RESEARCH ARTICLE

The Effects of Conjugated Linoleic Acid and Berberine Supplementation on Markers of Allopurinol Activated Oxidative Stress in Broiler Chickens

Authors
Vincent Dartigue[1], Knox Van Dyke[2], and Hillar Klandorf*[1]

Affiliations
[1]Division of Animal and Nutritional Science, West Virginia University, Morgantown, WV
[2]Department of Biochemistry, West Virginia University School of Medicine, Morgantown, WV

***Corresponding author**
Hillar Klandorf
E-mail: hillar.klandorf@mail.wvu.edu

Abstract
Selection for rapid growth in poultry can be linked to an exaggerated state of oxidative stress (reactive oxidative species). Reactive oxidative species are kept in balance by endogenous and exogenous antioxidants. Two compounds conjugated linoleic acid (CLA) and berberine a purified compound from plant root extract have been suggested to ameliorate oxidative stress. A six-week study examined the effect of CLA and berberine supplementation on markers of oxidative-stress in poultry. Day old broiler chickens (n=60) were equally divided into six groups; a control, a CLA group where half of the regular oil used in a standard was substituted for a CLA oil mixture, a berberine group consisting of berberine supplementation, an allopurinol group, a CLA and allopurinol with the same dose as the CLA and allopurinol groups and a berberine and allopurinol group. The allopurinol was added to induce an oxidative stress state. Body weight, plasma uric acid, plasma glucose, and relative gene expression of six endogenous liver antioxidants were measured during the course of the study. The addition of allopurinol to the diet induced an oxidative stress state as measured by a significant reduction in plasma uric acid. There were no significant changes in BW and blood glucose concentrations. There was a 10-fold increase in the relative mRNA expression of superoxide dismutase 2 and 3 as well as glutathione peroxidase 1 and 3 in CLA+ALLO and BRB+ALLO treatment groups. Notably, CLA increased the expression of uncoupling protein 19-fold compared to control, while the addition of allopurinol blocked these changes. In contrast, there was a slight increase in the uncoupling protein in the BRB+ALLO treatment. Despite the increase in mRNA expression of the antioxidants genes, these results suggest that at the dosages administered, CLA and berberine were not effective in reversing the oxidative stress induced by allopurinol.

Introduction

Inflammation is of significant concern in the poultry industry, it impacts production worldwide and the overall health of the chicken. Inflammation can arise from both environmental and internal factors, the former can be usually traced back to an increase in ambient temperature whereas the latter has a broader range of origin from species to gender and metabolism. Both environmental and internal factors are characterized by an increase in oxidative stress[1,2,2]. Oxidative stress results from an imbalance between reactive oxidative species (ROS), which are highly reactive oxygen base molecules, and antioxidants (Aox). A major source ROS is the electron transport chain (ETC)[4,5] and in an imbalanced physiologic state they represent a danger to aerobic organisms because of their high reactivity and tendency to form covalent bonds with lipids, proteins and DNA[6,7,8]. Any structural alterations in molecules can lead to a change in cell state, distancing itself from cell homoeostasis, which can be characterized by overexpression of oncogenes, formation of mutagens, and overall inflammation[9].

Birds in general have higher level of plasma glucose; however, they do not exhibit any noticeable side effects associated with classic diabetic complication due to the elevated levels of the endogenous antioxidant plasma uric acid. Plasma uric acid is a potent Aox for circulating ROS in species lacking uricase and is associated with prolonged life span[10]. Previous studies in poultry have demonstrated that allopurinol supplementation reduces plasma uric acid concentrations thus establishing a controllable oxidative stress model[11]. In a normal state, ROS generation is balanced by antioxidants and other molecules having Aox-like properties, which target and neutralize the oxidative species. Antioxidants can be characterized as endogenous or exogenous. The main families of endogenous Aox are the superoxide dismutase and the glutathione family while the exogenous ones are mostly characterized by the presence of a phenol group[9,11,12].

Berberine, an exogenous Aox isolated from plant root extract, has been used in traditional Chinese herbal medicine. This compound is a quaternary ammonium salt that possesses several benzene rings and methoxy functional groups, which allows the electron to circulate within the conjugated double bond system. In addition to increasing the activity of endogenous antioxidants, berberine is reputed to possess anti-diabetic, anti-inflammatory and anti-carcinogenic properties[13,14,15,16]. Neutralization of ROS may also be achieved by activation of uncoupling protein (UCP). Uncoupling protein is a member of the family of the transmembrane proteins located in the inner membrane of the mitochondria homologues involved in thermogenesis and ROS balance. Its main mode of action is to uncouple respiration (ETC) from oxidative phosphorylation and does so by moving protons from the inner mitochondrial membrane space into the matrix[17,18]. This action increases the probability of any pair of protons reacting with superoxide radicals, thereby, creating metabolic water from catalase or glutathione peroxidase. Berardi et al.[19] has suggested that UCP can be activated by the binding of fatty acid (C14 to C18) to the receptor whereas purine nucleotides act as allosteric inhibitors. For example, *trans*-10 *cis*-12 conjugated linoleic acid (CLA) has been shown to affect energy balance through up-regulation of UCP activity[18,20,21,22]. CLA refers to a collective of fatty acids, each 18 carbons long. The primary source of CLA is from dairy products by hydrogenation of dietary linoleic acid and stearic acid. Of the 28 conjugated system, the *cis*-9 *trans*-11 and *trans*-10 *cis*-12 have been shown to have the most prominent metabolic effects.

The purpose of this study was to determine if the addition of berberine or CLA to the diet of broiler chickens could reverse the induction of an oxidative stress state induced by allopurinol. We hypothesized that the addition of the two compounds should increase the endogenous antioxidants possibly reverting the redox state induced by the help of the allopurinol.

Material and Methods

All procedures and experiments were approved by West Virginia University's Institution Animal Care and Use Committee (ACUC #15-0301.1).

Experimental Design

Sixty mixed sex day old Cobb/Cobb 500 broiler chicks were obtained from Pilgrim's Pride, Moorefield, WV. Chicks were housed within pens and maintained under a twelve-hour light-dark cycle with temperature set at 27°C. On the third day, chicks were divided in two pens until they reached one week of age, at which time they were tagged with leg bands and divided into six pens based on weight. The study lasted for a total of six weeks. The chicks were maintained on a starter diet for the first four weeks. At week five of the study, the broiler chicks were started on different treatments. The treatments were based on a standard diet (see Table 1) with the supplementation of either berberine or the partial of conjugated linoleic acid oil. The six treatments were composed of a control group, a conjugated linoleic (CLA) diet in which half of the regular oil used in the normal diet was substituted for a mixture of 50/50 *cis*-9 *trans*-11 and *trans*-10 *cis*-12 isomers CLA oil (0.80% CLA oil diet)[23]. The berberine group (BRB) was supplemented with berberine (200mg/kg of feed)[14], the ALLO group was provided allopurinol (25mg/kg of body weight) supplemented in the diet[24], in addition to a CLA+ALLO and BRB+ALLO group where ALLO was provided for the last two weeks of the study. The ALLO, CLA+ALLO and BRBA+ALLO were treated with allopurinol to induce a redox state[25,26]. Weights were recorded weekly over the six-week experimental period. Berberine (chloride hydrate, purity >99%) and Allopurinol were purchased from Sigma-Aldrich (Saint Louis, MO, USA). The CLA was purchased commercially (Vitamorph Labs, Highland, IN, USA) as 1200 mg capsules. Food and water were provided ad libitum.

Table 1. Ingredient and percentage composition of diet fed to broilers

Item		
Ingredient %	Control Oil Diet	CLA oil Diet
Soybean meal	28.3	28.3
Corn	66.2	66.2
Regular oil	2.2	1.1
CLA	N/A	1.1
Dicalcium phosphate	1.7	1.7
Calcium carbonate	0.8	0.8
Salt	0.25	0.25
Nutra blend mix[a]	0.25	0.25
Methionine	0.2	0.2
Monensin	0.1	0.1
Total %		
M. Energy[b] (kcal/kg)	3130.5	3130.5
Protein %	19.5	19.5
Fat %	4.9	4.9
Fiber %	2.67	2.67
Calcium %	0.78	0.78
Phosphorous % (total)	0.67	0.67
Phosphorous % (available)	0.44	0.44
Methionine %	0.48	0.48
Methionine + Cysteine %	0.82	0.82
Lysine %	1.04	1.04

[a] Nutrablend premix is from Premix NB 3000 used at a rate of five lbs/ton Nutra Blend LLC (Neosho,MO) consisted of manganese (4%); zinc (4%); iron (2%); copper, 4.500ppm; iodine, 600ppm; selenium, 60ppm; vitamine A, 1.4E10e5 IU/lb; vitamin D3, 5.E5 IU/lb; vitamin E 3.E3 IU/lb; vitamin B12, 2mg/lb; menadione, 150mg/dl; riboflavin, 1200mg/dl; thiamine, 200mg/dl; D-pantothenic acid, 1200mg/dl; niacin; 5000mg/dl; vitamin B6 250mg/dl; folic acid 125mg/dl; choline, 700000mg/dl; Biotin 6mg/dl.
[b] Metabolizable energy

Sampling procedure

After two weeks on treatment, blood samples (4ml) were obtained from six birds per pen from the brachial (wing) vein. Each sample was transferred to a heparinized tube and immediately placed on ice. The same birds were sampled for the next three subsequent bleedings: for a total of four bleedings. The samples were centrifuged at 2000 rpm for 20 minutes at 4°C to collect plasma and stored at -20° C, pending analysis. On the last day of the study, the birds were euthanized and a sample of liver (~1g) was snap frozen in liquid nitrogen and stored in a -80°C freezer for further analysis.

Sample analysis

Plasma samples were used for measurement of plasma glucose and plasma uric acid. Plasma glucose was measured using amperometric measurement of hydrogen peroxide (H_2O_2) on a platinum electrode (YSI 2700 Biochemistry analyzer)[24,27]. Plasma uric acid (PUA) was

measured using *Infinity™ Uric Acid Liquid Stable Reagent* assay from Thermo Scientific (Waltham, MA, USA; Klandorf et al., 2001).

Liver samples (75-100 mg) were used to study the relative mRNA expression of six antioxidant related genes in the superoxide dismutase (SOD) family, glutathione peroxidase (GPX) family and uncoupling protein (UCP) family. A Trizol-Reagent (*Invitrogen™*, Carlsbad, CA) based method was used to extract and isolate total RNA. The isolated RNA was resuspended in nuclease free water and treated with DNase (Ambion®, Thermo Scientific Waltham, MA, USA). Four microliters of DNase treated RNA were reverse transcribed to cDNA utilizing an iScript™ cDNA synthesis kit from Bio-Rad (Hercules, CA). A SYBR® Green Master Mix kit (Bio-Rad, Hercules, CA) method was used on a BIORAD CFX96 Real Time System (C1000 Touch Thermal cycler) to quantify the expression of SOD1, SOD2, SOD3, GPX1, GPX3 and avUCP using designed primers. The gene sequences were obtained from the ncbi data base (https://www.ncbi.nlm.nih.gov) and designed using the Primer3 Input (version 4.0 http://bioinfo.ut.ee/primer3-0.4.0/primer3/) (see Table 2). Results of real time PCR were normalized to the housekeeping gene GAPDH. Calculation and analysis of mRNA expression was performed using the efficiency corrected delta-delta Ct method[28].

Table 2. Oligonucleotide primers for real-time RT-PCR

Gene	Forward (5'-3')	Reverse (5'-3')	Annealing (°C)	Accession Number
*GAPDH	Gagggtagtgaaggctgctg	cggatcaaaggtggaagaat	60	NM_204305.1
[1]GPX1	Ggaggctctgcagtaaagga	cttcgaggctttggaagatg	60	NM_001277853.2
[1]GPX3	Gagatcctccctgcactgaa	cacatccccttctggaaga	60	NM_001163232.2
[a](Cu-Zn)SOD1	Tgctggacctcgtttagctt	ctcatttcccactgccatct	60	NM_205064.1
[b](Mn)SOD2	Aatggtgggggtcatatcaa	agtcacgtttgatggcttcc	60	NM_204211.1
[a](Cu-Zn)SOD3	Tgtgatccatgagcaggaag	gtttgccagcatttccattt	60	XM_015285700.1
avUCP	Gagaaacagagcgggatttg	ctcctggctcacggatagag	60	NM_204107.1

*GAPDH is glyceraldehyde 3- phosphate dehydrogenase
[1]GPX is Glutathione Peroxidase
[a]SOD1 and 3 are soluble and extracellular respectively, using Copper-Zinc (Cu-Zn) as electron acceptor
[b]SOD2 is mitochondrial superoxide dismutase using manganese (Mn) as an electron acceptor

Statistical analysis

The effects of experimental treatments on bodyweight, plasma uric acid, and plasma glucose and gene expression were analyzed using ANOVA with treatment as main effect. Tukey's multiple comparison test was used for specific pair wise comparison. Statistical analysis for gene expression was done on ΔCt, however, the ΔΔCT measurement were used in the figures. The values presented are the LSM+SE. Statistical analysis was done using JMP program (SAS Institute Inc., Cary NC). Statistical significance was set at $P < 0.05$.

Results

There were no significant differences in body weight (BW) gain between the

treatments (data shown in Dartigue et al (unpublished)). There was a significant ($P < 0.05$) decrease in plasma uric acid concentrations at weeks 5 and 6 of the study in groups administered allopurinol compared to controls (Fig 1). The decrease was more pronounced ($P < 0.05$) in the CLA+ALLO and BRB+ALLO groups, 0.17±0.04mM and 0.16±0.04mM respectively, compared to both ALLO (0.27 ± 0.04mM) and controls (0.43 ± 0.04mM). Neither BRB nor CLA had a measurable effect on plasma glucose concentrations compared to controls (Figure 2). However, the combination of CLA+ALLO significantly decreased plasma glucose concentrations compared to the ALLO group. Liver to bodyweight ratio in the BRB group was significantly decreased compared to controls (Fig 3). Allopurinol administration in ALLO, CLA+ALLO and BRB+ALLO groups increased ($P < 0.05$) the liver/BW ratio as compared to the controls, Fig 3. However, there was a numerical decrease in the liver/bw ratio in the CLA+ALLO and BRB+ALLO groups compared to the ALLO group.

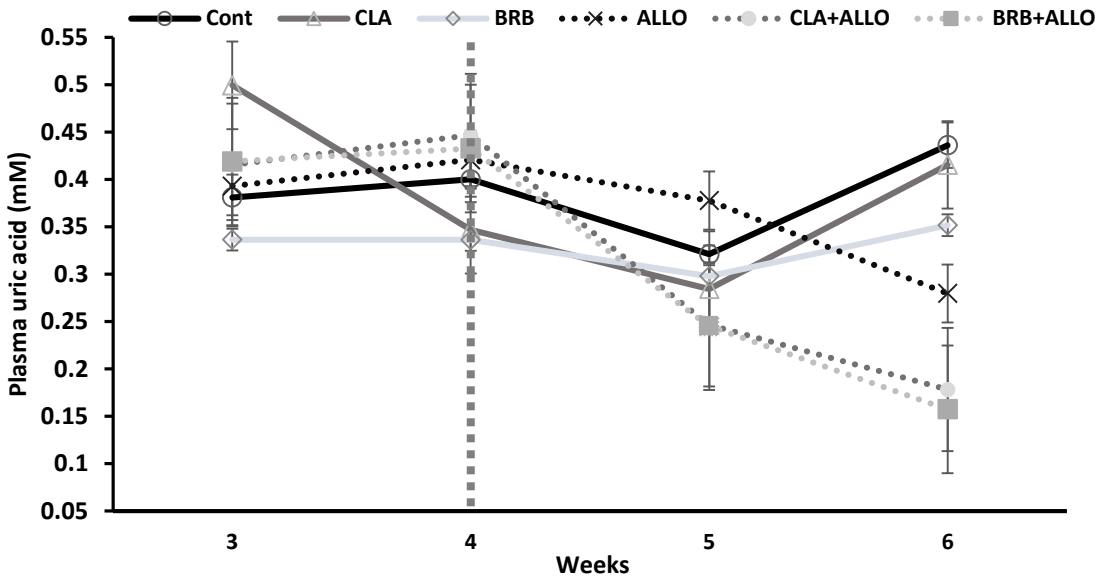


Fig 1. Change in plasma uric acid (mM) over the last four weeks of treatments. The vertical dashed line represents the time at which allopurinol was added to the respective diets. Controls, conjugated linoleic acid (CLA) and berberine (BRB) groups are shown as solid lines as compared to the Allopurinol (ALLO), conjugated linoleic acid+allopurinol (CLA+ALLO) and berberine+allopurinol (BRB+ALLO), which are represented by dotted lines. The notation **a** represents significant differences compared to controls whereas **b** represents significant differences compared to the ALLO group. $P < 0.05$

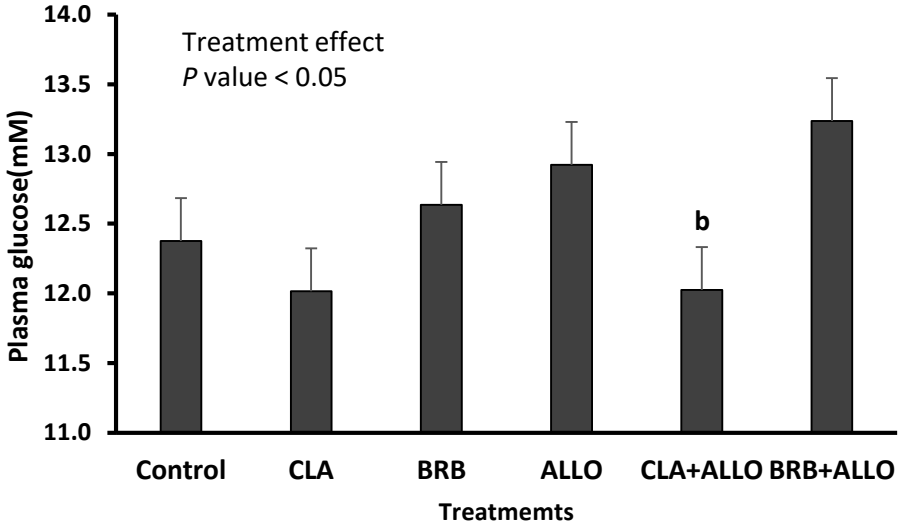


Fig 2. **Effect of treatment on plasma glucose concentrations**. The control, conjugated linoleic acid (CLA) and berberine (BRB) represent the non-redox state. Allopurinol (ALLO), conjugated linoleic acid+allopurinol (CLA+ALLO) and berberine+allopurinol (BRB+ALLO) are the redox state groups. The data represents least square mean ± standard error. The notation **a** represents significant differences compared to controls whereas **b** represents significant differences compared to the ALLO group. $P < 0.05$

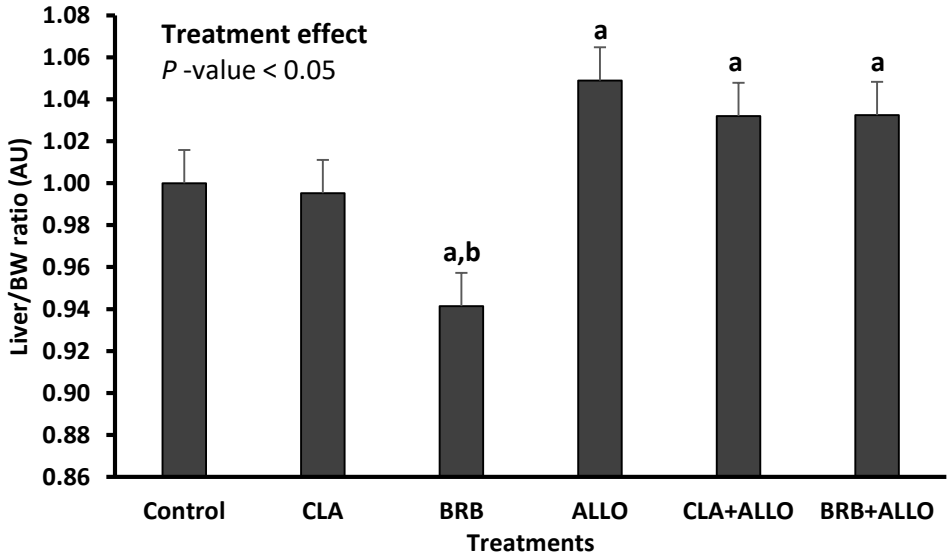
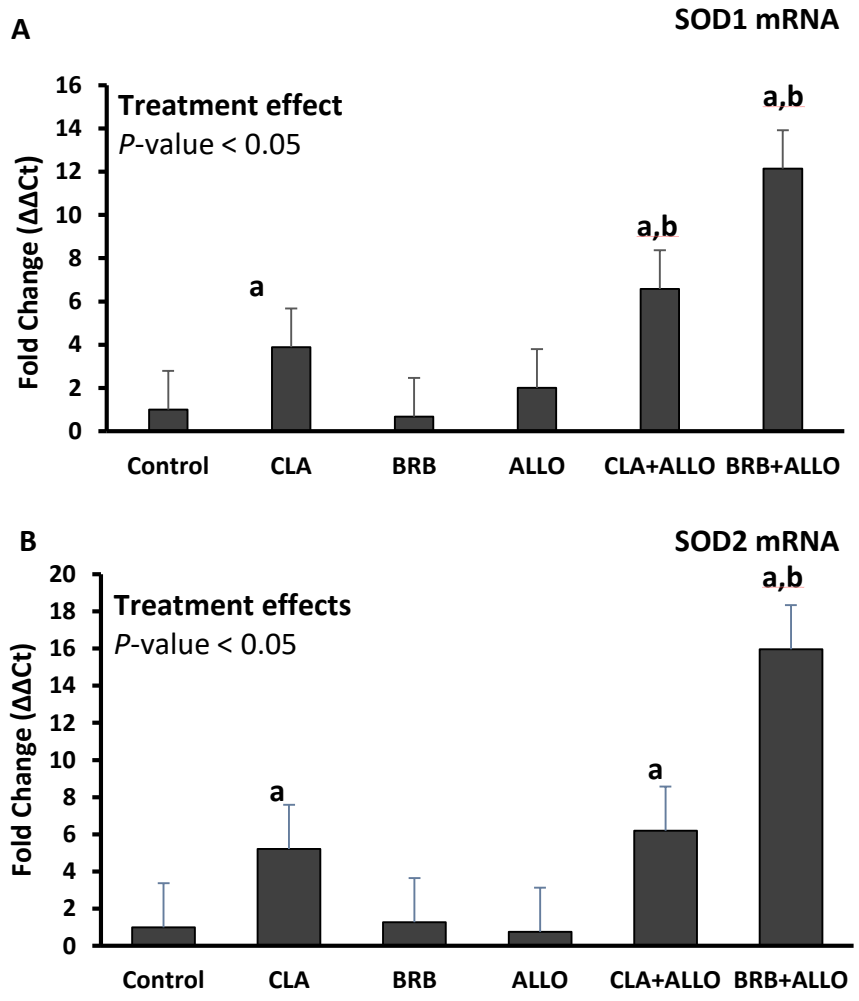
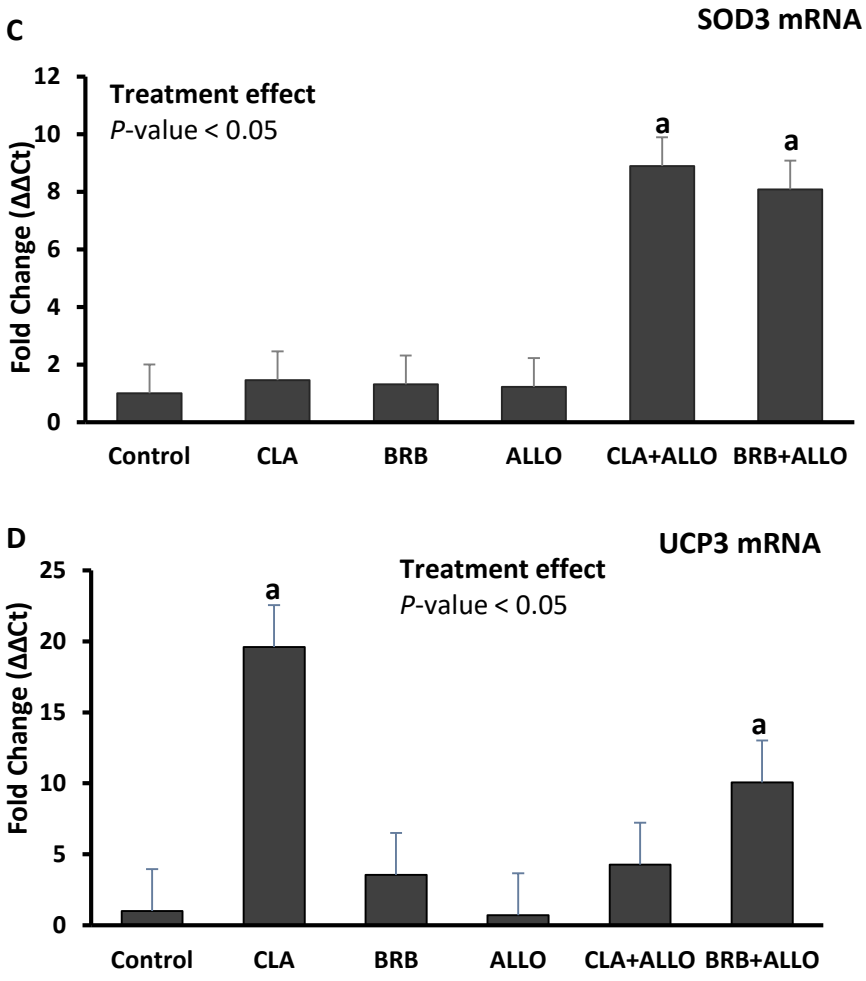


Fig 3. Effect of treatment on liver to body weight ratio. The control, conjugated linoleic acid (CLA) and berberine (BRB), represent the non-redox state. Allopurinol (ALLO), conjugated linoleic acid+allopurinol (CLA+ALLO) and berberine+allopurinol (BRB+ALLO) are the redox state groups. The data represents least square mean ± standard error. The notation **a** represents significant differences compared to controls whereas **b** represents significant differences compared to the ALLO group. $P < 0.05$

The relative expression of superoxide dismutase (SOD) 1 through 3, uncoupling protein (UCP) and glutathione (GPX) 1 and 3 are shown in Fig 4. The genes were chosen based on their location and their targets. SOD 1 and 3 uses Cu/Zn as electron acceptors and were expressed in the outer mitochondrial membrane and in the cytoplasm, respectively. SOD2 has manganese as an electron acceptor, which is located in the mitochondrial matrix. GPX 1 and 3 differed on their physical location, intracellular and plasma soluble respectively. Only one homologue of UCP was studied. Control units were expresses as arbitrary units and set as 1. In the baseline groups composed of the CLA group and BBR group, CLA led to a 4-fold increase in SOD1, whereas BRB slightly decreased SOD1 expression below that of controls (Fig 4, A). ALLO numerically increased SOD1 expression by 3-fold, which was not significant. The redox state however, caused by allopurinol led to a 6- and 12-fold increase in expression ($P < 0.05$) in both the CLA+ALLO and BRB+ALLO groups.





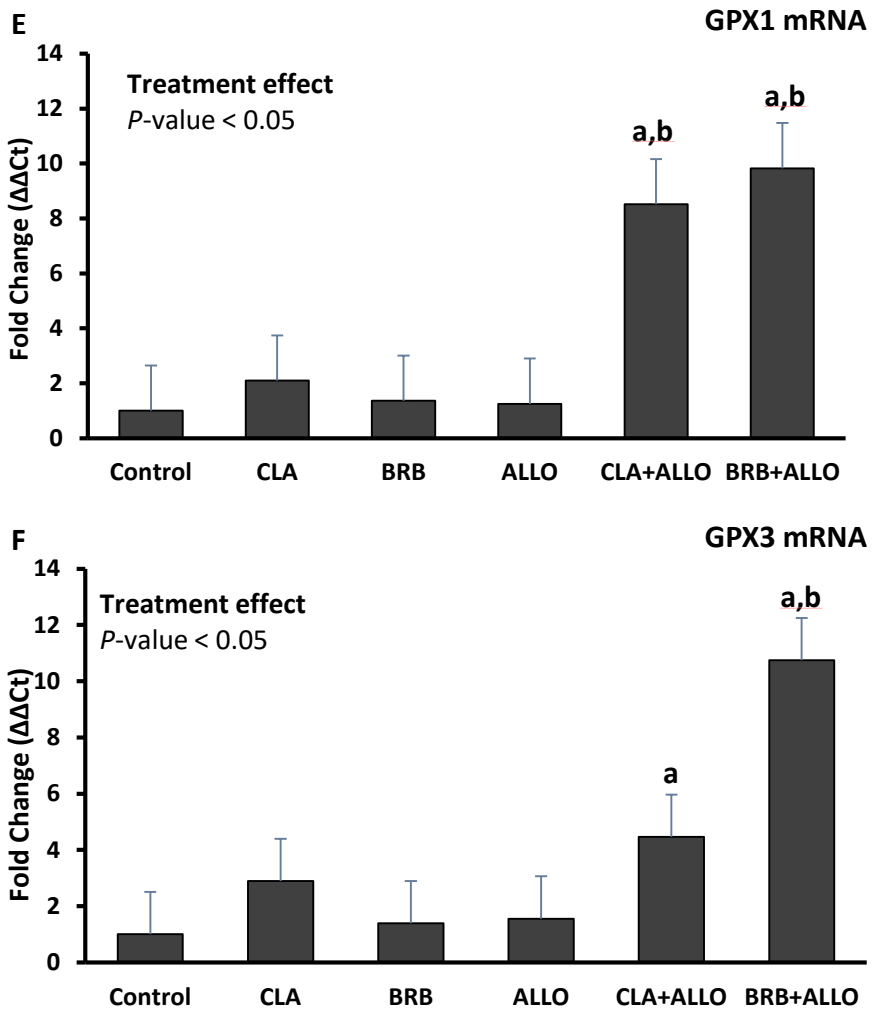


Fig 4. Relative mRNA expression of hepatic endogenous antioxidant enzymes in broiler chickens. Control, conjugated linoleic acid (CLA) and berberine (BRB) represent the non-redox groups. Allopurinol (ALLO), conjugated linoleic acid+allopurinol (CLA+ALLO) and berberine+allopurinol (BRB+ALLO) are in the redox state. Panel **A**, **B** and **C** represent the expression of superoxide dismutase (SOD) 1 through 3. (**D**) The effect of treatments on uncoupling protein (UCP3) expression. Panels **E** and **F** represent Glutathione peroxidase 1(GPX1) and GPX3 expression across treatments. The data was calculated using the δ-δ Ct method. GAPDH was used as the housekeeping gene to normalize expression. The data represents the mean ± standard error. The notation **a** represents significant differences compared to controls whereas **b** represents significant differences compared to the ALLO group. ($P < 0.05$).

SOD2 expression across treatments was comparable to the changes observed in SOD1 (Fig 4, B). In the non-redox state, CLA increased SOD2 six-fold, whereas BRB increased the expression two-fold. However, SOD2 expression decreased in the ALLO group in contrast to the increase observed in SOD1. CLA and BRB, when exposed to allopurinol, increased the expression of SOD2. CLA+ALLO also significantly increased SOD2 relative expression 7-fold and BRB+ALLO 16-fold ($P < 0.05$). In contrast to SOD1 and SOD2, SOD3 expression in CLA, BRB and ALLO groups were not significantly different compared to the controls (Fig 4, **C**). In the redox state,

however, there was an increased expression of SOD3 in CLA+ALLO and BRB+ALLO groups, at 10 and 9-fold, respectively.

A marked increase in uncoupling protein (avUCP) expression occurred in response to the CLA treatment (20-fold increase) whereas BRB marginally affected avUCP expression (3-fold increase) as compared to controls (Fig 4, **D**). avUCP expression decreased in the ALLO group as compared to controls. In contrast to CLA, CLA+ALLO increased UCP expression 4-fold whereas there was a 10-fold increase in UCP3 expression in the BRB+ALLO group.

Glutathione peroxidase 1 (GPX1) expression was similar to that observed in the SOD3 group (Fig 4, E). In the non-redox state, CLA and BRB did not undergo significant changes in gene expression. In the redox state, ALLO did not change the expression of GPX1, whereas CLA+ALLO and BRB+ALLO treatments led to 9- and 10-fold increases ($P < 0.05$) in expression, respectively. Glutathione peroxidase 3 (GPX3) expression closely resembled that of SOD 2 in both the non-redox and redox state (Fig 4, **F**). There was a small increase in GPX3 expression in the CLA and BRB groups, as well as in the allopurinol treated birds. CLA+ALLO and BRB+ALLO modulation were pronounced with increases in expression 5 and 11-fold, respectively ($P < 0.05$).

Discussion and Conclusions

Plasma uric acid is a potent antioxidant in birds and its inhibition has been linked to an increase in ROS production in birds[24,30]. Previous studies have established that allopurinol is a potent inhibitor of plasma uric acid in birds, which leads to the establishment of a redox state in treated chickens[11,24]. The degree or severity of the redox state can be varied depending on the dosage of allopurinol administered, resulting in a somewhat controllable model of oxidative stress for redox study. Making use of this model, we set out to determine if berberine and/or CLA could restore the bird to its pre-redox state caused by the administration of allopurinol.

One week after administration allopurinol, PUA declined in all treated groups. By week 10, the ALLO group had a level of plasma uric acid of 0.27 ± 0.04mM compared to the controls at 0.40 ± 0.037 mM. However, this decline was unexpectedly more pronounced in both the CLA+ALLO and BRB+ ALLO groups as they reached levels of 0.17±0.04 and 0.16±0.04mM respectively ($P < 0.05$). Kong et al[31] suggest that berberine may have an inhibitory effect on xanthine oxidase; if so, this would explain the negative synergistic effect on plasma uric acid concentrations.

CLA is known to increase the activity and expression of avUCP[32] whereas berberine is suggested to reduce blood glucose concentrations in Type 2 diabetes by increasing insulin sensitivity[33] as well as enhance the expression of endogenous antioxidants such as SOD[14]. UCP is a transmembrane protein with its active site exhibiting flippase activity to move protons between mitochondrial inner membrane space and the matrix. The accumulation of protons in the matrix increases the probability that a proton interacts with an electron as they cycle through complex III and IV. As the animal transitions from a normal to a redox state, there should be an increase in antioxidants to neutralize the ROS and potentially dampen the effect of the shift. With this basic premise, the addition of CLA and berberine was expected to increase the expression of antioxidant genes. To test this hypothesis, we measured the mRNA expression of six endogenous antioxidants genes. Berberine administration led to an increase in the relative expression of SOD 1, 2 and 3 by 13, 16 and 8-fold respectively. These compounds also increased mRNA

expression of UCP, glutathione 1 and 3, by a 10-fold change in both avUCP and GPX1 and an 11-fold change in GPX3. Although, CLA was hypothesized to affect avUCP, it also increased the relative expression of other endogenous antioxidants, albeit less pronouncedly. Comparable results were reported by Cantwell et al.[34] in human cell line exposed to cow milk containing CLA.

Reactive oxygen species are kept in balance by antioxidants. The superoxide dismutase (SOD) family and glutathione are among the principal endogenous antioxidants. SOD catalyzes superoxide to hydrogen peroxide, which is then degraded to in water and oxygen by glutathione[34.] In this study, CLA led to a 7, 6- and 8-fold increase in SOD 1, 2 and 3, respectively. Cellular glutathione's expression increased 9-fold whereas the increase in extracellular glutathione was 5-fold. However, the addition of CLA to allopurinol did not result in a further increase in UPC expression. Allopurinol is a purine analogue which may have inactivated UCP, which is down-regulated by purines. It is possible that allopurinol also masked the effect of CLA on avUCP. This would explain the 3-fold increase in the redox state in contrast to the 19-fold increase in avUCP expression without allopurinol. Furthermore, CLA modulation of avUCP expression could be an additional means to maintain ROS balance

An increase in ROS has been linked to liver inflammation due to leukocyte infiltration which, in turn, leads to additional ROS production[35]. In the current study, we measured an increase in liver size in the ALLO group as well as an increase in the liver to BW ratio compared to controls. The addition of the BRB and CLA to the allopurinol treated birds was able to numerically decrease liver weight but not back to control levels. These results are in agreement with Hwang et al[36] who observed in rats that berberine decreased ROS production and the incidence of liver lesions by an increase glutathione production. The results suggest that at the dosages used in this study CLA and berberine were not able to completely restore to a non-redox state in birds administered allopurinol.

The use of the allopurinol redox model in birds to study the effect of CLA and berberine on markers of oxidative stress was not conclusive. The liver to BW ratio suggested that the addition of CLA and berberine were able to reduce inflammation of the liver but not to that measured in control levels. The further reduction in plasma uric acid by these two compounds in allopurinol fed birds was not anticipated, which suggests that additional ROS may have been produced. CLA and berberine were able to increase the relative mRNA expression of the selected endogenous antioxidant genes but without measurement of the gene products, we cannot establish whether these changes have been translated/expressed. Additional studies are required to establish this relationship. Finally, at the dosage of allopurinol used we were not able to completely restore the redox state. We conclude that the allopurinol model can be used to measure the ability of alternative compounds to ameliorate the induced oxidative stress.

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References

1. Federico A, Morgillo F, Tuccillo C, Ciardiello F, Loguercio C. Chronic inflammation and oxidative stress in human carcinogenesis. *International Journal of Cancer*. 2007 Dec 1;121(11):2381-6.
2. Cerutti P, Amstad P. Inflammation and oxidative stress in carcinogenesis. In *Eicosanoids and Other Bioactive Lipids in Cancer*, Inflammation and Radiation Injury 1993 (pp. 387-390). Springer US.
3. Marku, L. and Klandorf, H. (2021). Plasma Urate and Neurodegenerative Disease. *J. of Drug Design and Research 8(1):*1078-1080.
4. Chen Q, Vazquez EJ, Moghaddas S, Hoppel CL, Lesnefsky EJ. Production of Reactive Oxygen Species by Mitochondria: CENTRAL ROLE OF COMPLEX III. *Journal of Biological Chemistry* 2003 September 19;278(38):36027-36031.
5. Murphy MP. How mitochondria produce reactive oxygen species. *Biochem J* 2009 Jan 1;417(1):1-13.
6. Martin KR, Barrett JC. Reactive oxygen species as double-edged swords in cellular processes: low-dose cell signaling versus high-dose toxicity. *Hum Exp Toxicol* 2002 02;21(2):71-75.
7. Wu W. The signaling mechanism of ROS in tumor progression. *Cancer and Metastasis Reviews* 2006;25(4):- 695-705.
8. D'Autreaux B&, Michel. ROS as signaling molecules: mechanisms that generate specificity in ROS homeostasis. *Nature Reviews Molecular Cell Biology* 2007;8(-10):- 813.
9. Pisoschi AM, Pop A. The role of antioxidants in the chemistry of oxidative stress: A review. *Eur J Med Chem* 2015 6/5;97:55-74.
10. Cutler RG. Urate and ascorbate: their possible roles as antioxidants in determining longevity of mammalian species. *Archives of gerontology and geriatrics*. 1984 3(4):321-48.
11. Simoyi MF, Falkenstein E, Van Dyke K, Blemings KP, Klandorf H. Allantoin, the oxidation product of uric acid is present in chicken and turkey plasma. *Comparative Biochemistry and Physiology* Part B: *Biochemistry and Molecular Biology* 2003;135(2):325-335.
12. Larson RA. The antioxidants of higher plants. *Phytochemistry* 1988;27(4):969-978.
13. Meeran SM, Katiyar S, Katiyar SK. Berberine-induced apoptosis in human prostate cancer cells is initiated by reactive oxygen species generation. *Toxicol Appl Pharmacol* 2008 5/15;229(1):33-43.
14. Li Z, Geng Y, Jiang J, Kong W. Antioxidant and Anti-Inflammatory Activities of Berberine in the Treatment of Diabetes Mellitus. *Evidence-Based Complementary and Alternative Medicine* 2014;2014:12.
15. Liu S, Lee H, Hung C, Tsai C, Li T, Tang C. Berberine attenuates CCN2-induced IL-1β expression and prevents cartilage degradation in a rat model of osteoarthritis. *Toxicol Appl Pharmacol* 2015 11/15;289(1):20-29.
16. Xie J, Xu Y, Huang X, Chen Y, Fu J, Xi M, et al. Berberine-induced apoptosis in human breast cancer cells is mediated by reactive oxygen species generation and mitochondrial-related apoptotic pathway. *Tumor Biol* 2015;36(2):1279-1288.
17. Teulier L, Rouanet J, Letexier D, Romestaing C, Belouze M, Rey B, et al. Cold-acclimation-induced non-shivering thermogenesis in birds is associated with upregulation of avian UCP but not with innate uncoupling or altered ATP efficiency. *J Exp Biol* 2010 The Company of Biologists Ltd;213(14):2476-2482
18. Abe T, Mujahid A, Sato K, Akiba Y, Toyomizu M. Possible role of avian

uncoupling protein in down-regulating mitochondrial superoxide production in skeletal muscle of fasted chickens. *FEBS Lett* 2006 9/4;580(20):4815-4822.
19. Berardi M, Chou J. Fatty Acid Flippase Activity of UCP2 Is Essential for Its Proton Transport in Mitochondria. *Cell Metabolism* 2014 9/2;20(3):541-552.
20. Mollica MP, Trinchese G, Cavaliere G, De Filippo C, Cocca E, Gaita M, et al. c9,t11-Conjugated linoleic acid ameliorates steatosis by modulating mitochondrial uncoupling and Nrf2 pathway. *J Lipid Res* 2014 May;55(5):837-849.
21. Viladomiu M, Hontecillas R, Bassaganya-Riera J. Modulation of inflammation and immunity by dietary conjugated linoleic acid. *Eur J Pharmacol* 2015.
22. Yang B, Chen H, Stanton C, Ross RP, Zhang H, Chen YQ, et al. Review of the roles of conjugated linoleic acid in health and disease. *Journal of Functional Foods* 2015;15:314-325.
23. Ramiah SK, Meng GY, Sheau Wei T, Swee Keong Y, Ebrahimi M. Dietary Conjugated Linoleic Acid Supplementation Leads to Downregulation of PPAR Transcription in Broiler Chickens and Reduction of Adipocyte Cellularity. *PPAR Res* 2014;2014:137652.
24. Klandorf H, Rathore DS, Iqbal M, Shi X, Van Dyke K. Accelerated tissue aging and increased oxidative stress in broiler chickens fed allopurinol. *Comparative Biochemistry and Physiology* Part C: Toxicology & Pharmacology 2001 6;129(2):93-104.
25. Settle T, Carro MD, Falkenstein E, Radke W, Klandorf H. The effects of allopurinol, uric acid, and inosine administration on xanthine oxidoreductase activity and uric acid concentrations in broilers. *Poultry Science* 2012 November 01;91(11):2895-2903.
26. Settle T, Falkenstein E, Klandorf H. The effect of allopurinol administration on mitochondrial respiration and gene expression of xanthine oxidoreductase, inducible nitric oxide synthase, and inflammatory cytokines in selected tissues of broiler chickens. *Poultry Science* 2015 October 01;94(10):2555-256.
27. Hall SB, Khudaish EA, Hart AL. Electrochemical oxidation of hydrogen peroxide at platinum electrodes. Part 1. An adsorption-controlled mechanism. *Electrochim Acta* 1998;43(5–6):579-588.
28. Barnes KM, Winslow NR, Shelton AG, Hlusko KC, Azain MJ. Effect of dietary conjugated linoleic acid on marbling and intramuscular adipocytes in pork. *Journal of Animal Science*. 2012 Apr 1;90(4):1142-9.
29. Van Dyke K, Castranova V. Cellular chemiluminescence. *CRC*; 1987 Jul 1.
30. Kong LD, Yang C, Ge F, Wang HD, Guo YS. A Chinese herbal medicine Ermiao wan reduces serum uric acid level and inhibits liver xanthine dehydrogenase and xanthine oxidase in mice. *Journal of Ethnopharmacology*. 2004 Aug 31;93(2):325-30.
31. Takahashi Y, Kushiro M, Shinohara K, Ide T. Dietary conjugated linoleic acid reduces body fat mass and affects gene expression of proteins regulating energy metabolism in mice. *Comparative Biochemistry and Physiology* Part B: Biochemistry and Molecular Biology 2002;133(3):395-404.
32. Xing L, Zhang L, Liu T, Hua Y, Zheng P, Ji G. Berberine reducing insulin resistance by up-regulating IRS-2 mRNA expression in nonalcoholic fatty liver disease (NAFLD) rat liver. *Eur J Pharmacol* 2011;668(3):467-471.
33. Cantwell H, Devery R, O'shea M, Stanton C. The effect of conjugated linoleic acid on the antioxidant enzyme defense system

in rat hepatocytes. *Lipids*. 1999 Aug 1;34(8):833-9.
34. Halliwell B. Antioxidants in human health and disease. *Annu Rev Nutr* 1996;16(1):33-50.
35. Greenhalgh SN, Thompson AI, Henderson NC, Iredale JP. Oxidative Stress and Liver Inflammation. Studies on Hepatic Disorders*: Springer*; 2015. p. 123-147.
36. Hwang J, Wang C, Chou F, Tseng T, Hsieh Y, Lin W, et al. Inhibitory effect of berberine on tert-butyl hydroperoxide-induced oxidative damage in rat liver. *Arch Toxicol* 2002;76(11):664-670.3

