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RESEARCH ARTICLE

Role of cardiac hypoxia in the pathogenesis of sudden death syndrome in broiler chickens – A metabolic and molecular study

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ABSTRACT

Sudden death syndrome (SDS) is an economically important disorder in broiler chickens with unknown aetiology. The aim of the present study was to evaluate the metabolic and molecular alterations related to hypoxia in the myocardium of broiler chickens with SDS. Samples from the cardiac muscle of internal control broiler chickens (ICs) ($n = 36$) and chickens having died of SDS ($n = 36$) were obtained during the rearing period. The activities of lactate dehydrogenase (LDH) and creatine phosphokinase (CPK) and the concentration of lactate were measured in the cardiac tissue using available commercial kits. The expression of hypoxia-inducing factor 1α (HIF1α), glucose transporter 1 (GLUT1), pyruvate dehydrogenase kinase 4 (PDHK4) and monocarboxylate transporter 4 (MCT4) genes was determined in the myocardium by real-time PCR analysis. The results showed the elevation of lactate level and activities of LDH and CPK in the cardiac muscle of SDS-affected chickens compared with the IC birds ($P < 0.05$). The cardiac muscle expression of HIF1α, MCT4 and GLUT1 genes was increased, while the PDHK4 mRNA level was decreased in the SDS-affected group compared to those in the IC chickens ($P < 0.05$). Our results showed that metabolic remodelling associated with hypoxia in the cardiac tissues may have an important role in the pathogenesis of cardiac insufficiency and SDS in broiler chickens.

KEYWORDS

sudden death syndrome, broiler chicken, cardiac tissue, hypoxia, metabolism

INTRODUCTION

Sudden death syndrome (SDS) or flip-over disease is a metabolic disorder that commonly affects well-nourished chickens with high growth rate (Siddiqui et al., 2009; Crespo and Shivaprasad, 2013). The prevalence of SDS has been reported to be 0.5–4% with the greatest losses occurring from 2 to 4 weeks of age (Siddiqui et al., 2009; Crespo and Shivaprasad, 2013; Basaki et al., 2016). Affected chicks do not show any specific clinical sign or abnormal behaviour and exhibit a sudden attack characterised by loss of balance, shouting, violent wing flapping, strong muscular contraction and apparent seizure only immediately prior to death (Crespo and Shivaprasad, 2013; Basaki et al., 2016).

Although a variety of nutritional and environmental factors including vitamins, fats, protein in the diet and lighting have been suggested to influence the incidence of SDS, the

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exact aetiology and pathogenesis are still either entirely or partly unknown (Scott, 2002). Some researchers suggest that SDS may be a cardiovascular dysfunction (Olkowski, 2007; Olkowski et al., 2008). Recently, three novel mutations (K289R, P308S, D310H) concomitant with downregulation of the calsequestrin 2 gene in the cardiac muscle of chickens having died of SDS have been determined (Basaki et al., 2016). Rupture of the right atrium and elevation of serum enzymes associated with cardiac lesions have also been reported in broilers with SDS, demonstrating that SDS in broilers may be a disease of cardiac origin (Imaeda, 1999; Qujeq and Aliakbarpour, 2005; Olkowski, 2007).

Over the last two decades, a defective mitochondrial function associated with hypoxia has been discovered to be involved in diverse cardiac disorders of humans (Ostadal et al., 1999; Budev et al., 2003). Numerous regulatory mechanisms have been proposed for heart compatibility during chronic hypoxia, including reduction of fatty acid uptake, inhibition of the transfer system of fatty acids into mitochondria, elevation of glucose uptake and anaerobic glycolysis, and increase of endogenic lactate production in cardiac cells (Hurford et al., 1990; Ngumbela et al., 2003; Essop, 2007).

At the molecular level, the redox-sensitive transcriptional modulator, hypoxia-inducible factor-1 (HIF-1) has the central role in regulating the expression of numerous adaptive genes in hypoxia (Ong et al., 2014). Under hypoxic conditions, the HIF-1α binds to *cis*-acting hypoxia-response elements (HREs) to induce the expression of target genes (He et al., 2014; Ong et al., 2014). It has been shown that cardiac fuel substrate switching in the cardiac muscle of animals or humans under chronic hypoxia can improve substrate utilisation in low-oxygen medium and help sustain cardiac output (Hurford et al., 1990; Ngumbela et al., 2003; Cowburn et al., 2017). Cardiac metabolic remodelling under hypoxic conditions is characterised by increased glucose transporter 1 (GLUT1) expression and glucose utilisation, enhancement of the uptake and release of myocardial lactate due to increased expression of lactate shuttle components such as monocarboxylate transporter 4 (MCT4), and elevation of lactate production due to the downregulation of transcript levels of pyruvate dehydrogenase kinase 4 (PDK-4), an inhibitor of pyruvate dehydrogenase and glucose oxidation (Malhotra et al., 2002; Kim et al., 2006).

The broiler chicken has been intensely selected for a higher growth rate, which requires a higher oxygen supply. Impaired oxygen supply to sustain a continuous fast growth rate may increase the risk for cardiac dysfunction and SDS incidence (Olkowski et al., 2005). Hypoxia has been known as a main pathophysiological factor of cardiac dysfunction in similar cases of SDS in humans (Rubart and Zipes, 2005), but metabolic changes related to hypoxia have not been determined within the cardiac muscle of chicken with SDS.

The aim of the present study was to evaluate the probable metabolic and molecular compensatory mechanisms related to hypoxia in the heart tissues of broiler chickens having died of SDS.

MATERIALS AND METHODS

Study design

Broilers (Cobb 500) in the commercial farm of the Faculty of Veterinary Medicine of Shiraz University with a rearing capacity of 7,000 chicks were closely monitored daily for the occurrence of SDS between the 2nd and 5th weeks of two rearing periods. Broilers suspected to have died of SDS were collected daily and the carcasses were examined by necropsy. Carcasses with lesions indicative of disorders other than SDS were discarded. Factors related to feed intake and growth in the herd were normal and there were no signs or complications of infectious and non-infectious diseases during the rearing period. Feed and water were provided *ad libitum* throughout the trial. Lighting consisted of a period of 18 h light and 6 h darkness from day 0 until the end of the experimental period. The ambient temperature was decreased by 2 °C per week, from 33 °C in the first week to 25 °C on day 28 and then kept constant. The SDS attack was characterised by failure of balance, violent flapping and strong muscular contractions. Death observed within 1–2 min in the case of birds lying on their backs with outstretched wings was suspected to being due to SDS. Carcasses were removed and evaluated by necropsy for the confirmation of SDS in suspect birds in the Poultry Diseases Research Center, School of Veterinary Medicine, Shiraz University. Healthy birds of similar age and weight were randomly selected as an internal control group (IC). All birds were used according to the guidelines for the care and use of laboratory animals published by the National Academy of Sciences (National Institutes of Health Publication No. 86-23). All protocols of the present study were approved by the research ethics committee of Shahid Chamran University of Ahvaz (EE/97.03.02.1357/scu.ac.ir).

Sample collection

During the two rearing periods, 36 broilers with SDS attack (28 males and 8 females) were found. Left and right ventricles were excised from chickens having died of SDS. Heart samples were obtained from 36 healthy broilers after slaughter by cervical dislocation. The laboratory data of this group were used as control. Tissue samples were stored at −70 °C until use.

Analysis of cardiac muscle biochemical factors

About 200 mg of combined right and left ventricle were homogenised in 800 µL cold phosphate buffer (pH 7.5) using Silent Crusher S Homogenizer (Heidolph, Germany). Samples were centrifuged at 12,000 × rpm for 10 min at 4 °C and the supernatant was used for biochemical assays. The activities of lactate dehydrogenase (LDH; Art No. 122050) and creatine phosphokinase (CPK; Art No. 116050) were measured using commercial kits (Pars Azmoon Co., Iran) and reported in units of IU/mg tissue protein. Cardiac lactate concentration was determined using a lactate colorimetric assay kit as recommended by the manufacturer

(Biovision Inc., Switzerland; Art No. K667) and reported in units of mmol/mg tissue protein. Tissue protein level was measured by the Bradford method using bovine serum albumin as standard (Bradford, 1976).

Total RNA isolation and cDNA synthesis

Total RNA was isolated from 100 mg of ventricles using the RNX™ RNA isolation kit (SinaClon Inc., Iran; Art No. EX6101). The samples were treated with DNase I enzyme (SinaClon Inc., Iran; Art No. MO540) to avoid DNA contamination. Finally, optical density (A_{260}/A_{280} and A_{260}/A_{230}) and concentration were measured using Eppendorf μCuvette G1.0 microvolume measuring cell (Eppendorf BioPhotometer D30, Eppendorf, Germany). RNA samples with a ratio of more than 1.8 were used for cDNA synthesis. For each sample, 1 μg of total RNA was reverse transcribed by YTA cDNA synthesis kit using random primers as described by the manufacturer (Yektatajhiz, Iran; Art No. YT4500). The reaction volume was 20 μL and the components used included 1 μg of total RNA as template (10 μL), YTA RT 200 U/μL (1 μL), YTA RNase Inhibitor 20 U/μL (1 μL), Random Hexamer primer (1 μL), dNTP Mix 10 mM (2 μL) and Reaction Buffer 5 × (4 μL). Then, samples were incubated for 10 min at 25 °C, 90 min at 42 °C and 5 min at 75 °C.

Quantitative real-time PCR data analysis

To determine the relative expression of HIF1-α, PDK-4, MCT-4 and GLUT-1 in the cardiac muscle of collected samples, real-time PCR analysis was done. Real-time PCR assay was performed using Ampliqon Real Q Plus Master kit for SYBR Green I® (Ampliqon, Denmark; Art No. A325402) on a Lightcycler® Detection System (Roche, USA). The relative expression level of the target transcript was compared to chicken β-actin (chβ-actin) as housekeeping gene. Specific sets of primers (Bioneer, South Korea) designed for this study are shown in Table 1. Reactions were prepared in a 12.5 μL mixture containing 6.25 μL master mix kit, 0.25 μL of each primer (200 nM), 3 μL cDNA (100 ng) and 2.75 μL nuclease-free water. The PCR protocol used consisted of a 5-min denaturation at 94 °C followed by 45 cycles of 94 °C for 15 s and 60 °C for 30 s. Two separate reactions without cDNA or with RNA were performed in parallel as controls. Relative quantification was performed according to the comparative $2^{-\Delta\Delta Ct}$ method and using Lightcycler 96® software. Validation of the assay to check that the primer for the target genes and chβ-actin had similar amplification efficiencies was performed as described previously (Tabandeh et al., 2012). All qPCR analysis was performed according to The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQEs) guideline (Bustin et al., 2009).

Statistical analysis

Statistical analyses were performed with GraphPad Prism 8.0 software (GraphPad Software, Inc., San Diego, CA). All experimental data were presented as the mean ± SEM. The Shapiro–Wilk or Kolmogorov–Smirnov tests were used to determine the normality of data or equality of error variances All parameters were analysed by two-way analysis of variance (ANOVA) with times (4 levels) and disease condition (2 levels; healthy vs. SDS) as factors. When interaction and/or the main effects were significant, means were compared between different experimental groups at different time points using Tukey's multiple-comparison *post hoc* test. Statistically significant differences between control and SDS-affected chickens at each sampling time were represented as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Statistically significant differences between different sampling times in SDS-affected birds were indicated as follows: $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$ and $\#\#\#\#P < 0.0001$.

RESULTS

At necropsy, birds that had died of SDS were in good body condition with a full gastrointestinal tract. The liver was enlarged, pale and fragile and the gallbladder was empty. The heart was elongated, the ventricles were contracted and the atria were dilated and filled with blood in the SDS-affected birds in comparison to those in the healthy chickens. Using strict diagnostic criteria, 36 SDS cases from the 2nd to the 5th week of the rearing period were detected. Most losses due to SDS were seen during the 2nd and 3rd weeks. Out of the 36 SDS cases (100%), 11 (31%), 17 (47%), 5 (14%) and 3 (8%) were found in weeks 2, 3, 4 and 5 of the

Table 1. Description of primers used in the present study (F: forward primer, R: reverse primer)

Target gene	Sequences	Size of amplicon (bp)	GenBank Accession No.
β-actin	F: GAAACATGTTGGAGCGAACG R: CACAGAGGCGAGTAACTTCC	135	NM_205518.1
GLUT-1	F: CTGTTGTTTCGCTCTTCGTG R: ATGCTGAGGTAGGACATCCA	145	NM_205209.1
MCT-4	F: CCTTCTTTGCTGGGATAGCA R: AGGCTCCAAGAAGAAGGAGA	141	XM_0049348722
PDHK-4	F: CCGAGGCACATTGGAAGTAT R: GCTTCAGTTCTGGAGACGTT	119	NM_001199909.1
HIF1-α	F: GACCTGCCCACTGTATTCTG R: CTTTGAGTAGCCCAGAGCAG	143	NM_204297.1

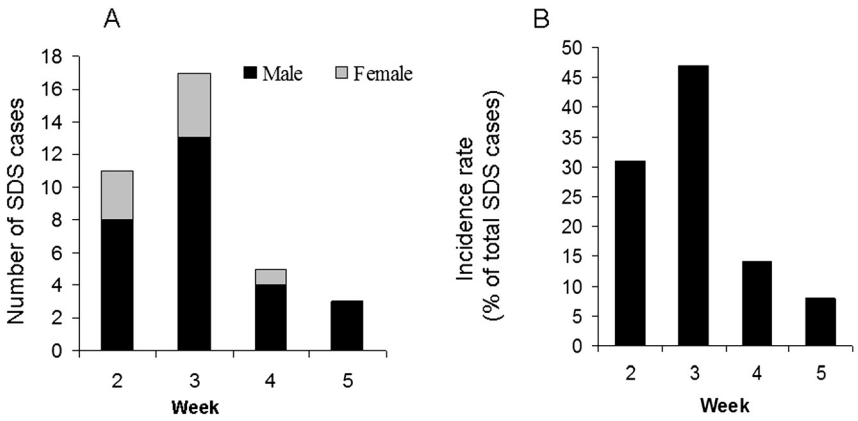

Fig. 1. The incidence of sudden death syndrome (SDS) among chickens during weeks 2–5 of the rearing period according to age and sex

rearing period, respectively (Fig. 1A and B). The number of male broilers showing SDS signs (28 cases, 77.8%) was higher than that of female chicks with an SDS attack (8 cases, 22.2%).

Based on the results of analysis of cardiac lactate concentrations by two-way ANOVA, significant effects were found for the main effect of disease condition ($P < 0.0001$) and the disease condition × sampling time interaction ($P = 0.027$), but the main effect of sampling time had no statistical significance ($P > 0.05$). Based on multiple-comparison analysis between variables, cardiac lactate concentrations were significantly higher in SDS-affected chickens than in the IC group ($P < 0.05$) in all weeks of the rearing period (Fig. 2). The lactate concentration in the cardiac muscle of SDS-affected chickens showed no significant differences at different weeks of age ($P > 0.05$).

The cardiac LDH and CPK activities were analysed in a 2 × 4, disease condition × sampling time design by two-way ANOVA. The results showed a significant main effect of disease condition ($P < 0.0001$), sampling time ($P < 0.0001$) and disease condition × sampling time interaction ($P < 0.001$) on cardiac LDH activity. As shown in Fig. 3A, chickens having died of SDS exhibited significantly increased levels of LDH activity in the cardiac muscle at all weeks of sampling when compared with healthy chickens ($P < 0.05$). LDH activity in the cardiac muscle of SDS-affected birds showed a maximal level at 3 and 4 weeks of age ($P < 0.05$) (Fig. 3A).

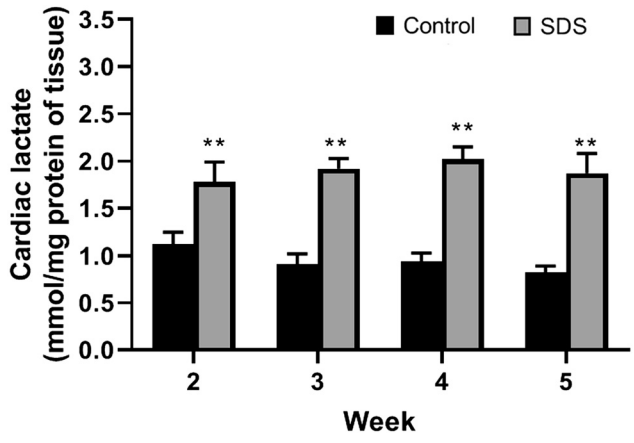

Fig. 2. Lactate concentration in the cardiac muscle of chickens having died of SDS from weeks 2–5 of the rearing period. Cardiac muscle samples ($n = 36$) were obtained from chickens having died of SDS and from healthy control chickens ($n = 36$) at the same age. **represents significant difference between control and SDS chickens at each sampling time at $P < 0.01$

A significantly higher cardiac muscle activity of CPK was observed in SDS-affected birds than in healthy chickens at all weeks of the rearing period ($P < 0.05$) (Fig. 3B). CPK activity in the heart of SDS-affected chickens showed no significant variation at different weeks of age ($P > 0.05$) (Fig. 3B).

Two-way ANOVA showed significant main effects of disease condition ($P < 0.0001$), sampling time ($P < 0.0001$) and disease condition × sampling time interaction ($P < 0.0001$) on the expression of all the genes studied. Real-time PCR analysis showed that HIF1α, MCT-4 and GLUT-1 transcription was increased in the cardiac muscle of SDS-affected chickens compared with the IC birds at 3 and 4 weeks of age ($P < 0.05$) (Fig. 4A–C). The mRNA level of PDHK-4 in the cardiac muscle of SDS-affected chickens was downregulated in a time-dependent manner, and showed the lowest level at 2–4 weeks of age compared with the healthy birds ($P < 0.05$) (Fig. 4D).

DISCUSSION

In the present study, the metabolic and molecular switching related to the inadequacy of oxygen supply to the cardiac muscle of SDS-affected chickens was studied between weeks 2 and 5 of the rearing period. The results showed increased LDH activity and lactate concentration in the cardiac muscle of SDS-affected chickens compared with healthy birds. Fatty acids are the main fuel substrate for heart cells under normal conditions and provide 60–80% of the energy needed for the heart (Tran and Wang, 2019). Exposure to hypoxia led to the launch of transcription modulators sensitive to oxygen, and cardiac cells switched on the catabolism of fatty acids to

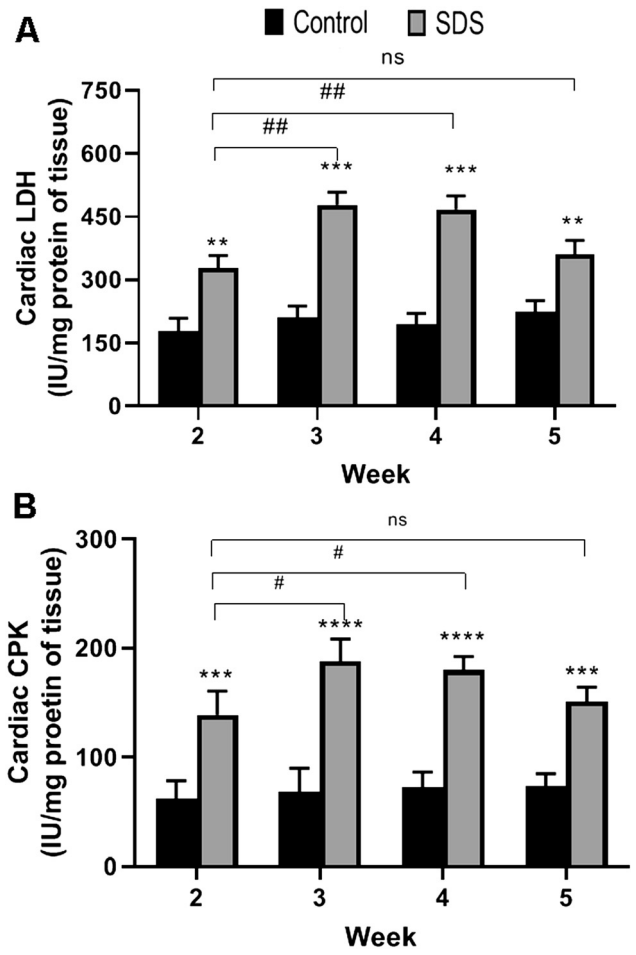

Fig. 3. Changes in lactate dehydrogenase (LDH) (A) and creatine phosphokinase (CPK) (B) activities in the cardiac muscle of chickens having died of SDS from weeks 2–5 of the rearing period. Cardiac muscle (n = 36) samples were obtained from chickens having died of SDS and from healthy control chickens (n = 36) at the same age. The data represent means ± standard error of the mean (SEM). The levels of significance between the control and the SDS groups at each sampling time are presented as $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. $^{\#,\#\#}$ represent a significant difference between SDS groups of different ages at $P < 0.05$ and $P < 0.01$, respectively

carbohydrates for efficient energy production. Under hypoxia, pyruvate-to-lactate conversion (anaerobic glycolysis) by LDH is accelerated, resulting in the production of high levels of lactate in the heart and an increase in the amount of glucose consumption (Adamo et al., 2017). Higher LDH activity and lactate production in the heart of SDS-affected chickens may be a molecular mechanism for adaptation to hypoxia. It has also been found that high concentrations of lactate in the circulation may have an inhibitory effect on lipolysis under hypoxic conditions. Accordingly, previous studies reported decreasing free fatty acid (FFA) concentrations in response to increasing lactate levels, implying that high circulating lactate content may have a direct inhibitory effect on lipolysis (Corbett et al., 2004). Although the analysis of FFA absorption in the cardiac muscle was practically impossible in our study, we hypothesise that lactate may have a regulatory role on fuel switching in the cardiac muscle of SDS-affected chickens through the possible inhibition of FFA bioavailability in the cardiac muscle. This hypothesis is supported by a previous study which has shown that attenuated sarcolemmal or mitochondrial fatty acid uptake by cardiac muscle is a putative regulatory step directing fuel substrate switching in response to chronic hypoxia (Neary and Breckenridge, 2014).

We observed, for the first time, that HIF1α expression was upregulated in the heart of SDS-affected chickens. The first transcriptional adaptive response to hypoxia is mediated by the HIF1α signalling pathway, which upregulates genes that restore oxygen and energy homeostasis. HIF drives an adaptive response to hypoxia by promoting the expression of genes including those that regulate glycolysis, in particular LDH. Taken together, these findings suggest that SDS is associated with high anaerobic glycolysis, a metabolic adaptation to hypoxia that exchanges the energy fuel from fat to glucose and increases lactate production and, thus, may have an important role in the incidence of cardiac dysfunction.

Previous studies have shown that hypoxia and lactic acidosis are the main pathophysiological findings in cardiac diseases of humans (Adamo et al., 2017; Gjesdal et al., 2018). To confirm the imbalance of lactate turnover in chickens with SDS, we determined the expression level of some genes related to lactate production and glucose transport in the cardiac muscle of SDS-affected chickens. Our results showed higher expression of MCT-4 and GLUT-1 and lower expression of PDHK-4 in the cardiac muscle of SDS-affected chickens compared with healthy birds at 3–4 weeks of age. Recent findings have shown that MCT-4 endows cells with the ability to export lactate in high-lactate microenvironments (Contreras-Baeza et al., 2019). PDHK-4 is one of the most important regulators of the PDH complex that inhibits the activity of the PDH complex by its phosphorylation at Ser293 and Ser300, resulting in an inhibition of the glucose oxidation. PDHK-4 expression is upregulated in tissues with high rates of fatty acid synthesis, such as the cardiac muscle, suggesting a critical role in lipid metabolism (Trinidad et al., 2017). The upregulation of GLUT-1 and MCT-4 and the downregulation of PDHK-4 along with increased LDH activity and lactate concentration in the cardiac muscle of chickens with SDS indicates the possible incidence of anaerobic glycolysis and hypoxia in the cardiac muscle that may have a detrimental role in the development of SDS in chickens.

In vitro studies have found that mammalian cells augment glycolytic capacity in response to hypoxia by increasing the glucose uptake. Recent findings indicate that prolonged hypoxia in mammals reduces blood glucose (Chen et al., 2007). In fast-growing broilers, high oxygen demand and low cardiac output may lead to lowered arterial blood pO_2 and general hypoxia (Olkowski et al., 2005). Thus, it remains unresolved whether cardiac metabolic remodelling in SDS-affected chickens is secondary to local hypoxia or, rather, generalised hypoxia that affects glucose uptake in various organs.

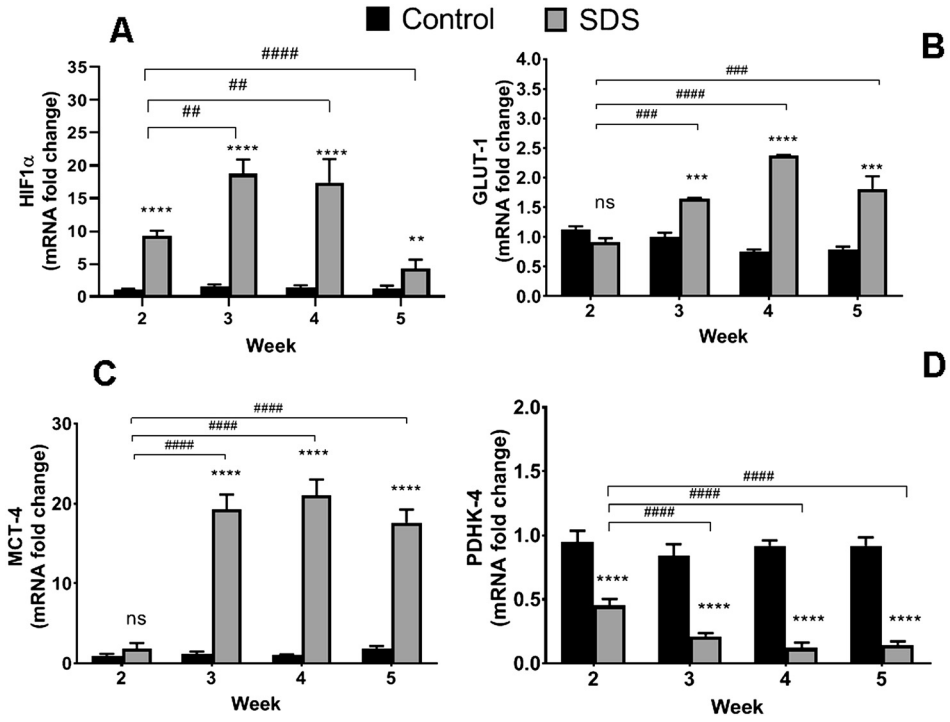

Fig. 4. The expression levels of hypoxia-inducing factor 1α (HIF1α; A), glucose transporter 1 (GLUT-1, B), monocarboxylate transporter 4 (MCT-4, C) and pyruvate dehydrogenase kinase 4 (PDHK-4, D) in the cardiac muscle of chickens having died of SDS during weeks 2–5 of the rearing period. mRNA was isolated from the dissected cardiac ventricles (right and left) of birds having died of SDS (n = 36) and from control chickens (n = 36) at different ages during the growing period, and analysed using real-time PCR. mRNA fold changes were calculated relative to the values obtained for chicken β-actin (GenBank: NM_205518.1). Data are reported as means ± SEM. The levels of significance between control and SDS groups at each sampling time are presented as $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$. $^{\#\#}$, $^{\#\#\#}$, $^{\#\#\#\#}$ represent significant differences between SDS groups of different ages at $P < 0.01$, $P < 0.001$ and $P < 0.0001$, respectively

In our study, CPK activity was higher in the cardiac muscle of SDS-affected chickens compared to healthy birds, in particular from the 3rd and 4th weeks of the rearing period when a higher incidence of SDS was observed. Previous studies have reported higher rates of myocardial phosphocreatine synthesis and cardiac ATPase activity in response to chronic hypoxia (Heather et al., 2012; Zervou et al., 2016). We suggest that elevation of creatine phosphate synthesis in the cardiac muscle of SDS-affected chickens may increase the efficiency of energy production and augment mitochondrial bioenergetic capacity to sustain cardiac function despite hypoxia.

Although hypoxia may be an important underlying mechanism for inadequate performance of the heart that limits blood oxygenation in chickens with SDS, the causes of individual variations in susceptibility to SDS among broilers are unclear. In accordance with our findings, a potentially wide role of cardiac hypoxia has been suggested as the main aetiological factor for the incidence of sudden death in humans. Currently, approximately 1 in 2,000 infants are victims of sudden death, and this rate decreased after the continuing success of the 'back to sleep' campaign started in 1994. A common mechanism transforms a seemingly disparate series of advice to parents into one simple message around avoiding a low oxygen environment and sets in motion more obvious preventative measures (Neary and Breckenridge, 2013).

In conclusion, the regulatory mechanisms directing cardiac metabolic remodelling in response to chronic hypoxia are complex and the subject of ongoing research work in broiler chickens. Our findings showed, for the first time, the incidence of hypoxia-related changes in the cardiac muscle of chickens having died of SDS, which were characterised by a progressive increase of LDH and CPK activities and lactate production and up- or downregulation of the expression of genes related to metabolic remodelling during hypoxic conditions in the cardiac muscle.

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