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Evaluating Oxidative Stress, Nitric Oxide Production and Mitochondrial Activity Trend in Broiler Blood Cells under Progressive Cold Stress

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ABSTRACT

Background: Broiler production is crucial for global food security as a high-quality protein source. However, climate change causes extreme temperature fluctuations, including severe cold stress. Sudden ambient temperature drops impair broiler growth and health. This study investigates how continuously decreasing ambient temperature affects oxidative stress and mitochondrial activity in broiler blood cells.

Methods: Blood cells pooled from two broiler chickens were diluted and subjected to a gradual temperature reduction from 42°C to 0°C, with 3°C decrements at each step. Mitochondrial activity, malondialdehyde (MDA), total antioxidant capacity (TAC), hydrogen peroxide (H_2O_2), reduced glutathione (GSH), catalase activity and nitric oxide (NO) levels were subsequently measured.

Result: Mitochondrial activity and NO levels showed significant quadratic trends ($P<0.05$), increasing at moderate cold before declining at lower temperatures. From 42°C to 21°C, mitochondrial activity rose significantly at 39-33°C and 21°C ($P<0.05$), while MDA levels increased at 39-27°C and 21°C ($P<0.05$). TAC declined significantly from 42°C to colder points ($P<0.05$). Between 18°C and 0°C, mitochondrial activity and NO fluctuated, suggesting an adaptive response. Conversely, H_2O_2, GSH and catalase levels remained stable across all temperatures ($P>0.05$). These findings highlight a complex cellular response to cold stress, emphasizing the need for proper cold stress management in broiler production.

Key words: Adaptation, Antioxidants, Climate change, Cold stress, Reactive oxygen species.

INTRODUCTION

Broiler production is a key contributor to global food supply. In 2022, global output reached 102.9 million tons and is expected to rise to 152.8 million tons by 2031 (Oke *et al.,* 2024). Broilers provide high-quality protein at low cost due to rapid growth and superior feed efficiency, supporting rising demand, especially in developing regions (Nguyen *et al.,* 2025). However, climate change, notably abrupt cold exposure, poses major challenges. Cold stress impairs growth, weakens immunity and elevates mortality in broilers (Aarif *et al.,* 2014; Liu *et al.,* 2022).

Cold stress induces oxidative stress, a condition in which the generation of reactive oxygen species (ROS) exceeds the antioxidant defense capacity (Afzal *et al.,* 2023). ROS can damage cellular proteins, lipids and DNA, posing a threat to cell viability (Jomova *et al.,* 2023). Understanding the oxidative stress response is therefore critical for mitigating cold-induced damage in broilers (Abo-Al-Ela *et al.,* 2021; Sahib *et al.,* 2024). Several biomarkers are used to assess oxidative stress: malondialdehyde (MDA), which indicates lipid peroxidation and cellular damage (Cordiano *et al.,* 2023; Srinontong *et al.,* 2024); total antioxidant capacity (TAC), which reflects the overall antioxidant status (Surai *et al.,* 2019); hydrogen peroxide (H_2O_2) accumulation, which signals redox imbalance (Ponnampalam *et al.,* 2022); reduced glutathione (GSH), which maintains redox homeostasis (Liu *et al.,* 2021 and catalase, which detoxifies H_2O_2 (Baker *et al.,* 2023). Nitric oxide (NO) regulates intracellular signaling and responds to oxidative cues (Xu *et al.,* 2022), while mitochondria, as the central site of cellular energy metabolism, modulate ROS generation under stress conditions (Casanova *et al.,* 2023).

Previous studies reported that dietary supplementation with glutamine, L-carnitine and betaine enhances TAC and GSH levels while reducing MDA concentration (Liu *et al.,* 2021).

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In addition, Baker *et al.* (2023) emphasized that catalase and GSH are major antioxidants depleted during cold exposure. Alterations in NO during stress have been linked to cellular injury (Semenikhina *et al.,* 2022) and Casanova *et al.* (2023) highlighted mitochondria as a key modulator of oxidative responses.

Although the effects of cold on oxidative stress, NO variation and mitochondrial activity are recognized, data on dynamic shifts in oxidative markers, NO and mitochondrial function under exposure to 0°C remain limited. Clarifying broiler cellular adaptation to extreme cold is essential. Therefore, the objective of this study was to investigate the responses of MDA, TAC, H_2O_2, GSH, catalase, NO and mitochondrial activity in broiler blood cells as ambient temperature declined from 42°C to 0°C. This study will reveal the impact of cold stress on broilers by assessing cellular responses and adaptations, offering insights to mitigate potential damage caused by climate change, particularly under extreme cold conditions.

MATERIALS AND METHODS

This experiment was conducted in 2025 and was approved by the Institutional Animal Experimentation Ethics Committee, Mahasarakham University (Approval No. IACUC-MSU-009-055/2025).

Animals

Ten 28-day-old broilers were obtained from a commercial farm in Maha Sarakham Province and housed at the Faculty of Veterinary Sciences, Mahasarakham University. After a 7-day acclimation under a 16L:8D photoperiod, birds were provided ad libitum access to a standard grower diet and clean water. No additional vaccines were given beyond the routine schedule. All broilers remained clinically healthy, showing no signs of disease or abnormality. At 35 days of age, birds were considered physiologically stable for experimentation.

Experimental design

This study comprised two parts. The first examined changes in key biochemical markers in broiler blood cells as ambient temperature gradually declined from 42°C (normal broiler body temperature) to 0°C. The markers analyzed included mitochondrial activity, H_2O_2, MDA, TAC, NO, GSH and catalase activity. The second part investigated how lower temperatures affected the same markers and was divided into two sub-studies: sub-study 2.1 compared 42°C with 39-21°C and sub-study 2.2 compared 42°C with 18-0°C.

Experimental procedure

The sample size was based on the method of Ilyas *et al.* (2017). Blood was collected from two broilers (randomly selected from ten broiler chickens; 2 mL each) in heparinized tubes. For washing, blood was mixed with phosphate-buffered saline (PBS; pH 7.4) and centrifuged at 2,500 rpm (769 × g) for 5 minutes. The supernatant was discarded and the process repeated twice. The washed blood was then diluted 1:200 (v/v) with PBS and 10 mL of the diluted sample was aliquoted into test tubes. Tubes were arranged by temperature from 42°C to 0°C, decreasing 3°C per step, with four tubes per temperature. All tubes were placed in a temperature-controlled water bath monitored by a digital thermometer. Prior to cooling, blood cells were held at 42°C for 30 minutes. Then, the temperature was reduced stepwise to 0°C. At each step, cells were held for 20 minutes before collecting four tubes (replicates) for testing.

Determination of biochemical indicators

Total antioxidant capacity

TAC was assessed by the FRAP assay (Srinontong *et al.,* 2023). The working solution combined 10 mL of 300 mmol sodium acetate buffer (pH 3.6), 1 mL of 10 mmol TPTZ and 1 mL of 20 mmol $FeCl_3 \cdot 6H_2O$. Then, 20 μL of sample was mixed with 180 μL of the solution and incubated for 5 min at room temperature. Absorbance was read at 595 nm. Ferrous sulfate heptahydrate was used as standard.

Malondialdehyde

MDA was measured using the TBARS assay. A 0.1 mL sample was mixed with 0.45 mL of 0.09% NaCl, 0.2 mL of 0.67% TBA and 1 mL of 10% TCA in 0.6 M HCl. The mixture was heated at 100°C for 30 min, cooled, then 2 mL of deionized water was added, vortexed and centrifuged at 3,000 rpm (1,008 × g) for 10 min. Absorbance was read at 532 nm (Sürmen-Gür *et al.,* 2003).

Nitric oxide

NO levels were determined using Griess reagent, composed of 1% sulfanilamide, 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride and 2.5% phosphoric acid. Equal volumes of reagent and supernatant were mixed and incubated for 15 minutes. Absorbance was read at 540 nm. Sodium nitrite served as the standard (Giustarini *et al.,* 2008).

Hydrogen peroxide

H_2O_2 was measured per Orprayoon *et al.* (2020), with slight modifications. Sample was mixed with 2.25 mmol/L $FeSO_4$, incubated 5 min, then 4 mmol/L norfloxacin was added and incubated 3 min. Absorbance was read at 440 nm. H_2O_2 (0-40 μmol/L) was used for the standard curve.

Mitochondrial activity

Mitochondrial activity in blood cells was measured using the MTT assay. MTT (5 mg/mL in acetone) was filtered. One milliliter of diluted blood was centrifuged, the supernatant removed and the pellet incubated with MTT at 41.5°C for 75 minutes. Dimethyl sulfoxide (150 μL) was added and absorbance read at 540 nm (Bahuguna *et al.,* 2017).

Reduced glutathione and catalase

GSH and catalase levels were measured using colorimetric assay kits (Abbkine; KTB1600 for GSH, KTB1040 for

catalase), following the manufacturer's instructions. Absorbance was read using a microplate reader.

Statistical analysis

Normality was checked before the analysis using PROC GLM. The differences were tested for significance using Duncan's multiple range test and results were considered significant if the *P*-value was less than 0.05 (SAS® Studio).

RESULTS AND DISCUSSION

Trends in the changes of biochemical parameters in broiler blood cells when ambient temperature is reduced from 42ºC to 0ºC

As shown in Table 1, mitochondrial activity and NO levels showed significant quadratic trends ($P<0.05$), indicating complex response patterns. Mitochondrial activity rose at 39-33°C, dropped at 30°C, increased again at 18-15°C, declined at 12-6°C, then sharply increased at 3-0°C ($P<0.05$). The rise at 39-33°C may help broiler blood cells sustain energy under mild cold stress (Gong *et al.,* 2023). The drop at 30-27°C may reflect impaired electron transport and reduced energy, leading to ROS overproduction and oxidative stress (Casanova *et al.,* 2023). Activity rose again as temperature neared 0°C, suggesting adaptive responses. NO levels fell at 39-36°C, rose at 33-21°C, dropped at 18°C and peaked at 6°C ($P<0.05$), consistent with its signaling role (Semenikhina *et al.,* 2022). These changes may reflect impaired NO synthesis or oxidative stress response (Pappas *et al.,* 2023). In contrast, H_2O_2, MDA, TAC, GSH and catalase levels showed no significant changes ($P>0.05$; Table 1). Overall, broiler blood cells retained function and showed dynamic biochemical shifts even at 0°C.

The effects of decreasing environmental temperatures from 42ºC to 21ºC on biochemical parameters in broiler blood cells

Mitochondrial activity at 42°C was significantly lower than at 39-33°C and 21°C ($P<0.05$). Activity at 39-33°C was significantly higher than at 30°C and 27°C, which in turn were lower than at 21°C ($P<0.05$). No significant differences were found between 42°C and 30-24°C, or between 39-33°C and 21°C ($P>0.05$; Table 2). Elevated activity at 39-33°C suggests an adaptive response to moderate cold, aligning with reports of mitochondrial energy upregulation under mild stress (Park *et al.,* 2021). The drop at 30-27°C may indicate overwhelmed adaptive capacity, reducing efficiency (Flensted-Jensen *et al.,* 2024). This supports findings that severe cold impairs mitochondrial function *via* oxidative damage and electron transport disruption (Timkova *et al.,* 2016). The rebound at 21°C may reflect a compensatory mechanism for energy restoration (Mohan *et al.,* 2023).

MDA levels at 42°C were significantly lower than those at 39-27°C and 21°C ($P<0.05$). Likewise, levels at 24°C were significantly lower than at 39°C and 21°C ($P<0.05$; Table 2). However, no significant differences were found between 42°C and 24°C or among 39-27°C and 21°C ($P>0.05$). These findings support earlier studies reporting

Table 1: Changing of mitochondrial activity, hydrogen peroxide, malondialdehyde, total antioxidant capacity, nitric oxide, reduced glutathione and catalase activity of broiler blood cells when the ambient temperature continuously decreased from 42°C until to 0°C (3°C each time).

Parameters	Ambient temperatures (°C)													SEM	*P*-value		
	42	39	36	33	30	27	24	21	18	15	12	9	6	3	0		A	L	Q	C
MA	1.73	2.76	2.82	2.49	1.70	1.94	2.20	2.49	2.83	2.73	2.31	2.25	2.43	2.65	3.15	0.32	<0.01	0.61	0.04	0.10
H_2O_2 (µmol)	32.64	32.83	27.30	24.82	25.07	33.30	32.02	23.55	27.55	28.73	31.82	29.25	30.82	31.48	31.28	1.10	0.28	0.30	0.76	0.62
MDA (µmol)	27.48	39.05	38.44	37.23	36.88	36.42	31.33	38.77	38.79	39.12	37.82	37.95	37.31	35.87	36.03	1.03	0.04	0.36	0.65	0.91
TAC (µmol)	34.73	26.44	28.98	29.63	29.18	29.08	31.08	20.58	27.87	31.12	32.10	31.81	32.04	21.23	30.36	1.07	0.02	0.49	0.38	0.97
NO (µmol)	0.55	0.48	0.49	0.55	0.57	0.57	0.56	0.54	0.51	0.48	0.51	0.53	0.56	0.55	0.54	0.07	<0.01	0.03	0.04	0.31
GSH (µg/ml)	71.85	65.79	53.06	56.18	46.83	30.76	45.32	42.12	73.46	68.25	60.69	60.26	55.78	48.29	53.19	2.43	0.24	0.14	0.91	0.96
Catalase (µmol)	10.25	9.82	9.57	9.87	9.62	9.20	9.83	8.50	9.38	9.20	9.67	9.30	9.10	8.66	8.73	0.48	0.14	0.40	0.34	0.25

MA = Mitochondrial activity; H_2O_2 = Hydrogen peroxide; MDA= Malondialdehyde; TAC = Total antioxidant capacity; NO = Nitric oxide; GSH = Reduced glutathione; SEM = Standard error of the mean; A= Analysis of variance (model); L = Orthogonal polynomial contrasts test for linear effects; Q = Orthogonal polynomial contrasts test for quadratic effects; and C = Orthogonal polynomial contrasts test for cubic effects.

increased lipid damage and oxidative stress under cold conditions (Aksit *et al.,* 2008). The elevated MDA at 39-27°C suggests rising oxidative stress as temperatures fall, likely due to increased ROS production beyond antioxidant control (Cordiano *et al.,* 2023). Interestingly, MDA was lower at 24°C than at 39°C and 21°C, possibly reflecting an antioxidant response at moderate cold. However, this response seems limited at colder temperatures, as indicated by the higher MDA at 21°C.

TAC was significantly higher at 42°C than at 39-27°C and 21°C and higher at 24°C than at 21°C (P<0.05). No significant difference was found between 42°C and 24°C, or among 39-24°C (P>0.05). These findings suggest that TAC decreased as temperature dropped, likely due to antioxidant depletion from elevated oxidative stress (Liu *et al.,* 2021). This aligns with previous reports showing oxidative stress lowers TAC by consuming antioxidants like GSH and catalase (Saracila *et al.,* 2023).

NO levels at 39°C and 36°C were significantly lower than at 42°C and between 33°C and 21°C (P<0.05). The decrease at 39-36°C may reflect reduced production during mild cold stress. These findings contrast with Zhang *et al.* (2011), who observed NO elevation under cold stress. However, in this study, NO increased as the temperature dropped further, suggesting an adaptive response. The elevated NO at 42°C and 33-21°C supports its role in maintaining physiological stability under both normal and cold conditions.

However, H_2O_2, GSH and catalase activity remained stable between 42°C and 21°C (P>0.05; Table 2). This suggests that GSH and catalase defense may sufficiently control H_2O_2 during moderate cold stress (Ponnampalam *et al.,* 2022). The consistent levels of these markers indicate that broiler blood cells can maintain antioxidant capacity, helping to limit cold-induced damage.

Comparative analysis of biochemical parameter changes in broiler blood cells at 42°C and reduced temperatures from 18°C to 0°C

Mitochondrial activity at 42°C was significantly lower than at 18-15°C and 3-0°C (P<0.05), but not different from 12-6°C (P>0.05). Activity at 18-15°C and 3-0°C also did not

Table 2: Effect of decreasing ambient temperature on mitochondrial activity, hydrogen peroxide, malondialdehyde, total antioxidant capacity, nitric oxide, reduced glutathione and catalase activity of broiler blood cells when the ambient temperature continuously decreased from 42°C until to 21°C (3°C each time).

Parameters	Ambient temperatures (°C)								SEM	*P*-value
	42 (NBT)	39	36	33	30	27	24	21		
MA	1.72^{c}	2.82^{a}	2.82^{a}	2.48^{ab}	1.70^{c}	1.94^{c}	2.20^{bc}	2.50^{ab}	0.17	<0.01
H_2O_2 (µmol)	32.64	32.83	27.30	24.83	25.08	33.30	32.01	22.30	1.30	0.08
MDA (µmol)	27.48^{c}	39.05^{a}	38.44^{ab}	37.23^{ab}	36.88^{ab}	36.42^{ab}	31.34^{bc}	38.77^{a}	1.09	0.01
TAC (µmol)	36.84^{a}	26.44^{bc}	28.98^{b}	29.64^{b}	29.18^{b}	29.08^{b}	31.07^{ab}	20.58^{c}	1.14	0.01
NO (µmol)	0.55^{a}	0.49b	0.50^{b}	0.55a	0.57^{a}	0.57^{a}	0.56^{a}	0.54^{a}	0.09	<0.01
GSH (µg/ml)	71.85	65.79	53.06	56.18	46.83	30.76	45.32	42.12	2.67	0.39
Catalase (µmol)	10.25	9.83	9.57	9.87	9.62	9.20	9.82	8.50	0.54	0.41

^{a,b,c}Mean within row with no common superscript letter differ significantly different (P<0.05).

NBT= Normal body temperature of broilers; MA= Mitochondrial activity; H_2O_2= Hydrogen peroxide; MDA= Malondialdehyde; TAC= Total antioxidant capacity; NO= Nitric oxide; GSH= Reduced glutathione; Catalase= Catalase activity and SEM= Standard error of the mean.

Table 3: The effects of environmental temperatures of 42°C and a decrease in ambient temperature from 18°C to 0°C (3°C reduction each time) on the mitochondrial activity, hydrogen peroxide, malondialdehyde, total antioxidant capacity, nitric oxide, reduced glutathione and catalase activity of broiler blood cells.

Parameters	Ambient temperatures (°C)								SEM	*P*-value
	42 (NBT)	18	15	12	9	6	3	0		
MA	1.72^{b}	2.84^{a}	2.74^{a}	2.31^{ab}	2.25^{ab}	2.43^{ab}	2.65^{a}	3.15^{a}	0.40	0.03
H_2O_2 (µmol)	32.64	27.55	28.73	31.83	29.25	30.82	31.48	31.28	1.25	0.90
MDA (µmol)	27.48^{b}	38.78^{a}	41.36^{a}	37.82^{a}	37.95^{a}	37.30^{a}	35.87^{a}	36.02^{a}	1.17	0.02
TAC (µmol)	36.84	27.87	31.12	32.10	31.81	32.04	21.23	30.36	1.41	0.09
NO (µmol)	0.55^{ab}	0.51^{cd}	0.48^{d}	0.51^{bcd}	0.53^{abc}	0.56^{a}	0.55^{a}	0.54^{abc}	0.01	<0.01
GSH (µg/ml)	71.85	73.46	68.25	60.70	60.26	55.78	48.29	53.19	3.83	0.14
Catalase (µmol)	10.25^{a}	9.39^{bc}	9.20^{bc}	9.67^{ab}	9.30^{bc}	9.10^{bc}	8.66^{c}	8.73^{c}	0.35	0.01

^{a,b,c}Mean within row with no common superscript letter differ significantly different (P<0.05).

NBT= Normal body temperature of broilers; MA= Mitochondrial activity; H_2O_2= Hydrogen peroxide; MDA= Malondialdehyde; TAC= Total antioxidant capacity; NO= Nitric oxide; GSH= Reduced glutathione; Catalase= Catalase activity; and SEM= Standard error of the mean.

differ (P>0.05). The pattern differed from the steady decline between 42-21°C and may reflect reduced mitochondrial respiration under cold. The increased activity at 18-15°C and 3-0°C may indicate a compensatory response to generate energy during cold stress (Casanova *et al.,* 2023), though prolonged activation may impair mitochondria *via* excess ROS (Lennicke and Cochemé, 2021).

MDA levels at 42°C were significantly lower than those at 18-0°C (P<0.05), indicating greater oxidative damage under cold. The rise in MDA reflects excess ROS production during cold stress, causing lipid peroxidation as antioxidant reserves decline (Wei *et al.,* 2024).

NO levels at 6-3°C were significantly higher than at 18-12°C (P<0.05), while levels at 15°C were significantly lower than at 42°C and 9-0°C (P<0.05). No differences were found between 42°C and 12-0°C or between 18°C and 12°C (P>0.05). The drop at 18-15°C may reflect protection against NO-induced oxidative damage (Pappas *et al.,* 2023). The lower NO at 15°C versus 42°C and 9-0°C suggests temperature-dependent synthesis. In contrast to Su *et al.* (2020), who reported increased NO under cold, our results reveal a cyclic fluctuation, suggesting adaptive responses to falling temperatures.

Catalase activity at 42°C was significantly higher than at 18-15°C and 9-0°C (P<0.05) and activity at 12°C was higher than at 3-0°C (P<0.05). No differences were found between 42°C and 12°C or between 18-15°C and 9-0°C (P>0.05). The high activity at 42°C highlights catalase's role in H_2O_2 detoxification (Baker *et al.,* 2023), while reduced activity at lower temperatures may reflect weakened antioxidant defense. The peak at 12°C, followed by decline at 3-0°C, may indicate a short-lived adaptive response before enzyme inactivation or antioxidant depletion.

In contrast, the levels of H_2O_2, TAC and GSH remained constant from 18-0°C (Table 3), indicating that the GSH antioxidant system and TAC can counteract cold stress and reduce the production of H_2O_2.

CONCLUSION

This study examined how broiler blood cells respond to cold stress by analyzing key biochemical markers as ambient temperatures dropped from 42°C to 0°C. Cold exposure caused notable changes in mitochondrial activity, MDA, NO, H_2O_2, TAC, GSH and catalase activity. Quadratic trend analysis revealed complex cellular responses. Mitochondrial activity and NO increased under moderate cold but declined in severe cold, reflecting adaptive mechanisms. In contrast, H_2O_2, GSH and catalase levels stayed stable, indicating strong antioxidant defense. These results enhance our understanding of cold stress effects and support strategies to reduce its impact in poultry.

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Disclaimers

The views and conclusions expressed in this article are solely those of the author and do not necessarily represent the views of their affiliated institutions. The author is responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Conflict of interest

All authors declare that they have no conflict of interest.

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