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Muscle Creatine Kinase (M-CK), Aldolase A and Voltage Dependent Anion Channel 2 (VDAC2) as Potential Biomarkers for Identification of Electrically Stimulated Chicken Muscle

Azura Amid[1-3]*, Norshahida Abu Samah[1] and Faridah Yusof[2]

[1]Department of Biotechnology Engineering, Kulliyyah of Engineering, International Islamic University Malaysia, P.O. Box 10, 50728 Kuala Lumpur, Malaysia.
[2]Bioprocess and Molecular Engineering Research Unit, Kulliyyah of Engineering, International Islamic University Malaysia, P.O. Box 10, 50728 Kuala Lumpur, Malaysia
[3]International Institute for Halal Research and Training, International Islamic University Malaysia, P.O. Box 10, 50728 Kuala Lumpur, Malaysia.

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Water bath stunning in commercial slaughter houses is a common practice and economical. However, the conditions applied for the stunning process may vary from one slaughterhouse to another and has opened up doors for food adulteration to take place. In this study, proteomics approaches have been used to study the effect of different current and voltages during stunning on the protein expression of the chickens. Protein profiles of the chickens muscle were constructed in order to detect any differences in protein expression and modifications. The protein muscle profiles of stunning conditions were compared to the non-stunned. After analysis using 2D Platinum ImageMaster 6.0 software and mass spectrometry identification, Muscle creatine kinase (M-CK), AldolaseA and Voltage Dependent Anion Channel 2 (VDAC2) were identified to be over expressed in the muscle sample of stunned chicken. The over expression of candidate proteins was confirmed at the transcriptional level of RNA expression by Real Time PCR. In conclusion, all three candidate proteins may serve as potential biomarkers for electrically stimulated chicken's muscle. The existence of these biomarkers will help to monitor the slaughtering and stunning process in future.

Key words: Aldolase A, Muscle Creatine Kinase, Stunning, Proteomics, Voltage Dependent Anion Channel 2.

Stunning renders animals insensible during slaughtering procedures. The efficiency of electrical stunning depends on the control of parameters such as voltage, amperage, current frequency and immersion time. Main adverse effects associated with high current or voltage electrical stunning are wing hemorrhages, red skin conditions including red wing tips and pygostyles, poor plucking, broken bones and blood blemishes or blood coagulates in breast muscle[1].Kuenzeland co-workers[2]reported that bleeding efficiency declined when the frequency of a pulsed direct current square waveform was increased beyond 240 Hz. According to Gregory and co-workers[3] high frequency currents during stunning might be expected to cause less physical spasm at the beginning of the current flow. The duration of the initial tonic phase can be reduced for frequencies greater than 75 Hz[4].

Gregory and co-workers[3]studied the effect of using unipolar square wave frequencies (direct current of 50, 200 and 350 Hz) with currents between 71 to 206 mA with an average of 131 mA.

* To whom all correspondence should be addressed.
E-mail: azuraamid@iium.edu.my

They found that the number of defects present in the carcasses was not affected at these frequencies and current range. A current with intensity less than 130 mA would reduce the incidence of convulsions and hemorrhages in birds[1]. However, using a current intensity of 150 mA at 50 Hz as observed by Santeand coworkers [5] will result in more birds being killed by ventricular fibrillation instead of rendering them unconscious. It was shown that blood loss was drastically reduced by cardiac arrest and it was studied that increasing the current frequency during stunning could reduce cardiac arrest. Gregory and co-workers[3]determined the effect of the stunning current frequency during stunning on broilers and hens. When a sinusoidal alternate current was used, 50 Hz killed some of the birds, whereas with corresponding root mean square currents, 1500 Hz caused no killing due to ventricular fibrillation. Lee and colleagues[6]indicated that the breasts of stunned birds would be tendered than those from unstunned birds, showing 30 % lower shear force values. In another study conducted by Papinaho and Fletcher[7], the effect of constant amperage electrical stunning was determined on broiler blood loss, post mortem breast muscle rigor development and breast meat quality. Those broilers were individually stunned for 5 seconds at 0, 50, 100, 150 and 200 mA. In this study, it was found out that stunning amperages between 0 and 200 mA had effects on the rate of early rigor development but there were no consistent effects on final breast meat quality. It was observed that stunning amperage had no effect on percentage blood loss and the most rapid post mortem reactions was shown by samples on non-stunned control group.According to Gregory and co-workers[3] 99 % of the birds would show ventricular fibrillation with currents greater than 110 mA at 50 Hz. Contreras and Beraquet[9] reported that in a study ranged from 20 to 125 mA, only birds stunned at 100 V, 60 Hz at 125 mA could experience ventricular fibrillation.

During electrical stunning, currents travel through the paths of least resistance from skin, skeletal breast muscle, cardiac muscle and leg muscle[10]. As a result, muscle contractions that include tonic spasms and tremors are stimulated. Simultaneously, heart and respiratory rates decrease and blood pressure increased[2]. In recent years, there has been a tendency in Europe to implement high voltage stunning primarily based on humanitarian grounds. When compared to low voltage stunning (in the range of 30 to 60 V, 20 to 45 mA/bird), high voltage stunning (150 V, 100 mA/bird) induces heart fibrillation and cardiac arrest and therefore results in rapid death[11].

In the United States of America, out of 329 poultry plants surveyed, 92.1 % utilized electrical stunning as the method of pre-slaughter immobilization. Low voltage (10 to 25 V) and high frequency (500 Hz) systems were used in 77.4 % of these plants. Such low voltage is in contrast to high voltage and current systems utilized in Europe and other parts of the world. In a study conducted by Contreras and Beraquet[9], it was found that the most efficient stunning condition for blood loss is at 40 V which resulted in 55.3 % blood loss that was higher than the 35 to 50 % reported by Newell and Schaffner[12] and the 35 to 50 % blood losses obtained by Potsubay and Duduck[13]. It was also observed that only when currents used were above 60 mA, 80 V, the birds showed symptoms corresponding to the end of respiration, retention of muscular tonus and death. The proportion of birds showing these conditions appeared to be greater for the 100 V treatments inferred that the reduction in blood loss with higher voltages could be due to a higher incidence of birds with ventricular fibrillation. However, rapid death before slaughtering caused by ventricular fibrillation is not allowed for certain community. Therefore, this study aims to identify biomarkers that would differentiate chicken muscle produced from unconscious chicken during stunning from those that already dead during the procedure.

MATERIALS AND METHODS

Experimental Design

The experimental design and most methods were similar to our previous manuscript[14]. Female broiler chickens aged 35 days and weighing approximately 1.5 kg were collected from a farm in Kuang, Selangor, Malaysia, electrically stunned, and slaughtered.The two parameters studied for stunning were current and voltage with three levels each: 0.25 A, 0.5 A, and 0.75 A for the current and 10 V, 40 V, and 70 V for the voltage. Combinations of the two parameters resulted in nine different

treatments: (0.25 A, 10 V), (0.25 A, 40 V), (0.25 A, 70 V), (0.5 A, 10 V), (0.5 A, 40 V), (0.5 A, 70 V), (0.75 A, 10 V), (0.75 A, 40 V), and (0.75 A, 70 V). These treatments of stunning were compared to the control which is the non-stunned sample (0 A, 0V). The amount of current was monitored by an ammeter. The treatments for stunning were designed using 3 level factorial design (Design Expert Software Version 6.0.8). Two biological replicates were studied with three replicates for each sample. A water stunner was designed to supply different sets of current and voltage. A glass aquarium was equipped with copper plate in the size of 56.5 cm x 28.5 cm x 28.5 cm. Positive terminal of the circuit was immersed in the copper plate of the aquarium while negative terminal is attached to the shackle which is used to hang the chicken's leg. Each chicken was stunned for duration of 5 seconds.

Sample Preparation

Muscle sample was homogenized in 10 volumes of phosphate buffer (250 mM of PO_4^+ buffer, pH 7.5, 0.01 % Triton X-100). The homogenate was centrifuged at 4ºC, 10 000 x *g* for 15 minutes and the supernatant was retained and stored at -80ºC until needed. The protein content of each sample was assayed using Bradford Assay[15].

Two-Dimensional Gel Electrophoresis (2-D GE)

Ten microgram (10 µg) of sample was added to the sample buffer (7 M Urea, 2 M Thiourea, 4 % CHAPS, 3 mg/ml DTT, 0.5 % Pharmalyte) resulting in a total volume of 170 µl. After that, 170 ìl of rehydration buffer (7 M Urea, 2 M Thiourea, 4 % CHAPS, trace amount of Bromophenol blue, 3 mg/ml DTT, 0.5 % Pharmalyte) was added and the mixture was left at room temperature for 30 minutes. Isoelectric focusing was conducted onIPGphor 3(GE Healthcare, Sweden) according to Amidand co-workers[14]. After electrophoresis, the gel was carefully removed from its cassette and stained with silver nitrate solution for gel visualization. After staining was completed, the gels were scanned by using ImageScanner III LabScan 6.0 software (GE Healthcare, Sweden). The scanner was set at 300 dpi, transparent mode with green filter.

Proteomic AnalysisofImageMaster Platinum 6.0

Protein spots were detected and analyzed using ImageMaster 2D Platinum 6.0 software (GE Healthcare, Sweden). After proteomics software analysis, the spots of interest were identified. The sample was run again on the same conditions and stained with Coomassie Brilliant Blue prior subjected to MALDI-TOF mass spectrometry analysis. The excised spot from the gel was digested with trypsin and subjected to Zip Tip purification.

MALDI TOF Mass SpectrometryAnalysis

After ZipTip treatment, the spot was analyzed by MALDI TOF/TOF Mass Spectrometer (Applied Biosystems, USA). Both MS and MS/MS spectra were recorded in the combination mode. In MS spectrum, 1800 shots were accumulated and 4500 shots were accumulated for each MS/MS spectrum. Protein was identified from their accumulated MS and MS/MS spectra by manual searching using a locally implemented MASCOT server and compared to SwissProt (http://web.expasy.org/docs/swiss-prot) and NCBI (www.ncbi.nlm.nih.gov) database.

Validationof Proteomics Resultby Real Time PCR

In order to confirm the identified candidate proteins through proteomics analysis, a validation was conducted on the transcriptional level of RNA. RNeasy Lipid Tissue Mini Kit (Qiagen, German) was used for the extraction of total RNA. After the extraction of total RNA, the complementary DNA was synthesized using SuperScript III CellsDirectcDNA Synthesis System (Invitrogen, USA). The single-stranded cDNA concentration was measured and stored at -20°C for further PCR amplification.Potential proteins that were identified to be positively expressed in relation to stunning treatment were further studied at the RNA level. The genes of interests identified from muscle sample areMuscle creatine kinase (M-CK), Aldolase A and Voltage dependent anion channel 2 (VDAC2). The housekeeping gene chosen for this study was 18S rRNA. The complete sequence for each gene was obtained from NCBI database and primers of the potential biomarkers as well as the housekeeping gene were designed using Primer3 free software (hhtp://Frodo.wi.mit.edu/primer3/). Real Time PCR was conducted using SYBR Green as the detection method with dissociation curve. Dissociation curve will determine whether the amplification is free from contaminants or not. The results were analyzed using MxPro software (Stratagene, USA).A sample

of known concentration is used to generate a standard curve that gives the equation of Ct value versus Initial Quantity (relative) of the amplified gene. This equation for standard curve is applicable in calculating the fold change of the targeted gene[16]. For each experiment, 18S rRNA gene was used as the reference gene, and the non-stunned chicken muscle cDNA was the control sample.

RESULTS

It was observed that in 0.75 A, 70 V stunned chicken (Figure 1 a), more verified spots are detected in the sample suggesting that higher current and voltage induced the over expression of proteins in muscle. These verified proteins exclusively present in stunned samples but absent in the non-stunned samples (Figure 1 b). These proteins were also detected at lower protein intensity in the sample of 0.5 A, 40 V (Figure 1 c). Therefore, these verified proteins could be considered as proteins where expression is influenced by the amount of electrical current and voltage given. To confirm the identified spots are true biomarkers, scatter plot analysis was carried out.

In Figure 2, for instance, gel number 97584 is the reference gel for 0.75 A, 70 V stunned chicken and displayed on the Y axis while gel number 97567 which is plotted on the X axis represents the non-stunned (0 A, 0V) chicken. The number of matched spot displayed when gel number 97584 is plotted against gel number 97567 is 214 with data correlation of 0.907. Therefore, the two gels are

Table 1. List of primers designed for genes related to potential proteins identified from muscle sample

Target gene	Left primer	Right primer	Product size
M-CK	5'-tgttcgataaacccgtctcc-3'	5'-tcaggtggtcctcctcattc-3'	129
Aldolase A	5'-agggagaaaaaggggactctg-3'	5'-cacgagaacacgacatgagc-3'	150
VDAC2	5'-tggattccaccgcttctatc-3'	5'-cagcattgatgctctttcca-3'	132

Table 2. Intra class report of potential proteins from muscle sample

Protein	Mean (100 %)	M.S.D	Variation	Protein Intensity 1	Protein Intensity 2	Protein Intensity 3
h	0.7292	0.2309	0.3166	0.7721	0.4275	0.9880
i	0.3001	0.2313	0.7707	0	0.5628	0.3374
l	0.4552	0.3318	0.7289	0.5840	0.7815	0

Table 3. MALDI-TOF analysis of potential proteins from muscle sample

Spot ID	Protein name	Accession No	Protein Mw	Protein Score	Protein Score C.I %	Total Ion Score	Total Ion Confirmed C.I %
h	M-CK	gil45382875	43529.1	452	100	336	100
i	Aldolase A	gil409191	4438.1	223	100	206	100
l	Voltage-dependent Anion Channel 2	gil46048903	30293.3	460	100	394	100

Table 4. Real Time PCR of potential proteins from muscle sample

Spot ID	Protein Name	Gene name	Ct Value Sample	Ct Value Control	Ct Value Difference	A value	Fold Increment
h	M-CK	M-CK	16.26 ± 0.26	16.73 ± 0.16	1.09	-3.258	4.65
i	Aldolase A	Aldolase A	26.44 ± 0.73	27.53 ± 0.29	1.09	-3.671	13.10
l	VDAC2	VDAC2	22.74 ± 0.24	24.18 ± 0.24	1.44	-3.237	59.87

highly correlated as correlation near to 1. Any new spot emerged from gel number 97584 is a true biomarker.

The results were further verified by performing intraclass analysis (Table 2). It is shown that protein h has a mean of 0.7292 with mean standard deviation of 0.2309 and variation of 0.3166. The intensities of protein h were determined to be 0.7721, 0.4275 and 0.9880. Whereas, the mean of the spot intensity for protein i is 0.3001 with mean standard deviation of 0.2313 and variation of 0.7707. The spot intensities detected on two out of three

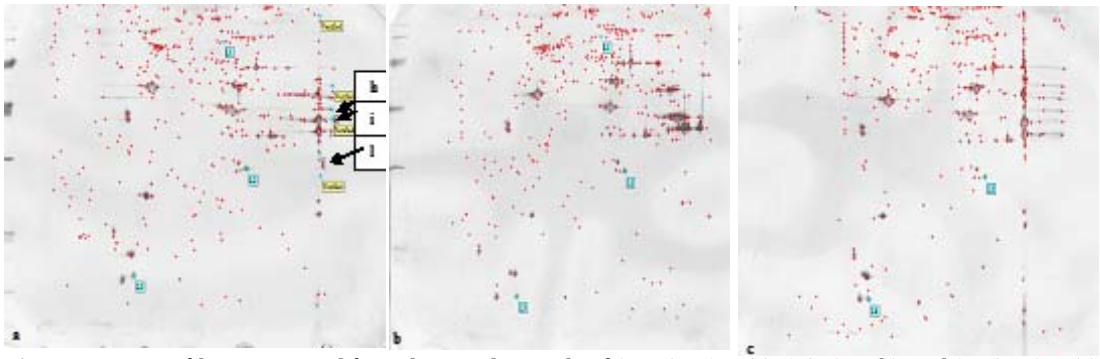

Fig. 1. Protein profiles constructed from the muscle sample of 0.75 A, 70 V (a), 0 A, 0 V (b), and 0.5 A, 40 V (c) chickens respectively. Each protein spot is represented by a red crossed sign in the protein map. The crossed spot shape draws a cross in the centre of gravity of each spot. The protein map was matched to other maps of different stunning treatments by indicating few landmarks to ensure correct positioning of the gel maps. Landmark (L1, L2, L3, and L4) are predefined label category displayed in blue background to mark spots in the gels which serve as reference points. This is purposely for gel alignment or matching and two annotations are considered as referring to the same point in different gels when they bear identical labels of the same category.

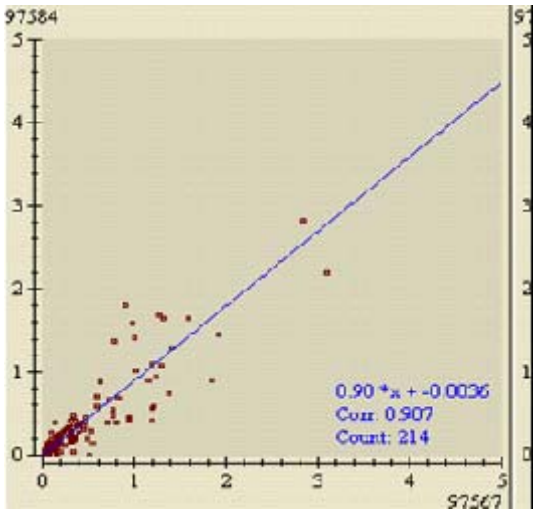

Fig. 2. Scatter plot of muscle sample. Scatter plot is a feature used to analyze gel similarities or experimental variations such as disparities in stain intensity or sample loading for matched spots in sets of gels. The correlation value near to 1 indicates that the data of the two gels are highly correlated. The Y axis plotted gel number 97584 which is the reference gel for 0.75 A, 70 V stunned chicken while gel number 97567 which is plotted on the X axis represents the non-stunned (0 A, 0V) chicken

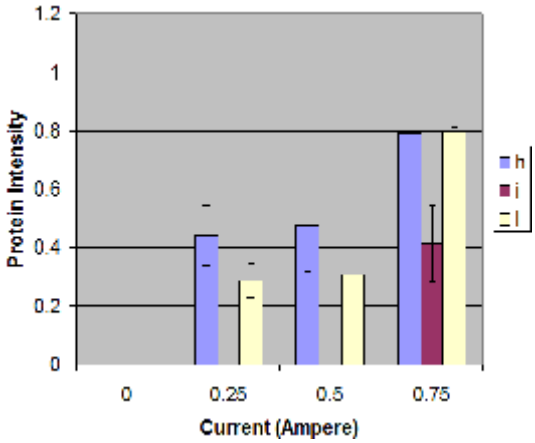

Fig. 3. Bar chart for the three verified protein spots identified from muscle samples of electrically stunned chickens. The intensity of the protein was determined to be directly related to the amount of current given in the stunning treatment. Protein h shows increment from 0.44 to 0.47 to 0.79 as the current is increased from 0.25 A to 0.5 A and finally to 0.75 A. Meanwhile, protein i is only detected in 0.75 A sample with protein intensity of 0.41. Protein l showed 2.76-fold increment in intensity from 0.29 to 0.31 and finally to 0.80 as the current is increased from 0.25 A to 0.75 A.

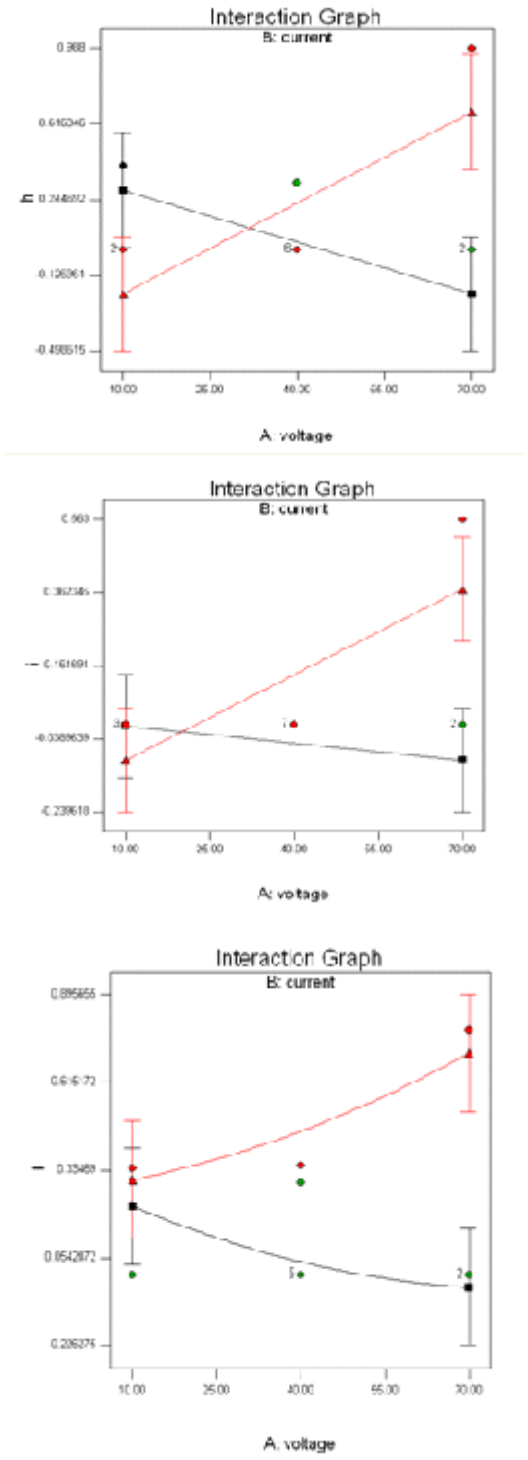

Fig. 4. Interaction graph of (a) protein h (b) protein i (c) protein l. The interaction graph plots Protein Intensity versus Voltage (V). The red plot represents 0.75 A, while the black plot represents 0.25 A

over stunned gels are 0.5628 and 0.3374. Meanwhile, all three replicates of non-stunned samples show disappearance of protein i (Figure 1). Protein l has a mean of 0.4552 with mean standard deviation of 0.3318. The variance for protein l is 0.7289 with intensity of the protein spot detected in two out of gel triplicates at 0.5840 and 0.7815.

After proteomics analysis, there are three potential spots that shown consistent pattern in term of spot intensity to the current given in stunning treatment. The spots were identified as Muscle creatine kinase (M-CK), Aldolase A and Voltage dependent anion channel 2 (VDAC2) via MALDI TOF mass spectrometry. Protein h was identified as Muscle creatine kinase with molecular weight of 43,529.1 Da. Meanwhile, protein i was identified as Aldolase A of 4,438.1 Da and, protein l was identified as Voltage dependent anion channel 2 with molecular weight of 30,293.3 Da. All three identified proteins shown 100 % of total ion confirmed (Table 3).

Figure 3 shows protein h spot intensity increased from 0.44 to 0.47 and eventually to 0.79 as the current was increased from 0.25 A to 0.5 A and finally to 0.75 A. Meanwhile, protein i was only detected in samples obtained from chicken's muscle treated with 0.75 A with protein intensity of 0.41. A complete disappearance was observed in

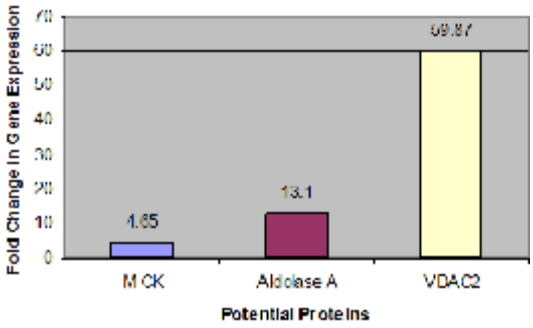

Fig. 5. Fold change of Muscle creatine kinase (M-CK), Aldolase A and Voltage dependent anion channel 2 (VDAC2) genes expression. The fold change was calculated based on the RNA expression determined by Real Time PCR. *M-CK* gene shows fold change of 4.65 while *Aldolase A* gene shows fold change increment of 13.1. *VDAC2* exhibits the highest fold change increment with 59.87 in over stunned chicken. Gene expression on all three genes confirmed the findings from MALDI-TOF analysis. An exclusive upregulated genes in 0.75 A, 70 V stunned chicken muscle suggests high potential of such proteins as biomarkers for detection of stunned chicken meat

samplesobtained from chicken's muscle treated with0 A (non stunned), 0.25 A and 0.5 A stunned. Protein l intensity increased from 0.29 to 0.31 and finally to 0.80 as the current is increased from 0.25 A to 0.5 A and to 0.75 A.

Interaction Graph was employed to further validate the potential proteins identified in the proteomic analysis. Figures 4 (a), (b), and (c) show the interaction plots of protein h, i, and l, respectively. The graphs were generated from the Design Expert software via 3-level factorial design. It is shown that the intensity of protein h, i, and l is increasing with the increase of voltage when current was maintained at 0.75 A. Meanwhile, when the current applied is 0.25 A, the pattern is decreasing. The analysis of variance (ANOVA) shows that for protein h, only interaction of voltage and current significantly influenced the protein intensity with p value of 0.0104. Interaction of voltage and current also shows significant influence on protein i intensity (p value = 0.0300). As for protein l, both current and interaction of voltage and current show significant effect with p value of 0.0048 and 0.0336 respectively.

After MALDI TOF identification, the expression levels were further confirmed at the transcriptional level by Real Time PCR (Figure 5). Primers for the MALDI TOF identified proteins were designed (Table 1).*M-CK* gene shows fold change of 4.65 while *Aldolase A* gene shows fold change increment of 13.1 in over stunned chicken. Meanwhile, *VDAC2* has shown the highest gene upregulation with fold change increment of 59.87 (Table 4). Gene upregulation on the three genes confirmed the findings from MALDI-TOF analysis, thus, suggesting the proteins as promising biomarkers in identification of electrically stunned chickenmuscle or meat.

DISCUSSION

There are three potential biomarkers identified from muscle sample which are Muscle Creatine Kinase (MCK), Aldolase A, and Voltage-dependent Anion Channel 2 (VDAC2). The muscle specific form of creatine kinase (MCK) is critical for energy metabolism of skeletal muscle. This enzyme acts as the key enzyme in the phosphorylcreatine shuttle between mitochondria and the myofibrils[16]. The M-form of this enzyme (MCK) is a characteristic of an adult skeletal muscle and it has specific binding properties to the M-line of myofibrils[18]. A rapid increase in MCK protein has been observed to follow a parallel increase in MCK mRNA levels[19]. An increase in MCK has been correlated to the increase of α-actin, myosin heavy (MHC) and light chains, troponins, and tropomyosin[20]. All of the above mentioned proteins are classified as contractile proteins. Their expressions are interrelated suggesting that there is a single regulatory event or complex series of events that coordinate the protein regulation[21]. Previously, α-actin cardiac muscle and troponin I were observed to have upregulation in expression following electrical stimulation of the hearts of chickens[14].

Crosand co-workers[22] discovered that MCK and Glyceraldehyde-3-phosphate dehydrogenase have been upregulated in case of muscle disuse. Muscle disuse is a very common situation that occurs each time a muscle remains inactive for an extended period such as during limb immobilization or bed rest. In studies using animal models, physical immobilization is considered as one of the treatment for muscle disuse. One of the methods of physical immobilization is the use of electrical treatment in stunning. This physical immobilization will result in a spectacular loss of muscle mass especially in slow-twitch muscle which is normally counteracts gravity[23]. Atrophy occurs following muscle disuse as an adaptation consequence. Such adaptations cause the muscle to lose strength but gain some fast-twitch features such as an increase in maximum unloaded shortening velocity, higher myosin adenosinetriphosphatase activity, and faster contraction/relaxation times[24,25]. Progression of atrophy will control the size of muscle fibers and their fast or slow twitch properties. Fast twitch is capable in generating short but intense contractions while slow twitch is responsible for the weaker but longer types of contraction. These contractile protein modifications occur concomitantly with increases in the ratios of glycolytic to oxidative enzyme activities[26,27].The oxidative capacity of skeletal muscle is normally reduced with a marked increase in the glycolytic metabolism. Crosand co-workers[22]found that MCK is the earliest marker at both mRNA and protein levels. This protein is categorized as metabolic

proteins and upregulatedprior to the upregulation of the contractile proteins such as actin and myosin. MCK protein upregulation was observed to be exactly paralleled to the progression of atrophy, therefore, constituting an excellent marker for skeletal muscle adaptations to disuse.

Meanwhile, Aldolase A is a glycolytic enzyme cleaving D-fructose 1,6-biphosphate to dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate by retro aldol reaction. This protein is approximately 39.3 kDa and the most common localization of aldolase A is in the cytosol, but recent studies have detected aldolaseA associated with the plasma membrane of erythrocytes under hypoxic conditions[28]. Increased expression of aldolase A under iron deprivation could be involved in the upregulation of ATP production by glycolysis because iron deprivation can lead to impaired oxidative phosphorylation due to the impaired function of iron-containing enzymes of oxidative phosphorylation[29]. In addition, Kondohand co-workers[30]showed that glycolytic enzymes play an important role in cell protection from oxidative stress when oxidative phosphorylation is impaired. Semenzaand co-workers[31]reported that pharmacological manipulation of glycolytic pathway such as in Aldolase A by targeting Hypoxia-inducible factor 1 (HIF-1) may provide a novel approach to the treatment of cancer and ischemic heart disease. Aldolase A is a type of glycolytic enzyme which will be induced in case of hypoxia. The induction of glycolytic gene expression in response to hypoxia may be relevant to the phenomenon of myocardial hibernation, a local contractile defect observed in patients with chronic myocardial ischemia in which affected tissue is akinetic but viable[32]. In line with this finding, it is relevant to have an elevated amount of expression in Aldolase A which is a glycolytic enzyme following an electrical treatment on chicken which will trigger enhanced rate of heartbeat.

Voltage dependent anion channel (VDAC) has several isoforms which are VDAC-1, VDAC-2 and VDAC-3. This protein is a major component of the outer mitochondrial membrane, and highly conserved large conductance anion channel involved in fluxes of ions and metabolites across the outer mitochondrial membrane[33]. VDAC accumulation has been associated with dystrophic neurites of â-amyloids plaques in Alzheimer's disease. VDAC provides the main trans-membrane transport of ions, ATP and other metabolites through the outer mitochondrial membrane. Therefore, it can be suggested that altered VDAC results in disablement of bidirectional energy fluxes across the mitochondrial membrane[34].

Valisand co-workers[35]showed that Aldolase A and VDAC2 levels were upregulated under iron deprivation in K562 (Human erythromyeloblastoid leukemia) cells by high resolution 2D electrophoresis and confirmed via Western blot analysis. Iron acts as transition metal enabling electron transport and redox reaction and thus, is essential in all living organisms. Iron is the key component of many cellular proteins including enzymes and regulatory proteins and it is required for cellular processes such as DNA synthesis and ATP production[36,37].Aldolase A and VDAC2 are two membrane proteins. VDAC2 is a relatively small protein with approximately 30 to 38 kDa. VDAC can exist in multiple conformational states which are able to transport anions such as chloride, phosphate, citrate, succinate, ATP, and superoxide. The cations that transported via VDAC are Ca^{2+}, Mg^{2+}, K^{+}, and Na^{+}[38,39,40].

VDAC2 is capable of transporting citrate and divalent cations, and thus, could play a role as ferric citrate receptor, a ferric citrate NADH-dependent reductase, and a transporter of Fe^{2+} and citrate. Iron deprivation is known to stimulate hypoxia and hypoxia induces membrane lipid peroxidation[41]. Stark[42]studied that membrane lipid peroxidation results in the activation of VDAC-mediated transport. VDAC is also useful in regulating some enzymes that involved in redox status control, such as, copper and zinc containing superoxide dismutase[43].Cheng and co-workers[44]studied that VDAC2 co-evolved to regulate cell death and VDAC2 has a distinctive physiological role *in vivo* as a specific inhibitor of BAK (B-cell leukemia/lymphoma 2 homologous antagonist/killer)-dependent mitochondrial apoptosis. Chandra and co-workers[45]suggested that an increase in VDAC2 complex formation in stimulated HCT116 (colonic epithelial) cells might be considered as a prosurvival mechanism activated by apoptotic stimuli. However, in HCT116-Bax-KO cells, the putative prosurvival mechanism of VDAC2 oligomerization is

diminished upon apoptotic stimulation, thus, allowing Bak to play a full-throttle role in mediating apoptosis.

In other studies conducted by Turko and Murad[46], Mostynand co-workers[47]and McCabe[48], VDAC has been associated with type 2 diabetes mellitus. Yuqiand co-workers[49]concluded that an increased expression of mitochondrial VDAC and subcellular co-localization of VDAC/Bax increases mitochondrial permeability and apoptosis. The increased of VDAC expression highlight the potential importance of mitochondrial genes in the etiology of hypertension. This is due to the function of VDAC as a highly conserved protein located on the outer membrane. All metabolites that enter and leave mitochondria must cross the mitochondrial outer membrane through VDAC. VDAC shows both ion selectivity and voltage dependence[50]. In open state, anions are favored over cations, but the selectivity is relatively weak. Both positive and negative membrane potentials close VDAC. The voltage effect is symmetrical, and half maximal closure occurs at about 50 mV[51].

VDAC closure very effectively blocks movement of organic anions, including respiratory substrates, creatine phosphate, ATP, ADP, and Pi[52]. VDAC closure also help to explain inhibition of metabolite movement during anoxia and the persistent suppression of mitochondrial function in cytopathic hypoxia during septic shock and multiple organ failure[53,54,55]. VDAC seems to act as a brake on mitochondrial metabolism. In other instances, dynamic regulation of VDAC conductance by glucose-metabolizing enzymes may control insulin secretion in pancreatic beta cells and aerobic glycolysis in cancer cells. However, excessive VDAC closure may also promote apoptosis, cytopathic hypoxia, fatty liver disease and other pathophysiological changes. Lemasters and Holmuhamedov[51] viewed VDAC as a governator that establishes set points for global mitochondrial activity that change in response to cellular needs and metabolic stresses.

Asadi and co-workers[56] reported the difference expression level of Vascular endothelial growth factor (VEGF), which is a potent angiogenic factor that stimulates wound healing in sensory (direct current, 600 μA) and motor (monophasic current, pulse duration 300 microseconds, 100 Hz, 2.5 – 3.0 mA) intensities of cathodal electrical stimulation in male Sprague-Dawley rats. On the seventh days after surgical incision, it was observed that the skin VEGF expression was significantly elevated in the sensory group. Arakawa and co-workers[57]proposed that electrical stimulation with 4 mA rectangular pulses of 0.5 ms duration at 2 Hz lasting for 1 hour would be a potential treatment for preventing atrophy of denervated skeletal muscles. Fujita and co-workers[58]reported that although electrical stimulation is effective to prevent muscle atrophy, the combination of electrical stimulation and isometric contractions have further effect. Brighton and co-workers[59] discovered that electrical stimulation of cultured cartilage explants resulted in significant increases in proteoglycan and collagen production and a highly significant upregulation of aggrecan and type II collagen mRNA expression. These findings support the contention that delivery of a specific, defined electrical field to articular cartilage could result in matrix preservation. Meanwhile, stimulation of higher current and voltage will result in upregulation of expression in proteins related to muscle disuse and hypoxia.

CONCLUSION

Muscle creatine kinase, Aldolase A, and Voltage dependent anion channel 2 have been seen to be elevated in the sample of chicken's muscle treated with 0.75 A, 70 V current. All three proteins are completely absent in sample of electrically non-stimulated chicken's muscle. Muscle creatine kinase showed gradual increment of protein intensity from 0.44 to 0.47 and eventually to 0.79 when current was increased from 0.25 A to 0.5 A and finally to 0.75 A. Meanwhile, Aldolase A was only detected in sample of 0.75 A with protein intensity of 0.41. Voltage dependent anion channel 2 showed increment of protein intensity from 0.29 to 0.31 and finally to 0.80 as the current was increased from 0.25 A to 0.5 A and to 0.75 A. When confirmed at the transcriptional level, Muscle creatine kinase showed gene upregulation of 4.65-fold change in 0.75 A, 70 V stunned sample while Aldolase A showed gene upregulation of 13.10-fold change. Voltage dependent anion channel 2 showed the highest gene upregulation of 59.87. Therefore, Muscle creatine kinase, Aldolase A, and Voltage

dependent anion channel 2 are positively expressed in correlation to the electrical stimulation during the stunning procedure before slaughtering.

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