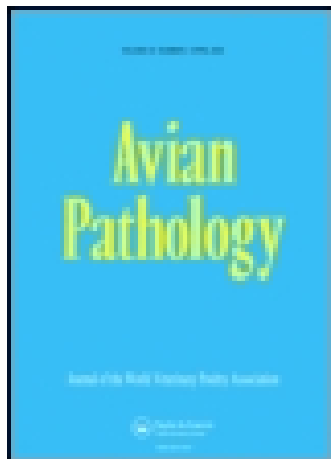


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The role of oxidative stress in the development of congestive heart failure in a chicken genotype selected for rapid growth

S. Nain^a, B. Ling^b, B. Bandy^b, J. Alcorn^b, C. Wojnarowicz^c, B. Laarveld^a & A. A. Olkowski^a

^a Department of Animal and Poultry Science,

^b College of Pharmacy and Nutrition,

^c Prairie Diagnostic Services, Department of Veterinary Pathology, University of Saskatchewan, Saskatoon, SK, S7N 5A8, Canada

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ORIGINAL ARTICLES

The role of oxidative stress in the development of congestive heart failure in a chicken genotype selected for rapid growth

S. Nain¹, B. Ling², B. Bandy², J. Alcorn², C. Wojnarowicz³, B. Laarveld¹ and A. A. Olkowski^{1*}¹Department of Animal and Poultry Science, ²College of Pharmacy and Nutrition, and ³Prairie Diagnostic Services, Department of Veterinary Pathology, University of Saskatchewan, Saskatoon, SK S7N 5A8, Canada

The present study examined the possible role of reactive oxygen species in the pathogenesis of heart failure in broilers. Data were collected from three groups of birds at various risk of heart failure: Leghorn chickens (resistant to heart failure), slow-growing feed-restricted broilers (low risk of heart failure), fast-growing *ad libitum* fed broilers (high risk of heart failure), and broilers with congestive heart failure (CHF). In the first part of the study, basic clinical parameters and ultrastructural changes were examined in the context of lipid peroxidation of the ventricular myocardium. This was followed by the study of *in vitro* changes in the activity of selected cytosolic enzymes (creatine kinase and lactate dehydrogenase) and mitochondrial enzymes (pyruvate dehydrogenase and α -ketoglutarate dehydrogenase) in the presence of oxidants (hydrogen peroxide or tertiary butyl hydroperoxide). The distinctive clinical feature in the fast-growing broilers and in the broilers with CHF as compared with slow-growing broilers or Leghorn chickens was a significantly lower heart rate ($P < 0.05$). Electron microscopy revealed marked morphological changes in myocardial mitochondria in these broilers (i.e. fast-growing broilers and broilers with CHF). The level of malondialdehyde equivalents, an indicator of lipid peroxidation subsequent to generated oxidative stress, was significantly higher ($P < 0.05$) in *ad libitum* fed broilers and was highest ($P < 0.01$) in broilers with CHF. *In vitro*, the presence of oxidants had a detrimental effect on creatine kinase and α -ketoglutarate dehydrogenase activity, while lactate dehydrogenase activity increased. The activity of pyruvate dehydrogenase was not altered by oxidants. Our results indicate that the deterioration of heart function in fast-growing commercial broilers in our experimental model is associated with oxidative stress leading to lipid peroxidation of cellular and mitochondrial membranes, and decreased activity of myocardial creatine kinase and α -ketoglutarate dehydrogenase enzymes critical for energy synthesis and transformation pathways.

Introduction

Modern fast-growing broilers are inherently more susceptible to heart diseases than laying strains. Previous studies from our laboratory revealed that a relatively large number of commercial broilers show evidence of hypoxemia associated with subclinical heart disease (Olkowski *et al.*, 2005; Nain *et al.*, 2008).

Oxidative metabolism is a normal process in all tissues. Cardiomyocytes require a constant supply of oxygen for normal cardiac functions. However, oxygen-associated metabolism in the myocardium sometimes can contribute to cardiac dysfunction, and may ultimately lead to heart failure (Giordano, 2005; Redout *et al.*, 2007). During the normal oxidative metabolic process, various reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced. During this normal metabolism, 1% to 2% of oxygen is converted to ROS (Sheeran & Pepe, 2006). Several specialized metabolic mechanisms normally dispose off these very harmful reactive species. However, under

some circumstances, increased ROS/RNS production or decreased antioxidant defenses may lead to oxidative stress where the generated reactive species can alter the properties of lipids, proteins, and nucleic acids, leading to cellular dysfunctions (for a review see Orrenius *et al.*, 2007). Recent research findings from different laboratories suggest that ROS and RNS play a critical role in development of human heart failure (Andreka *et al.*, 2004; Sam *et al.*, 2005; Nediani *et al.*, 2007).

Lipid peroxidation can alter the membrane properties of cellular and subcellular organelles (mitochondria and sarco-endoplasmic reticulum) crucial for maintenance of normal cardiomyocyte function. Broilers with congestive heart failure (CHF) show evidence of calcium overload in these subcellular components (Maxwell *et al.*, 1993; Li *et al.*, 2006), and evidence of breakdown and release of the protein of contractile apparatus, such as myosin and troponin T, into the circulation (Maxwell *et al.*, 1994).

*To whom correspondence should be addressed. Tel: +1 306 966 5848. Fax: +1 306 966 4151. E-mail: Andrew.Olkowski@usask.ca

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The role of oxidative stress has long been debated in the pathogenesis of heart failure in human and animal models of cardiomyopathy. However, limited research has been carried out to investigate the possible involvement of oxidative stress in CHF in broilers. Recent studies from our laboratory revealed that energy metabolism is compromised in broilers developing heart failure (Nain *et al.*, 2008; Olkowski *et al.*, 2007). In order to further understand the physiological and biochemical disturbances leading to CHF in fast-growing commercial broilers, we were interested to examine the possible role and molecular mechanisms of oxidative stress in the pathogenesis of chronic heart failure. In order to explain the molecular mechanisms underlying the pathogenesis of heart failure in commercial broilers we compared clinical, morphological, molecular and biochemical parameters among chickens that were categorized according to risk of developing heart failure as follows: (1) Leghorn chickens (resistant to heart failure); (2) feed-restricted, slow-growing broilers (low risk of heart failure); (3) *ad libitum* fed, fast-growing broilers (high risk and incidence of heart failure) and (4) broilers showing signs of congestive heart failure (broilers with CHF, observed within the *ad libitum* fed group). Firstly, oxidative stress associated with the deterioration of heart function was measured. Secondly, we examined the impact of simulated oxidative stress on the activities of essential enzymes involved in cardiac energy synthesis and transformation pathways.

Materials and Methods

Animal and management. The birds (all commercial broiler males; Aviagen) used to measure specific parameters represented a random sample obtained from basic flocks consisting of 48 Leghorns, 39 feed-restricted slow-growing broilers, and 83 fast-growing *ad libitum* fed broilers. The group of broilers designated as slow-growing was subjected to a feed restriction regime (70% *ad libitum*) from day 7 onwards. All other groups of birds were fed *ad libitum*. The birds were raised using the lowered environmental temperature protocol as described previously (Olkowski *et al.*, 1999). Briefly, during the first 7 days the temperature was maintained at 34°C followed by a gradual decrease to a level approximately 21°C by the end of the third week, and 17°C by the end of the fifth week. The lowered environmental temperature forces the birds to increase their metabolic rate, and this results in increased burden on the cardiovascular system, which effectively exposes metabolic weaknesses of cardiac tissue.

The experimental protocols were approved by the University of Saskatchewan Animal Care Committee. The procedures were performed as per the requirements of the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care, 1993).

Clinical evaluation. Birds were monitored daily for overt signs of heart disease such as fatigue, exercise intolerance, tachypnea, hypoxemia, generalized cyanosis, and ascites. Birds *in extremis* were euthanized. Detailed physical examination was performed prior to data collection. Heart rate measurements were obtained using electrocardiography as described previously (Olkowski & Classen, 1998) in 10 birds from each group at the end of the fifth week of the experiment. Birds showing the above listed signs were classified as broilers with CHF.

Transmission electron microscopy. To determine the ultrastructural changes in myocardium of birds at various risk of heart failure, hearts obtained from Leghorn chickens, fast-growing *ad libitum* fed broilers, and broilers with CHF were processed for transmission electron microscopy. Heart tissue samples from the mid portion of the left ventricular free wall were processed as described previously (Olkowski *et al.*, 2001).

Heart tissue procurement for biochemical analysis. Heart tissue samples used for biochemical analysis were obtained from five randomly selected birds from each group as described above, at the end of the sixth week of the experiment. Hearts were collected following euthanasia by cervical dislocation, and were snap frozen in liquid nitrogen. These were stored in liquid nitrogen until analyzed.

Thiobarbituric acid reacting substances assay. Thiobarbituric acid reacting substances (TBARS), an indicator of oxidative damage, were measured spectrophotometrically as described previously by Ohkawa *et al.* (1979) with modifications. Briefly, frozen/pulverized tissue samples from the mid portion of the left ventricular myocardium were homogenized first in KCl mixture (2 mM ethylene glycol tetraacetic acid, 0.02% butylated hydroxytoluene (BHT) in 1.15% KCl) using microtube homogenizer and then radioimmune precipitation assay (RIPA) buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM ethylenediamine tetraacetic acid, 1% Triton X-100, 1% sodium deoxycholate and 1% sodium dodecyl sulfate, with pH adjusted to 7.2 using NaOH) was added, and the assay was thoroughly mixed. Finally, 1 g heart tissue was present in 6 ml solution containing RIPA buffer and KCl mixture in a ratio of 4:5 v/v. The homogenate was centrifuged at 15 000 × g for 10 min at 4°C. Then 200 µl supernatant was mixed with 0.2 ml of 8.1% sodium dodecyl sulfate, 2500 µl of 30% acetic acid (pH adjusted to 3.5), 375 µl of 0.8% thiobarbituric acid (TBA) and 8.25 µl of 0.02% BHT aqueous solution followed by incubation at 95°C for 1 h. After incubation and subsequent cooling, an equal volume of *n*-butanol/pyridine mixture (15:1 v/v ratio) was added. After vigorous shaking, the assay was centrifuged at 4000 × g for 10 min. The organic layer obtained at the top following centrifugation was used for measuring thiobarbituric reactive substances at 532 nm. A calibration curve was prepared using a malondialdehyde (MDA) standard, and the dilution was made in such a way that the expected concentration of each sample falls in the middle range of the calibration curve. The coefficient of determination approached 0.99 for the standard curve. All samples were analyzed in duplicate in the same assay to avoid interassay variability.

***In vitro* enzyme inhibition.** The activity of selected cytosolic (creatine kinase [CK] and lactate dehydrogenase [LDH]) and mitochondrial (pyruvate dehydrogenase [PDH] and α -ketoglutarate dehydrogenase [α -KGDH]) enzymes was tested using an *in vitro* indicator of oxidative stress (i.e. hydrogen peroxide [H₂O₂] or tertiary butyl hydroperoxide [TBH] or both). Enzyme sensitivity measurements were performed in heart tissue obtained from the mid portion of the left ventricle free wall of fast-growing *ad libitum* fed broilers.

The activities of CK, LDH, PDH and α -KGDH were measured as described previously (Nain *et al.*, 2008). Briefly, aliquots of 300 mg frozen samples were homogenized in phosphate buffer (50 mM, pH 7.4) in the ratio of 1:10 (100 mg tissue:1 ml buffer) in test tubes pre-cooled with ice. The homogenate was centrifuged at 2500 × g for 10 min at 4°C and the suspension was further centrifuged at 12 000 × g for 10 min. The supernatant was used for CK and LDH measurements. The CK activity was measured using a CK kit (Roche Diagnostics, Indianapolis, Indiana, USA) while LDH activity was measured using LD-L10 (Sigma Diagnostics Inc., St Louis, Missouri, USA). The mitochondria-containing pellet was further washed three times with phosphate buffer to remove remnants of cytosolic enzymes, and finally this pellet was resuspended in Tris buffer (50 mM, pH 7.6 with 0.5% Triton). The PDH and α -KGDH activities were assayed in mitochondrial extracts. The PDH activity was measured using a cocktail, where the final components of the incubation media were 2 mM pyruvate, 2.5 mM nicotinamide adenine dinucleotide (NAD), 0.15 mM flavin adenine dinucleotide, 2 mM MgCl₂, 0.2 mM thiamine pyrophosphate, 0.13 mM coenzyme A, 2.6 mM dithiothreitol, and 30 mM Tris buffer at pH 7.2. The α -KGDH activity was measured using a cocktail containing 3.2 mM α -keto glutaric acid, 2 mM NAD, 0.5 mM coenzyme A, 0.7 mM thiamine pyrophosphate, and 1 mM MgCl₂. The assays were validated for the linearity of responses for time of reaction and protein content.

Initially, the inhibition study of all the above-mentioned enzymes was performed in presence of various concentrations of H₂O₂, but because the activities of PDH and α -KGDH were not affected by H₂O₂ we also

assessed the activity of these enzymes in the presence of serial dilutions of TBH.

Before performing final measurements, each component of the assay cocktail mixture for every enzyme was incubated in the presence of H₂O₂ or TBH, to establish that the individual components of these enzymes were not affected by the presence of oxidants—the results obtained are purely subsequent to enzyme oxidant interaction.

The reaction was initiated by adding 20 µl cytosolic or mitochondrial fraction in 200 µl cocktail plus 5 µl serially diluted H₂O₂ or TBH per well in a 96-well micro plate pre-incubated at 37°C for all of the above-mentioned enzymes. Enzyme activity measurements were performed at 340 nm using a microplate reader SpectraMax Plus (Molecular Devices, California, USA). The final measurements were performed during the linear phase of responses pre-established during the validation phase as described previously. The inhibition constant (IC₅₀) was used to evaluate the concentration of the oxidants required for 50% inhibition of an enzyme activity. IC₅₀ values were calculated using GraphPad Prism 3.03 (GraphPad Software, San Diego, California, USA).

Statistical analysis. Statistical analyses were carried out by analysis of variance from the microcomputer package Number Cruncher Statistical System (Hintze, 1995). The means were compared using Fisher's least significant difference (LSD) Replace test. Statistical significance was assumed to exist when the probability of making a type I error was less than 0.05.

Results

Incidence of heart failure. Overall, during the course of this study, fulminant CHF (based on mortality and morbidity data) was observed in 38 out of 83 (46%) broilers from the *ad libitum* fed group. None of the birds from the feed-restricted and Leghorn groups showed clinical signs indicative of heart disease.

Heart rate. The cardiac measurements revealed a markedly ($P < 0.05$) lower heart rate (308 beats/min) in the *ad libitum* fed group as compared with the feed-restricted (heart rate, 371 beats/min) and Leghorn chickens (heart rate, 372 beats/min). Profound bradycardia (heart rate, 236 beats/min) was observed in broilers with fulminant CHF.

Gross pathology and ultrastructural changes. Postmortem examination was performed on birds that were euthanized or died during the course of study. Broilers with CHF showed gross dilation of the ventricular chambers along with pulmonary congestion. Approximately 80% to 90% of broilers showing these changes also showed severe pericardial effusion and the accumulation of ascitic fluid in the abdominal cavity.

Ultrastructural examination revealed significant morphological changes in the cardiomyocytes' contractile apparatus and mitochondria. In some areas, disintegration or even total dissolution of myofibrillar elements were observed. Mitochondrial lesions were seen in some apparently normal broilers, but predominantly in broilers with CHF. Major morphological changes in the mitochondria include pleomorphism, variable degrees of swelling and vacuolization along with disintegration and loss of cristae.

A distinctive feature in fast-growing *ad libitum* fed broilers, as compared with Leghorn chickens, was that the inter-fibrillar mitochondria were numerous and appeared in large clusters. Careful evaluation of electron micrographs (Figure 1) revealed that changes were most pronounced in broilers with CHF.

Thiobarbituric acid reacting substances assay. Findings from the TBARS assay showed that lipid peroxidation was highest ($P < 0.05$) in broilers with CHF as compared with slow-growing feed-restricted broilers, fast-growing *ad libitum* fed broilers and Leghorn chickens (Table 1). The findings from this assay further revealed that fast-growing *ad libitum* fed broilers had higher ($P < 0.05$) lipid peroxidation as compared with slow-growing feed-restricted broilers and Leghorn chickens. The values from slow-growing feed-restricted broilers and Leghorn chickens did not differ significantly ($P > 0.05$).

Enzyme inhibition. In the first part of the study it was ensured that the enzymatic assay components (CK and LDH kits, α -KGDH and PDH cocktail components) were not affected by the presence of oxidants (Table 2).

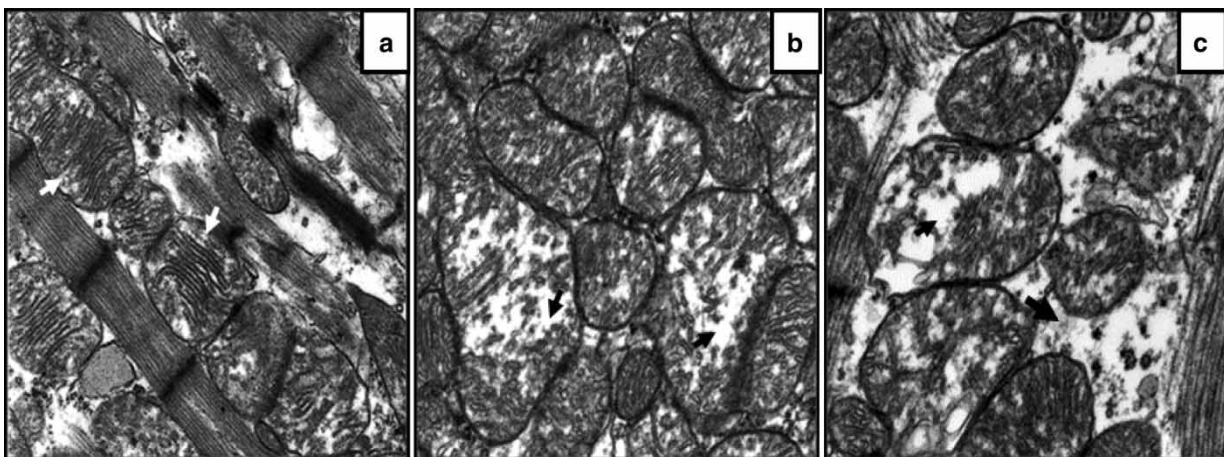


Figure 1. Representative transmission electron micrographs from left ventricular myocardium of (1a) a Leghorn chicken (resistant to heart failure), (1b) a fast-growing *ad libitum* fed broiler (high risk of heart failure) and (1c) a broiler that developed CHF. Original magnification $\times 24000$. Comparable presentation of electron micrographs from various categories of birds, revealing various degrees of mitochondrial lesions. 1a: Micrograph from the Leghorn reveals mitochondria are monomorphic with well-defined cristae (white arrow) and mitochondrial membrane is intact. 1b: Micrograph from fast-growing *ad libitum* fed broiler shows various degrees of changes to mitochondrial architecture; some mitochondria are normal while few revealed vacuolization and disintegration of cristae (black arrow). 1c: Micrograph from broiler with CHF shows mitochondrial swelling, vacuolization and disintegration of cristae (black arrow).

Table 1. Heart rate and MDA equivalent levels measured from Leghorn chickens, feed-restricted slow-growing broilers (Broiler-Res), fast-growing broilers fed ad libitum (Broiler-AL), and in broilers with CHF and ascites (Broiler-CHF)

Measurements	Leghorn	Broiler-Res	Broiler-AL	Broiler-CHF
Heart rate (beats/min)	372 ± 5.7 ^c	371 ± 3.6 ^c	308 ± 6.4 ^b	236 ± 8.2 ^a
MDA equivalents (nmol/mg heart)	0.91 ± 0.045 ^a	0.99 ± 0.038 ^a	1.19 ± 0.087 ^b	1.41 ± 0.025 ^c

Data presented as the mean ± standard error. Means within rows with different superscript letters are significantly different ($P < 0.05$).

In vitro enzyme inhibition studies revealed a decreased CK activity ($IC_{50} = 5.71$ mM) in the presence of increasing H_2O_2 concentrations (Figure 2). However, the LDH activity was increased in the presence of H_2O_2 at a concentration above 12.2 mM.

The PDH activity was not affected by the presence of H_2O_2 and TBH. The α -KGDH activity was not affected by the presence of H_2O_2 but decreased in the presence of increasing concentrations of TBH ($IC_{50} = 6.25$ mM).

Discussion

Several findings from the present study provide evidence that deterioration in heart function in broilers is associated with oxidative stress. The morphological changes observed in cardiac mitochondria in *ad libitum* fed broilers and in broilers with CHF are consistent with damage associated with oxidative stress, and this is well supported by biochemical evidence of damage to mitochondrial membranes caused by lipid peroxidation.

The Leghorn chickens in the present study did not show any sign of heart failure; hence measurements from the Leghorns were used as reference values. In order to understand the trends in the measured parameters (MDA equivalents and heart rate), the values from the slow-growing feed-restricted broilers (low risk of heart failure), fast-growing *ad libitum* fed broilers (high risk of heart failure) and broilers with CHF (observed within the *ad libitum* fed group) were plotted with respect to levels observed in Leghorn chickens (resistant to heart failure) taken as 100% (Figure 3).

The present findings further confirmed that fast-growing commercial broilers are at increased risk of heart failure. This increased risk of heart failure in broilers was associated with deterioration of heart functions as revealed by lowered heart rate observed in *ad libitum* fed broilers, and in broilers with CHF as

compared with feed-restricted slow-growing broilers and Leghorn chickens.

A declining heart rate is the most characteristic early pathophysiological feature of deterioration of heart pump function in fast-growing broilers. This has been reported in previous studies from our laboratory (Olkowski & Classen, 1998; Olkowski *et al.*, 2005, 2007, Nain *et al.*, 2008), as well as studies of others (Deng *et al.*, 2006; Druyan *et al.*, 2007), and has been confirmed in the present study. Interestingly, as demonstrated in Figure 3, the pattern of lowered heart rate appears to be strongly correlated with increasing levels of TBARS. This indicates that the deterioration of heart function is associated with oxidative damage in the myocardium.

Morphological changes observed in myocardial mitochondria in the present study are consistent with oxidative damage. Notably, mitochondria are the major source of ROS, but, because of their very high component of membranes, they are also a very sensitive target of ROS attack. The membrane lipids are very sensitive to oxidative damage due to the presence of polyunsaturated fatty acids, subsequently leading to lipid peroxidation (Halliwell & Gutteridge, 1985). Currently, one of the most common and well recognized approaches to measure the effects of free radicals is by measuring the oxidative damage (i.e. lipid peroxidation) to cellular membranes (Lykkesfeldt & Svendsen, 2007).

The measurements from the lipid peroxidation in the present study showed that, in fast-growing broilers, oxidative stress increases as the risk of heart failure increases (Figure 3). Moreover, the observed oxidative damage can readily be associated with morphological changes in the mitochondria.

The biochemical evidence of oxidative damage (elevated TBARS) corresponds well with the observed morphological changes in the mitochondria such as mitochondrial swelling, vacuolization, loss and disintegration of cristae. Earlier studies from our laboratory have reported insufficiency of high-energy phosphates in

Table 2. Effect of the presence of oxidants (H_2O_2 or TBH) on enzyme assay kit/cocktail components (CK and LDH kits, PDH and α -KGDH cocktails)

Reaction components	Enzyme activity (OD at 340 nm)			
	CK	LDH	PDH	α -KGDH
Kit/cocktail + H_2O_2 /TBH	ND	ND	ND	ND
Kit/cocktail + enzyme	100%	100%	100%	100%
Kit/cocktail + enzyme + H_2O_2 /TBH	INH	ACT	NO	INH/NO
ATP + H_2O_2 /TBH	ND	NA	NA	NA
NAD + H_2O_2 /TBH	ND	ND	ND	ND
NADH + H_2O_2 /TBH	ND	ND	ND	ND

ACT, increased activity in the presence of H_2O_2 ; INH, activity inhibition observed at various concentrations of H_2O_2 ; INH/NO, activity inhibition observed in the presence of TBH/no change in activity in the presence of H_2O_2 ; NA, not applicable; NADH, reduced nicotinamide adenine dinucleotide; ND, no detectable change; NO, no change in activity in the presence of H_2O_2 /TBH; OD, optical density.

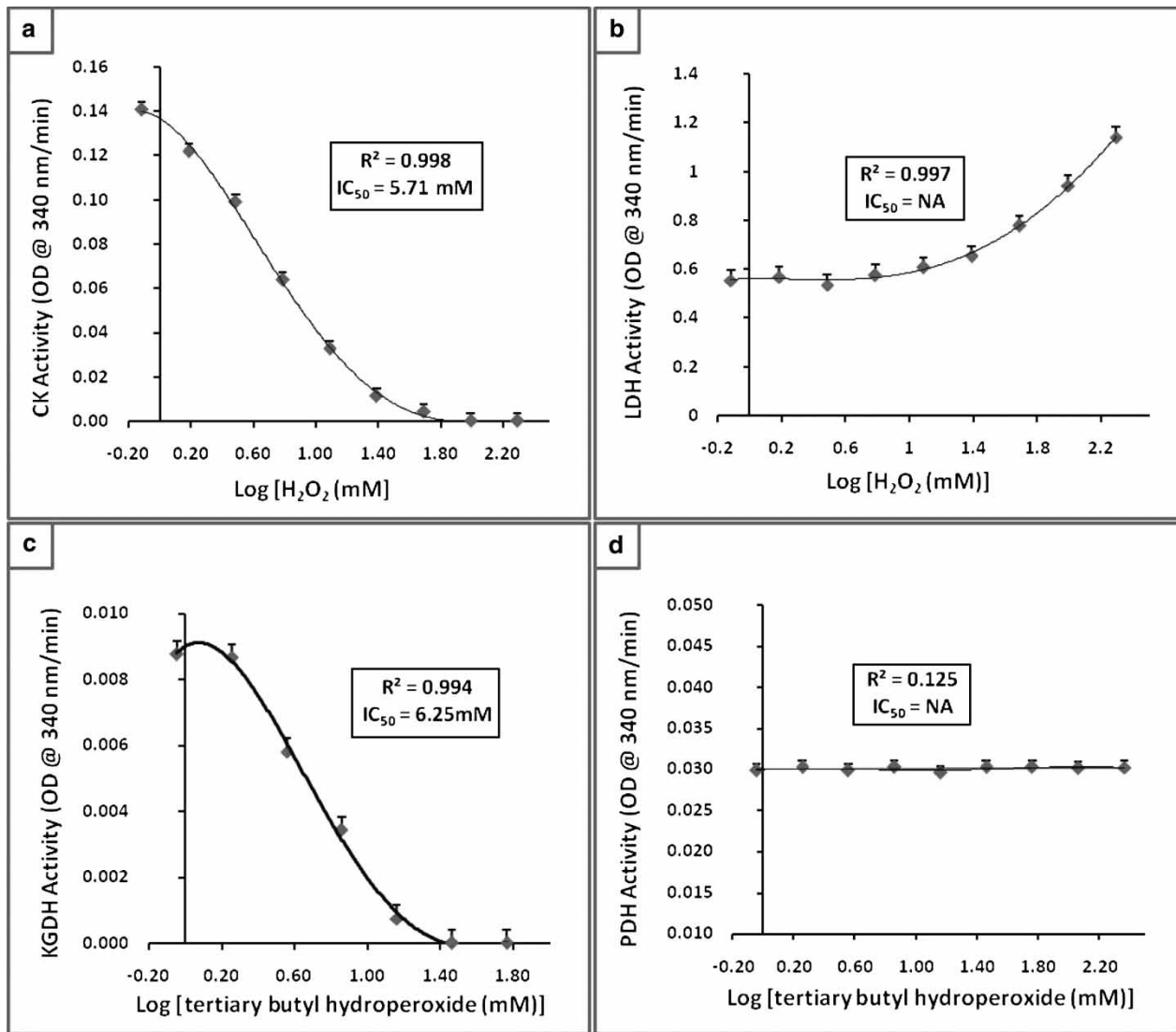


Figure 2. The CK, LDH, α -KGDH, and PDH enzyme sensitivity to oxidative stress tested in the presence of H_2O_2 or TBH. 2a: CK activity, note decreased activity ($\text{IC}_{50} = 5.71 \text{ mM}$) as the concentration of H_2O_2 increases. 2b: LDH activity, note activity of LDH increases with increased concentration of H_2O_2 . 2c: α -KGDH activity, note decreased activity ($\text{IC}_{50} = 6.25 \text{ mM}$) as the concentration of TBH increases. 2d: PDH activity, no affect on activity in the presence of TBH. Each data point represents the average of three measurements. Vertical bars indicate the standard error of the mean. OD, optical density.

the myocardium of broilers developing CHF (Nain *et al.*, 2008; Olkowski *et al.*, 2007). Hence, we can argue that mitochondrial damage corresponds with the lowered energy reserve in the myocardium as the mitochondrion is the power house of the myocardial cell.

The heart is one of the greatest energy-consuming organs in the body, which requires a constant supply of oxygen to maintain its metabolic functions (Giordano, 2005). In the cardiac tissue, mitochondria comprise 30% of the cardiomyocyte volume (Sheeran & Pepe, 2006). The major sites of ROS formation are at complex I and complex III of the electron transport chain located in the inner membrane of mitochondria (Turrens & Boveris, 1980; Turrens *et al.*, 1985). During normal metabolism, 1% to 2% of oxygen is converted to ROS. Hence, increased ROS or RNS production or decreased antioxidant defenses leads to oxidative stress.

α -KGDH, one of the key rate-limiting enzymes of the tricarboxylic acid cycle, is involved in energy synthesis pathways. Studies in rats have demonstrated that α -KGDH is a sensitive target of H_2O_2 (Nulton-

Persson & Szweda, 2001), but this enzyme can also act as a source of H_2O_2 in the mitochondria (for a review see Tretter & Adam-Vizi, 2005). It has been demonstrated that α -KGDH may generate ROS, leading to a state of oxidative stress (Starkov *et al.*, 2004; Tretter & Adam-Vizi, 2004). Although the activity of α -KGDH does not appear to be directly sensitive to H_2O_2 , it is notable that this enzyme was sensitive to TBH. In this context it is important to stress that TBH can produce both alkoxyl and hydroxyl radicals, two of the most potent ROS.

CK is involved in energy transformation pathways. A sizable fraction of CK is present inside mitochondria (mi-CK) in addition to that present in sarcoplasm. The ATP synthesized inside the mitochondria is converted to phosphocreatine by mi-CK, later transported to sarcoplasm and reconverted to ATP by CK present in sarcoplasm. As CK and α -KGDH are susceptible to oxidative damage, the mi-CK and α -KGDH are more likely to be affected *in vivo*, due to its closeness to the site of ROS production—that is, present inside the mito-

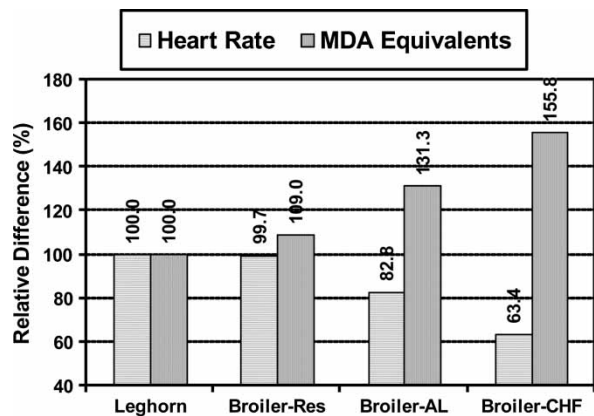


Figure 3. Relative heart rate and level of MDA equivalents in feed-restricted slow-growing broilers (Broiler-Res, low risk of heart failure), ad libitum fed fast-growing broilers (Broiler-AL, high risk of heart failure) and broilers with CHF (Broiler-CHF), expressed on a percentage basis of Leghorn chickens (Leghorn, resistant to heart failure).

chondria is the electron transport chain, a major source of free radicals.

In addition to the mitochondrial electron transport chain, xanthine oxidase is another important source of free radicals (Mekhfi *et al.*, 1996). Xanthine oxidase has the ability to produce H_2O_2 . *In vitro* studies have demonstrated that xanthine and xanthine oxidase had inhibitory effects on CK activity in heart tissues in a dose-dependent manner (Mekhfi *et al.*, 1996; Genet *et al.*, 2000).

In an anaerobic situation, LDH contributes to energy synthesis by anaerobic glycolysis. An increased production of ROS/RNS occurs during tissue hypoxia (Chen & Meyrick, 2004), which can negatively affect the activity of energy synthesis and transformation pathways. With hypoxia, activation of LDH enzyme by ROS may work as a force to counter the negative effect of other enzymes on energy synthesis and transformation pathways. Recently, we observed higher LDH activity in broilers developing CHF (Nain *et al.*, 2008). Hence, increased activity of LDH in broilers developing CHF is most probably due to generated oxidative stress in the broilers.

Insufficiency of creatine phosphate and ATP leads to deterioration in heart pump function in broilers (Nain *et al.*, 2008; Olkowski *et al.*, 2007). However, the decline in these energy substrates could not be explained by changes in the end point kinetic activities of the rate-limiting enzymes such as α -KGDH and PDH (Nain *et al.*, 2008). In view of the present findings it is entirely possible that the inhibition of α -KGDH and CK activity by oxidants can occur, regardless of their apparent kinetic activity *in vivo* (which may include compensatory up-regulation). This suggests that the observed decline in energy phosphates with deterioration in heart functions might be associated with the decreased activity of these enzymes during oxidative stress.

Our findings from the lipid peroxidation assay strongly suggest that oxidative stress is generated during development of heart failure. In fast-growing broilers, oxidative damage is probably associated with excessive generation of H_2O_2 . Maxwell *et al.* (1996) provide further supportive evidence as these authors demonstrated higher H_2O_2 activity in the cardiomyocytes from failing heart in broilers. However, as discussed above,

several other enzymatic pathways may generate oxidants. Among the most relevant to oxidative damage in broiler heart is xanthine oxidase complex, which has very high activity in birds (Edson *et al.*, 1936). This may be biochemically related to specific features of nitrogen metabolism in fast-growing broilers as uric acid is the end product of purine metabolism in all vertebrates, and in particular amino acid metabolism in birds. This reasoning is supported by the study of Enkvetchakul *et al.* (1993) who found that uric acid levels are higher in broilers with CHF, providing indirect evidence that xanthine oxidase may be involved in the pathogenesis of heart failure in broilers.

Based on the findings from the present study, we conclude that the deterioration in heart pump function and ultrastructural lesions observed in the mitochondria are subsequent to oxidative stress, which affects mitochondrial energy generation and synthesis pathways, leading to lowered energy reserve in the myocardium.

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