were performed using the IBM SPSS Statistics for Windows, version 20.0 (IBM Corp., Armonk NY, U.S.).

Results

Electric current during stunning and BW differed between genders

Fifteen male broilers and 34 female broilers were sacrificed for this study. Between genders, BW was significantly higher in the male broilers than the females (Figure 1A). Electric current flow during stunning was significantly lower in the females than the male broilers (Figure 1B). In contrast, chicken's body resistance was significantly higher in the females than the male broilers (Figure 1C). No difference of

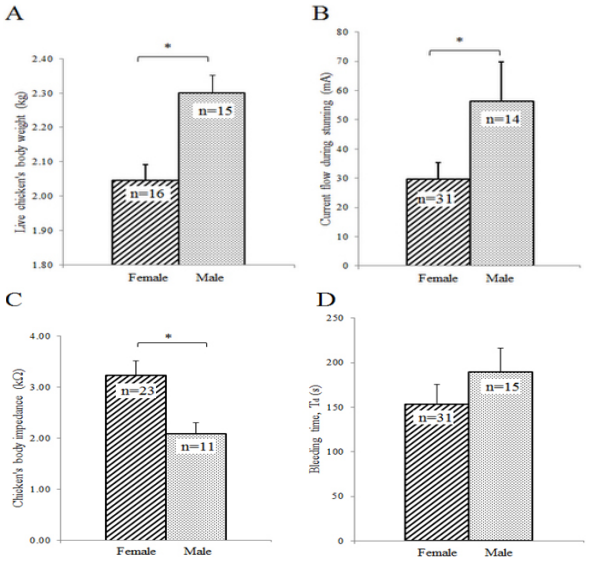

Figure 1. Differences between parameters of stunning in male and female chickens. A. Live body weight (BW) was significantly higher for male compared to female. B. Electric current flow during stunning was significantly higher for male compared to female. C. Chicken's body resistance during stunning was significantly lower in male than in female. D. The time for the chickens to become insensible upon neck cutting (T_d) was the same in both male and female. * shows $p < .05$ between male and female broilers; n, number of chickens.

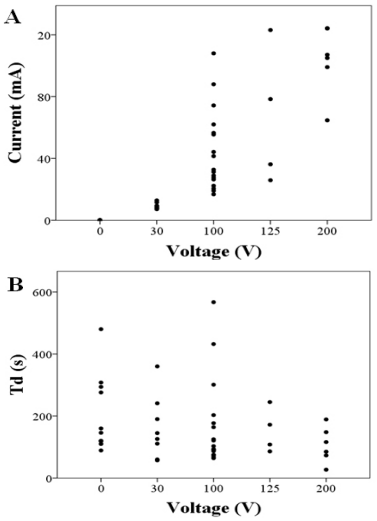

Figure 2. Dot diagrams showing the variation of: (A) current and (B) T_d when different voltages were applied during poultry stunning. T_d refers to the time between neck cutting and the point where the chicken becomes insensible, by the absence of wing flapping, pupil reflex, comb pinching response and heartbeat. No correlation was found between the T_d and the current and voltage applied during stunning. Dots represent the number of chickens slaughtered in this study.

T_d was found between genders of the broilers (Figure 1D).

Stunning did not affect T_d

When the same voltage was applied for stunning, a wide range of electric current had been observed (Figure 2A). Similarly, a wide variation of T_d was observed when chickens were stunned using the same stunning voltage (Figure 2B). Stunning parameters tested such as voltage/current and resistance were found to have no relationship with T_d (Table 1). Moreover, no correlation was found between chicken's BW and T_d either (Table 1).

Table 1. Correlations between the conditions of chickens at slaughter and stunning parameters during laboratory experiment

Parameter[1-5]	C	Volt	R	T_d	BW
T_d					
r	-.10	-.26	-.31	1	.32
P	.53	.08	.08	-	.08
N	45	46	34	46	31
BW					
r	16	.12	-.34	.32	1
P	.41	.53	.12	.08	-
N	30	31	22	31	31

[1] C = Electric current applied during stunning
[2] R = Resistance
[3] Td = Duration between neck cutting and death
[4] Volt = Voltage applied during stunning
[5] BW = Live weight of chickens
r, Spearman's correlation is significant at $p \leq .05$

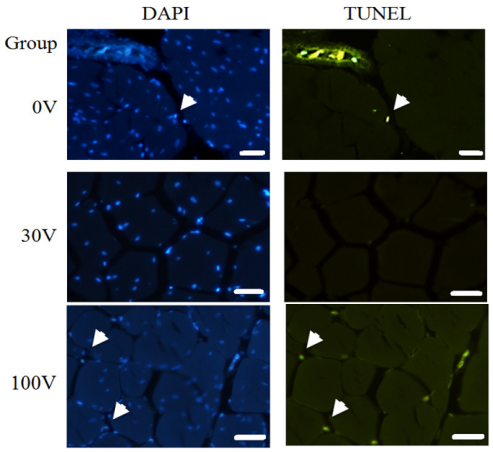

Figure 3. *In situ* terminal deoxyribonucleotidyl transferase nick end labelling (TUNEL) assay of pectoralis major muscle from stunned (30V and 100V) and unstunned (0V) chickens. Apoptotic myonuclei was not detected either in stunned or unstunned tissue sections. Nuclei were stained blue with 4',6-diamidino-2-phenylindole (DAPI), and apoptotic nuclei were stained green with fluorescence-conjugated antibody. Arrow heads indicate the apoptotic nuclei, which were mainly in the connective layers endomysium or perimysium (i.e. not myonuclei), suggesting that these apoptotic nuclei belong to interstitial cells. Bars, 25 µm.

DNA fragmentation was absent in myofibers of stunned and unstunned meat

DNA fragmentation in the muscle tissues was analysed using TUNEL assay. Myonuclei were differentiated from interstitial nuclei by the location of the nuclei distribution, i.e., any nuclei found outside the cytoplasm were considered as interstitial nuclei. Although a few of TUNEL-positive interstitial nuclei were present, no TUNEL-positive myonuclei could be detected immediately at postmortem, in either

stunned or unstunned muscle tissues (Figure 3).

Stunning did not affect expression of apoptotic proteins

Expressions of apoptotic proteins such as caspase 3, AIF and Hsp70 were analysed using immunohistochemical staining. Stunning chickens at different voltages did not affect the expression of these proteins. Expressions of housekeeping genes (β-actin and GAPDH) were also found to be similar in both stunned and unstunned tissues.

Discussion

Poultry stunning does not affect T_d

It is to be noted here that defining live or dead chicken may depend on the definition of signs of life. Referring to the state of living or dead upon slaughtering, researchers in the field of poultry science have been using the words sensible and insensible. However, drawing any conclusive statement on how to define live/sensible and dead/insensible is beyond the scope of this manuscript. In this study, T_d is defined as the time taken from neck cutting until the point when the chickens become insensible; where the latter was verified by the failing to exhibit signs of life such as wing flapping, pupil reflex, comb pinching response and heartbeat. Td therefore is an important determinant to set the time gap between neck cutting and scalding, in the commercial poultry meat processing plants, in order to prevent the processing of condemned chickens. Condemned chickens are chickens which enter the scalding tank alive.

Several reasons could be associated with the variation of Td such as chicken physiology, inefficient neck cutting or stunning parameters. In this study, we have sacrificed chickens of the same age (42 days) and with an average BW of 2.17 ± .24 kg per bird (Figure 2B). To ensure efficient neck cutting, all sacrifices were performed manually, by the severance of both jugular veins and carotid arteries.

Stunning parameters tested such as voltage/current and resistance were found to have no relationship with Td (Table 1). Although high electric current (120 mA) has been associated with 90% of instantaneous death in birds, via cardiac fibrillation (Kettlewell and Hallworth, 1990), there was no correlation between voltage/current of stunning with the T_d (Table 1).

In some religious slaughter, such as halal, chickens must be sacrificed by neck cutting, and no other means. In halal slaughtering, stunning may be allowed to facilitate the slaughtering process, provided that the chickens are still alive after coming out from the stunner. In order to avoid chickens being killed during stunning, many halal slaughter plants use low voltage/current setting instead. However, this study found that the application of low voltage/current of stunning, does not ensure rapid death of the chickens (Figure 2B).

Parameters of stunning varies between chicken's genders

Body resistance and BW of the chickens are significantly varied between genders (Figures 1A and 1C). The male broilers have larger BW ($p = .001$; Figure 1A) because of higher bone density, than the female broilers. Body resistance in the female is higher than the male broilers ($p = .015$; Figure 1C) because the females have higher fat contents, which act as electric insulator, thus reduces the electrical conductivity in the body. This is also true since the electric current flow during stunning was lower in the female than the male broilers ($p = .037$; Figure 1B).

Despite that males and female broilers have different BW and behave differently when electric current is introduced during stunning, the T_d was not significantly different between genders ($p=.33$; Figure 1D). Although the reason for this is unclear, this finding further emphasises that the determination of Td is not affected by the biophysical attributes of the chickens and the stunning parameters.

Stunning does not affect apoptosis of the meat

Electrical stimulation has been involved in increased signaling cascade activity in the cells, including the apoptotic pathway (Ugarte and Brandan, 2006; Huang *et al.*, 2011). Myofiber apoptosis can be detected by the presence of DNA fragmentation of the myonuclei. In this study, DNA fragmentation of the myonuclei by TUNEL assay was not observed in all of the muscle tissue sections, when comparing stunned and unstunned meat (Figure 3).

At cellular level, apoptosis can be induced by the activation of death receptors, or in response to critical conditions for cell survival, via either the mitochondrial pathway or receptor-mediated pathway (Figure 5). Both apoptotic pathways finally lead to caspase 3 activation. In the mitochondrial pathway, AIF is released from mitochondrion into sarcoplasm, before it enters nucleus and leads to DNA fragmentation and eventually apoptosis (Figure 5). In this study, no expression of AIF was detected in all of the myofibers when comparing stunned and unstunned meat at 1 d postmortem (Figure 4). Lack of AIF expression in all of the myofibers is consistent with the failure to detect DNA fragmentation of the myonuclei as observed in this study.

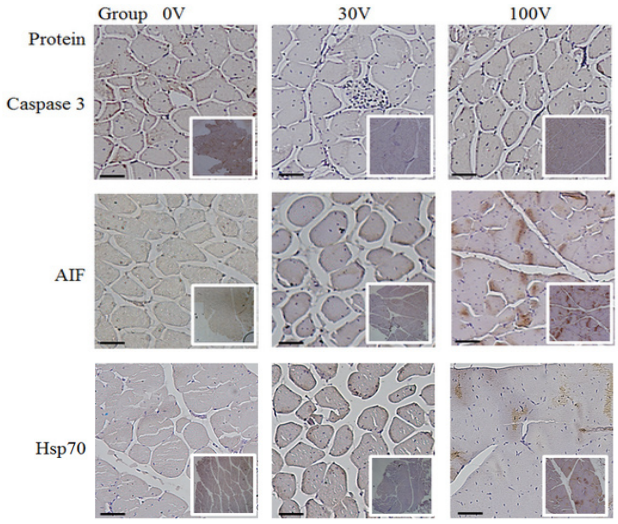

Figure 4. Presence of apoptotic regulating proteins (in 1:100 dilution) such as caspase 3, AIF and Hsp70 in myofibers at 1 d postmortem were detected using immunohistochemistry method. Expression of these proteins in unstunned chickens were similar with the muscle tissues from stunned chickens. Antibody-antigen complex were detected using 3,3'-diaminobenzidine tetrahydrochloride which produced brown-coloured substrate. Haematoxylin was used to produce purplish-coloured background staining and to distinguish the nuclei. Bars, 50 µm.

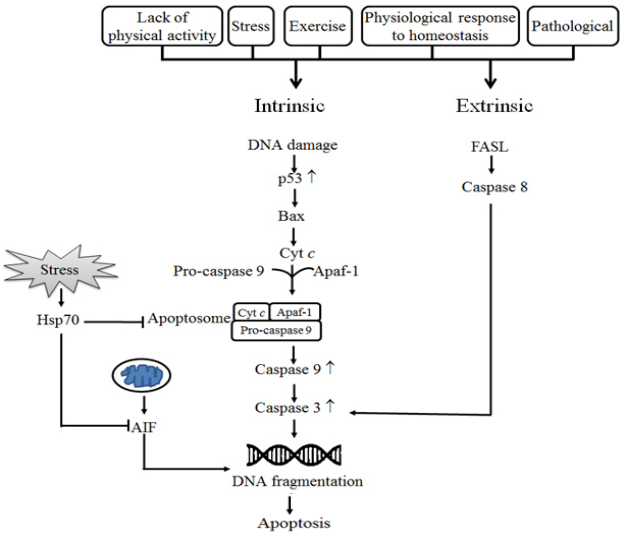

Figure 5. Apoptotic pathways in skeletal muscle. Apoptosis can be initiated by events occurring at the mitochondria and death receptors (e.g. FAS). Once initiated, this can result in the activation of proteolytic enzymes (i.e., caspases) or caspase-independent events, which can collectively lead to DNA fragmentation and cleavage of myofibrillar proteins and other cellular substrates. A number of proteins are present in the cell that can regulate these apoptotic pathways, including Bax, 70-kDa heat shock protein (Hsp70) and p53. Lack of physical activity, stress, strenuous exercise and injury in cells have been shown to increase skeletal muscle apoptotic signaling and DNA fragmentation, effects that may be mediated by several potential mechanisms, including: (i) upregulating the expression of several apoptotic proteins such as apoptotic protease-activating factor-1 (Apaf-1), Bax, caspases, Fas ligand (FasL), Hsp70 and p53; and (ii) influencing mitochondrial function such as permeability transition pore formation. Solid arrows represent activation events and solid T lines represent inhibitory events. Cyt *c*, cytochrome *c*; AIF, apoptosis-inducing factor.

Detection of cleaved caspase 3 is a sensitive indicator of apoptosis. The time of caspase 3 activation detected in paraffin tissue sections had been reported between 20 min to 48 h after the induction of apoptosis (Gown and Willingham, 2002). In this study, muscle samples were fixed within 30 min postmortem. However, lack of expression of caspase 3 suggested that apoptosis was inhibited in both stunned and unstunned chickens.

Hsp70 has a protective role to defend cells from physical stimuli, including electrical stimulation, and other biochemical changes during the process of slaughtering such as ischaemia and acidification of the meat. Hsp70 inhibits apoptosis by preventing the activation of AIF and apoptosome formation (Figure 4). Low voltage (13V) and short electrical stimulation has been shown to induce moderate Hsp70 response compared to prolonged electrical stimulation in mouse myoblasts (Wang, Liu, Jin, and Steinacker, 2010). Thus, since both AIF and caspase 3 expressions were inhibited (Figure 3), it was thought that Hsp70 was induced during the slaughtering process which subsequently prevented apoptosis. However, Hsp70 were also not overexpressed at 1 d postmortem, in stunned as well as unstunned meat. This could be due to the very low level of Hsp70, thus undetectable by the method used in this study. Hsp70 is also a known protein with short-life expand, which expression might have been missed during the time of preserving the tissues. Another possible reason is that other upstream proteins could be involved in the inhibition of apoptosis during stunning and slaughtering.

Conclusion

WES, a common step in poultry meat production, has been suggested to promote swift slaughtering. However, this study concluded that the time for the chickens to become insensible after neck cutting (T_d) was not affected by stunning. Moreover, analysis at cellular level to compare stunned and unstunned meat revealed that apoptosis was inhibited, at 0 h postmortem. Future work can analyse the Hsp70 mRNA level, or other stress proteins which may inhibit apoptosis. Postmortem changes of the meat can be investigated further by comparing the structure of the stunned and unstunned meat during chilled

storage period.

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