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Effects of Heat Conditioning and Dietary Ascorbic Acid Supplementation on Heat shock Protein 70 Expression, Blood Parameters and Fear-Related Behavior in Broilers Subjected to Heat Stress

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ABSTRACT

***Background*:** Heat stress is one of the most important environmental factors. Exposure of broilers to high temperature causes significant changes in physiological response. Early heat conditioning induces the heat tolerance of broiler chickens at later growth stage prior to marketing. Ascorbic acid supplementation has been reported to may alleviate the negative effects of heat stress on the performance of broilers. This study was carried out to investigate the effects of heat conditioning and dietary ascorbic acid supplementation on heat shock protein 70 expression, blood parameters and fear-related behavior in broilers subjected to heat stress.

***Material, Methods & Results*:** A total of 320 male broilers were used as the material of this study. Broilers were randomly assigned to four treatments. Each group contained four replicates with 20 chicks in each pen. Until the 21 days of age, all animals were raised at comfort temperature. The brooding temperature was maintained at approximately 32°C for the first 3 days and then decreased 3°C weekly until 21 days. Broilers in control group were kept under thermo-neutral conditions (24°C) and fed with the basal diet throughout experimental period. Other three groups were exposed to heat stress from 22 to 42 days of age. Heat stress was applied by exposing the broilers to a temperature of 35°C for 6 h/day between 10:00 h and 16:00 h. Ascorbic acid supplemented group was fed a diet supplemented with 500 mg of L-ascorbic acid/kg ration and exposed to heat stress from 22 to 42 days of age. Heat conditioned group was exposed to a temperature of 36°C for 24 h at the age of 5 days; fed with basal diet and exposed to heat stress from 22 to 42 days of age. Heat stress group was fed with the basal diet, no subjected to heat conditioning and exposed to heat stress from 22 to 42 days of age. The broilers in heat stress group had higher body temperature, H/L, TI duration, serum corticosterone, glucose, cholesterol, triglyceride concentrations and Hsp 70 expression in brain, liver and kidney tissues, but lower antibody titer against NDV than those in control group. The lower H/L, TI duration, serum corticosterone, glucose, cholesterol, triglyceride concentrations and Hsp 70 expression in brain, liver and kidney, whereas the higher antibody titer against NDV were obtained in heat conditioned and ascorbic acid supplemented diet fed groups, compared with heat stress group.

***Discussion*:** The results obtained in this study showed that heat stress caused to increase in H/L ratio, TI duration, body temperature, serum corticosterone, glucose, cholesterol, triglyceride concentrations and Hsp 70 expression in brain, liver and kidney, whereas it decreased antibody titer against to NDV. Heat conditioning and dietary ascorbic acid supplementation decreased H/L ratio, TI duration, serum corticosterone concentration and Hsp 70 expression and also increased antibody titer against to NDV, indicating the lower stress level in these groups. From these results, it can be said that heat conditioning and ascorbic acid supplementation alleviated the negative effects of heat stress in broilers. Heat conditioning was more effective than ascorbic acid supplementation in alleviating the adverse effects of heat stress on brain, liver and kidney tissues. In conclusion, heat conditioning of broilers by exposure to 36°C for 24 h at the age of 5 days and dietary ascorbic acid supplementation (500 mg/kg of diet) may offer a potential protective management practice in preventing heat stress in broilers.

Keywords: Broiler, heat stress, heat conditioning, ascorbic acid, blood parameters, Hsp 70.

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INTRODUCTION

Heat stress is one of the major stressors in poultry production and produces a wide range of physiological alterations, the nature of which depends upon the degree of heat stress imposed [13,30]. One of the hormonal responses to heat stress is an increase in blood levels of corticosterone, the primary glucocorticoid hormone produced by the avian adrenal gland [17]. It has been also reported that plasma protein concentration reduced and blood glucose and total cholesterol concentrations markedly increased during heat stress [22]. At the cellular level, heat stress cause to an increase in the synthesis of heat shock proteins (Hsp), also known as stress proteins. Increased Hsp defends cells against damage and protects them against apoptosis [13,29].

Early heat conditioning has been reported to induce the heat tolerance of broiler chickens at later growth stage prior to marketing [15,31]. Antioxidant nutrient supplementation; especially the addition of ascorbic acid to the diet can be used to alleviate the negative effects of heat stress in poultry [23,30]. It has been reported that ascorbic acid supplementation is associated with a lower plasma level of corticosterone in stressed poultry [17]. There is limited information on the effects of heat conditioning and dietary ascorbic acid supplementation on blood parameters, fear related behavior and especially Hsp 70 expression in broilers exposed to heat stress. This study was carried out to investigate the effects of heat conditioning and dietary ascorbic acid supplementation on heat shock protein 70 expression in brain, liver and kidney tissues, blood parameters and fear-related behavior in broilers subjected to heat stress.

MATERIALS AND METHODS

Animals, treatments and management

A total of 320 male broilers (Ross 308) were obtained from a local hatchery. Chicks were wing-banded, weighed and randomly assigned to four groups with four replicates. All chicks were fed with a starter diet from 0 to 21 days of age (3060 kcal ME/kg, 23% crude protein) and grower diet from 22 to 42 days of age (3200 kcal ME/kg, 21.5% crude protein). Ingredients and nutrient composition of basal diets used in starter and grower periods of the experiment are shown in Table 1.

Until the 21 days of age, all animals were raised at comfort temperature. The brooding temperature was maintained at approximately 32°C for the first 3 days and then decreased 3°C weekly until 21 days. Control group was kept under thermo-neutral conditions (24°C) and fed with the basal diet throughout experimental period. Other three groups were exposed to heat stress (35°C for 6 h/day between 10:00 h and 16:00 h) from 22 to 42 days of age. Ascorbic acid supplemented group was fed a diet supplemented with 500 mg of L-ascorbic acid/kg ration and exposed to heat stress from 22 to 42 days of age. Heat conditioned group was exposed to a temperature of 36°C for 24 h at the age of 5 days, fed with basal diet throughout experimental period and exposed to heat stress from 22 to 42 days of age. Heat stress group was exposed to heat stress from 22 to 42 days of ages, fed with the basal diet and no subjected to heat conditioning.

Body temperature and tonic immobility duration measurements

On day 42 day, immediately after heat challenge, the rectal temperature of 12 broilers (3 broilers per replicate) randomly chosen from each group were recorded using a digital thermometer. Immediately following recording of body temperature, tonic immobility duration of broilers was recorded. Placing a bird on its back induced tonic immobility. The bird was restrained for 10 s by maintaining a light pressure on its sternum. A stopwatch was started to record latencies until the bird righted itself. If the bird righted itself <10 s, the reastraining procedure was repeated. If the bird did not show a righting response over the 10-min test period, a maximum score of 600 s was recorded [2].

Sampling and blood parameters

At 42 days of age, blood samples were collected from brachial vein of 12 broilers (3 broilers per replicate) randomly chosen from each group. The bleeding procedure was limited to 1 min or less to minimize the influence of handling stress. Blood samples for biochemical analyses and antibody titers against to NDV were centrifuged and stored -20°C until assayed. Serum glucose, cholesterol, triglyceride and total protein levels were measured with an automatic analyzer using commercial test kits[1]. Serum corticosterone concentration was determined by enzyme-linked immunoassay (EIA) by using the EIA kits[2]. Serum antibody titers against NDV were determined by

haemagglutination-inhibition test [1]. Blood smears were prepared using May-Grunwald-Giemsa stain to obtain H/L ratio. One hundred leucocytes were counted on each slide, using a light microscope at X 1,000 magnification. The H/L ratio was calculated by dividing the number of heterophils by the number of lymphocytes [8].

At the end of study (42 days of age), 10 broilers randomly selected from each group were killed by cervical dislocation for necropsy and brain, liver and kidney tissues were collected for histopathologic and immunohistochemical evaluations.

Histopathology

Following necropsy, tissue samples taken from brain, liver and kidney were fixed in 10% buffered formalin, dehydrated in ethanol and embedded in paraffin wax, sectioned at 5 μm and stained by routine methods with haematoxylin and eosin (HE). Replicated sections were used for immunoperoxidase labeling.

Immunohistochemistry

Sections from the liver, kidneys and brain tissue samples were cut 5-7 μm and immunohistochemistry was performed by the standard streptavidin peroxidise kit[3] according to the manufacturer's instructions. Mouse monoclonal Hsp70 antibody[4] were used at dilutions of 1:1000 for 60 min. Control tissue sections were incubated with normal mouse serum.

The percentage of the area of HSP 70 positive cells was assessed semi-quantitatively under a light microscope with a 10 × ocular with grids and a 20 × objective. The labeling intensity in a given cellular compartment was assessed on a semi-quantitative basis.

Statistical Analysis

The data were analyzed by one-way analysis of variance using SPSS 13.0 software package program[5]. Significant differences among treatment means were determined using Duncan's multiple range test [4].

Table 1. Ingredients and nutrient composition of basal diets used in starter and grower periods of the experiment.

Ingredients (%)	Starter diet (0-21 d)	Grower diet (22-42 d)
Vegetable oil	1.42	3.04
Corn	53.58	55.46
Soybean meal	27	23
Full-fat soybean	14	15
Di calcium phosphate (DCP)	1.8	1.3
DL-methionine	0.2	0.1
Ground limestone	1.3	1.5
L-lysine hydrochloride	0.1	0
Salt	0.35	0.35
Vitamin-mineral premix[1]	0.25	0.25
Calculated chemical analyses		
Crude protein, %	23	21.5
ME, kkal/kg	3060	3200
Calcium, %	0.97	0.9
Total phosphor, %	0.44	0.35
Lysine, %	1.33	1.16
Methionine + cystine, %	0.92	0.78

RESULTS

Heat stress significantly affected body temperature, H/L ratio, TI duration and antibody titer against NDV in broilers ($P < 0.001$). The broilers in heat stress group had higher body temperature, H/L, TI duration, but lower antibody titer against NDV, compared with the broilers in control group. Heat conditioning and ascorbic acid supplementation significantly decreased body temperature, H/L ratio, TI duration, while significantly increased antibody titer against NDV in heat stressed broilers (Table 2).

Heat stress caused to increase in serum corticosterone, glucose ($P < 0.001$), cholesterol, triglyceride ($P < 0.05$) concentrations, but it significantly decreased serum total protein level ($P < 0.01$) of broilers. Serum corticosterone, glucose, cholesterol, triglyceride levels were decreased by dietary ascorbic acid supplementation and heat conditioning. There was no statistical different between ascorbic acid supplemented diet fed group and heat conditioned group in terms of serum corticosterone, glucose, cholesterol, triglyceride and total protein concentrations (Table 3).

Histopathological examination showed that the liver and kidneys of the chicks in heat stress group had remarkable degenerative changes, characterized by swelling, pale or finely granular cytoplasm of the hepatocytes of the liver and epithelial cells renal proximal tubules. In the brain, there were edema (characterized by vacant space surrounding the neurons and capillary) and neuronal necrosis (characterized by neuronal shrinkage) was detected in heat stress group. These findings were slight in ascorbic acid supplemented group and heat conditioned group.

Differencies in Hsp 70 expression by immunohistochemistry between groups are shown in Figure 1. Heat exposure significantly increased HSP 70 expression of brain, liver and kidney. Heat shock protein 70 expression of brain, liver and kidney in broilers exposed heat stress was significantly decreased by dietary ascorbic acid supplementation and heat conditioning. Heat conditioned broilers had less HSP 70 density of brain, liver and kidney than those fed a diet ascorbic acid supplemented diet ($P < 0.005$). At cellular level, HSP-70 immunolabeling was observed in hepatocytes of the liver, epithelial cells proximal and distal tubules of the kidneys, and neuronal cells and neurophil of the brain [Figures 2,3 & 4].

Table 2. Effects of heat conditioning and dietary ascorbic acid supplementation on body temperature, heterophil-lymphocyte ratio, tonic immobility duration, antibody titers against to NDV .

	Treatments				Effects
	Control	Heat Stress	Heat Conditioning	Ascorbic acid supplementation	
Body temperature (°C)	41.51 ± 0.12^{c}	42.79 ± 0.12^{a}	42.28 ± 0.13^{b}	42.10 ± 0.09^{b}	***
H/L	0.36 ± 0.03^{c}	0.71 ± 0.05^{a}	0.54 ± 0.04^{b}	0.59 ± 0.02^{b}	***
TI (sn)	156.75 ± 8.62^{b}	278.58 ± 10.38^{a}	202.25 ± 31.15^{b}	184.42 ± 16.50 ^{b}	***
Antibody titer against NDV (log_2)	6.90 ± 0.18^{a}	4.85 ± 0.21^{c}	6.21 ± 0.22^{b}	5.67 ± 0.15^{b}	***

H/L:Heterophil-lymphocyte ratio. TI: Tonic immobility duration. NDV: Newcastle disease virus. $^{a\,b,c}$Mean values within a row with no common superscript differ significantly ($P < 0.001$). ***$P < 0.001$.

Table 3. Effects of heat conditioning and dietary ascorbic acid supplementation on the serum concentrations of corticosterone, glucose, cholesterol, triglyceride and total protein

	Treatments				Effects
	Control	Heat Stress	Heat Conditioning	Ascorbic acid supplementation	
Corticosterone (ng/mL)	9.39 ± 1.07^{c}	17.46 ± 0.84^{a}	13.59 ± 1.16^{b}	12.72 ± 0.71^{b}	***
Glucose (mg/dL)	194,62 ± 3.54 ^{c}	227.22 ± 6.45 ^{a}	213.97 ± 3.64 ^{b}	211.83 ± 3.89 ^{b}	***
Cholesterol (mg/dL)	148.71 ± 11.03^{b}	209.06 ± 10.62^{a}	155.42 ± 10.54^{b}	161.65 ± 20.98^{b}	*
Trigliceride (mg/dL)	61.63 ± 4.24^{b}	78.76 ± 3.21^{a}	67.54 ± 2.70^{b}	65.93 ± 4.93^{b}	*
Total protein (g/dL)	4.64 ± 0.23^{a}	3.72 ± 0.10^{b}	4.21 ± 0.19^{ab}	4.16 ± 0.08^{ab}	**

$^{a\,b,c}$Mean values within a row with no common superscript differ significantly ($P < 0.05$). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

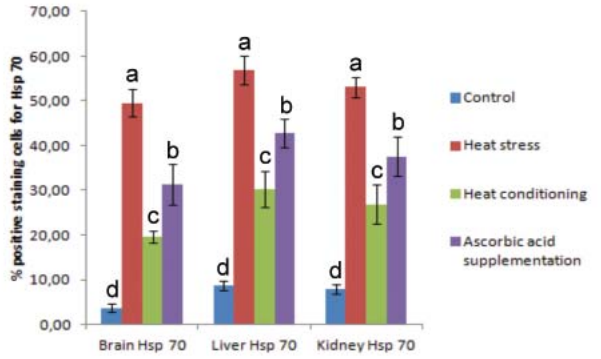

Figure 1. Effects of heat conditioning and dietary ascorbic acid supplementation on Hsp 70 level in the brain, liver and kidney of broilers exposed to heat stress. [a-d]Means ± SEM with no common letters differ at $P < 0.05$, and n=10 chickens/group.

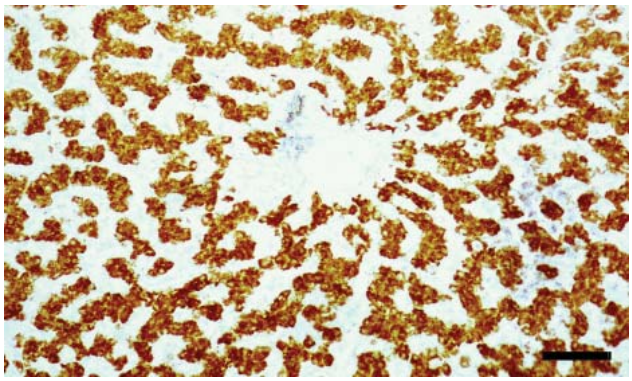

Figure 2. Immunreactivity for Hsp 70 in hepatocytes of the liver in heat stress group. Immunohistochemistry. Bar = 50 µm.

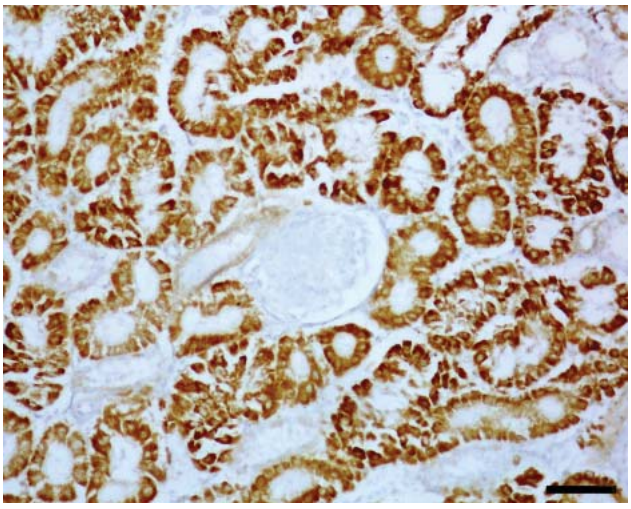

Figure 3. Immunreactivity for Hsp 70 in tubular epithelial cells of the kidney in heat stress group. Immunohistochemistry. Bar = 50 µm.

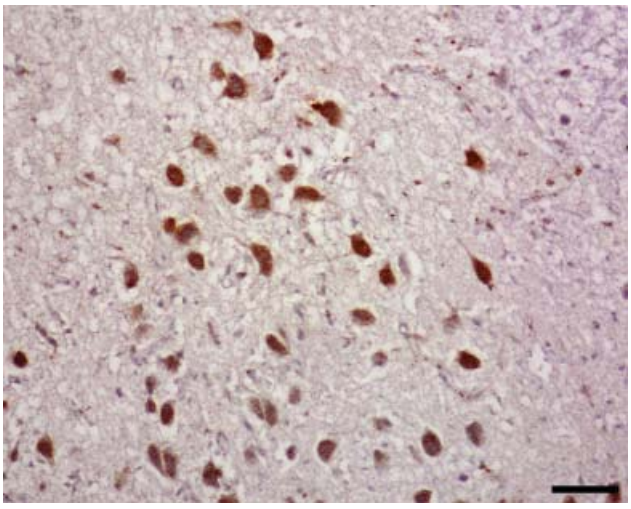

Figure 4. Immunreactivity for Hsp 70 in neurons of the brain stem in heat stress group. Immunohistochemistry. Bar = 50 µm.

DISCUSSION

The H/L ratio is a sensitive indicator of stress, and 0.2, 0.5 and 0.8 characterize low, optimum and high levels of stress, respectively [8]. In the present study, the highest H/L ratio was obtained for broilers in heat stress group, whereas the broilers in control group had the lowest H/L ratio. The H/L ratio of broilers in heat conditioned and ascorbic acid supplemented groups was lower than those in heat stress group. This result suggests that broilers in heat stress group displayed a high level stress; however heat conditioning and dietary ascorbic acid supplementation caused to decrease in stress status of animals exposed to heat stress. TI is an adaptive psycho-physiological response which is characterized by reduced responsiveness induced by physical restraint and it has been used as a measure of fearfulness and stress status in poultry [12]. In this study, broilers in heat stress group the longer TI duration displayed compared with other groups. However, there was no significant difference among control, ascorbic acid supplemented group and heat conditioned group. Similarly, Yalcin *et al*. [32] also reported that heat stress increased TI duration, whereas there was no significant difference between control and heat conditioned group in terms of this trait.

It is well known that stressors such as high environmental temperature increase production and release of corticosteroids primarily corticosterone, in birds [25]. In this study also it was determined that heat stress caused to increase in serum corticosterone concentration in broilers. Heat conditioning and ascorbic acid supplementation decreased serum corticosterone

concentration in heat stressed-broilers, indicating the lower stress level in broilers in these groups. Similarly, several researchers showed that ascorbic acid reduced corticosterone concentration in birds subjected to heat stress [17,24]. This finding could be attributed to suppressive effect of ascorbic acid on adreno-cortical steroidogenesis, thus depressing the plasma corticosterone levels [18]. Increased circulating levels of glucose, cholesterol and triglycerides have been shown as the physiological stress responses that occur most frequently in broilers [19,26]. In the present study, heat exposure resulted in an increase in serum glucose, cholesterol, triglyceride levels, whereas decrease in serum total protein concentration. The increase in serum glucose level may be related to increasing efforts to decrease heat load as a result of increasing glucocorticoid secretion that increased glucogenesis [7,27]. Ascorbic acid supplementation and heat conditioning caused to decrease in serum glucose, cholesterol, triglyceride levels in this study.

Disease resistance is complex and involves various factors of the individual, population and host pathogen levels. There is considerable evidence to indicate that response and coping with environmental fluctuations can modify biological defense systems. Stressors have been reported to may impede production of antibodies and effective cell-mediated immunity, thereby increasing susceptibility to viral diseases [14,25]. This situation could be due to the production of corticotropin releasing factor on hypothalamus under stress. Corticotropin releasing factor is known to increase adrenocorticotropic hormone from the pituitary; adrenocorticotropic hormone then stimulates corticosterone production from the adrenal gland. Corticosterone suppresses antibody production [9,28]. In this study, the broilers in heat stress group had the lower antibody titer against NDV as compared to those in control group. Ascorbic acid supplementation and heat conditioning increased antibody production against NDV in broilers under heat stress. This result was not surprising, because serum corticosterone concentration was lower in these groups than heat stress group.

Heat stress causes increased oxidative stress with enhanced production of reactive oxygen species (ROS) [16,20,29], causing damage to biomolecules such as lipids, proteins, and nucleic acids [10]. Hsp70 is associated with mechanisms protecting the body from the deleterious effects of ROS [5]. Enhancing of Hsp 70 expression in response to stressful environments may improve cell survival by protecting proteins from degradation and facilitating their refolding [11,21]. It has been reported that hyperthermia may increase the activity of the heat shock transcription factor, which enhances Hsp 70 mRNA synthesis and consequently Hsp 70 concentration [3]. In the present study, heat stress caused to an increase in Hsp 70 expression in brain, liver and kidney of broilers. Broilers fed a diet supplemented with ascorbic acid had lower Hsp 70 level than those fed basal diet under heat stress conditions. This result confirms the knowledge that ascorbic acid acts synergistically with antioxidant enzymes including superoxide dismutase, glutathione peroxidase and catalase, destroying hydroxyl, singlet oxygen and superoxide radicals [15].

The results of this study showed that heat conditioning of broilers by exposure to 36°C for 24 h at the age of 5 days significantly decreased HSP 70 expression as compared to those of heat stress group. This finding might be explained that broilers in heat conditioned group had lower body temperature than those in heat stress group [Table 3]. It has been reported that there is a positive correlation between the rate of HSP 70 synthesis and body temperature [6] and the synthesis of HSP 70 is dependent to temperature [30]. Contrary to the current study, Liew *et al*. [14] and Zulkifli *et al*. [33] determined that the HSP 70 response of heat conditioned broilers was not statistically significant from those of control group. These differences among the studies may be related to the differences in heat challenge protocol.

CONCLUSION

The results of the present study suggested that heat stress caused to increase in serum corticosterone concentration, H/L ratio, TI duration, body temperature, Hsp 70 expression in brain, liver and kidney tissues, whereas it decreased antibody titer against to NDV of broilers. Dietary ascorbic acid supplementation and heat conditioning alleviated the negative effects of heat stress. Heat conditioning was more effective than ascorbic acid supplementation in alleviating the adverse effects of heat stress on brain, liver and kidney tissues. Consequently, heat conditioning of broilers by exposure to 36°C for 24 h at the age of 5 days and dietary ascorbic acid supplementation (500 mg/kg of diet) may offer a potential protective management practice in preventing heat stress in broilers.

SOURCES AND MANUFACTURERS

[1]DDS Diagnostic systems-Istanbul, Turkey.

[2]Correlate-EIA for corticosterone, Assay Designs, Inc., Ann Arbor, MI, USA.

[3]Histostain-plus bulk kit, Zymed Laboratories, Inc., South San Francisco, CA, USA.

[4]Mouse monoclonal Hsp70 antibody, Clone: BRM-22, Sigma-Aldrich, St. Louis, MO, USA.

[5]SPSS 13.0, Release 2004. (Statistical Package for the Social Sciences for windows. Chicago, IL., USA.

***Ethical approval*.** This study was approved by the Animal Ethical Committee of the Adnan Menderes University (Approval No:64583101/2013/045).

***Declaration of interest*.** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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