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Effects of Supplementing Laying Hens with Purified Amino Acid Prepared from Animal Blood

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ABSTRACT

The objective of the experiment was to investigate the effects of supplementing laying hens with purified amino acids (PAA) derived from the blood of animal slaughter house on their egg production, egg quality, and immune response. The experiment was based on completely randomized design. A total of 144 Isa Brown laying hens (56-weeks old) were randomly allotted to 4 treatments with 4 pen replications (control, T1: 0.05%, T2: 0.1%, and T3: 0.5% PAA). Each pen housed nine laying hens. The laying hens were reared under a deep litter system. Once a week, a total of 12 eggs of each treatment were collected for egg quality analysis. At the end of the experiment, blood samples were collected for biochemical analysis and cytokines profiles using ELISA kit assays. Hen day egg production and hen house egg production increased significantly (P<0.05) along with the increase in PAA supplementation. The average egg weight increased significantly (P<0.05) with PAA supplementation. Significant differences (P<0.05) were also found in egg shell strength, shell thickness, and albumen ratio. Blood biochemical variables, such as glucose, total cholesterol, blood urea nitrogen (BUN), total bilirubin, and glutamate oxalate transaminase (GOT) were within the normal range. However, the glucose was highest in the control group. Whereas, the total cholesterol and total bilirubin were highest in T2 as compared to control group. Plasma immunoglobulin A (IgA) and immunoglobulin G (IgG), interleukin-1 (IL-1), interleukin-2 (IL-2) concentrations were not affected by PAA supplementation. Plasma interferon gamma (IFNγ) of PAA supplemented treatment groups was significantly (P<0.05) lower than the control group. However, tumor necrosis factor alpha (TNFα) was significantly (P<0.05) higher in T2. On the basis of these results, we conclude that PAA supplementation improved the production performance of laying hens without affecting their health.

Keywords: animal blood; purified amino acid; laying hens; egg production; egg quality

INTRODUCTION

Blood is one of the unavoidable by-products of slaughterhouses as it comprises 4% of live animal weight. The blood produced from slaughterhouses is considered a problem as it is an environmental pollutant if discarded directly (Bah *et al*., 2012; Fearon *et* al., 2014). However, blood collected from abattoirs can be processed and used as a valuable source of protein. The use of locally available alternative feed resources aids in producing cheaper animal proteins (Melesse *et al*., 2011; Mohammed *et al*., 2012; Moreki & Tiroesele, 2012). Blood and plasma proteins have also been utilized as high quality ingredients in feed for farm animals due to their nutritional benefits (Hejnfelt & Angelidaki, 2009), replacing increasingly expensive traditional protein sources.

In general, plant proteins are deficient in important essential amino acids for poultry, i.e. lysine and methionine, whereas blood meal is rich in both of these amino acids (Sklan & Noy, 2004). Feeding laying hens with high-protein diets is reported to improve body weight gain, egg production, and egg quality (Novak *et al*., 2004). The protein composition of egg varies considerably from plant and animal proteins; thus the availability of specific essential amino acid can have potential to affect the production performance of the laying hens (Ramsay & Houston, 1998).

The proteins found in animal bodies are composed of 22 amino acids, and all of these are physiologically essential. Among the 22 amino acids, 10 amino acids, collectively referred to as essential amino acids, cannot be synthesized by poultry, and thus must be supplied in their diet for maintenance, growth, and production (Ravindran & Bryden, 1999). The purified amino acid prepared from the blood collected from slaughterhouse is a good source of protein as its essential amino acids content is high (Ambardekar *et al*., 2009) and thus

improves the quality of primary protein sources in poultry diets. The purified amino acid has a positive effect on the growth performance of broiler chicken (Wandita, 2018). However, the effects of supplementing laying hens with purified amino acids from animal blood have not been previously reported. Moreover, the use of low crude protein, crystalline amino acid supplemented diets is gaining momentum as it reduces excretion of nitrogen (Osada *et al*., 2011). However, the cost of crystalline amino acids is relatively high; thus, the use of low protein, amino acid supplemented diets is relatively expensive compared to that of using conventional dietary protein levels (Kwshavarz & Austic, 2004). Previous studies have reported that, low protein; amino acid supplemented diets did not improve the egg production and egg weight (Harms & Russell, 1993). Tenesa *et al*. (2016) reported that diets with a high proportion of crude protein (17%) with amino acid supplementation had a positive impact on egg production and were beneficial to intestinal micro flora. Reducing the crude protein concentration in diets and supplementing diets with amino acids seems to have varied effects in laying hens. Therefore, the present study was designed to determine the effect supplementing laying hens with purified amino acids as a top dressing in corn soybean based basal diet. In this study, we evaluated the effects of purified amino acid supplementation on egg production and immune response of laying hens.

MATERIALS AND METHODS

Purified Amino Acids

Purified amino acids (PAA) were provided by Nanum Co., Eumsung-gun, Chungcheongbuk-do 27698, South Korea. PAA were prepared from animal blood collected hygienically from slaughterhouses. The collected blood was pulverized and then fermented and enzymatically digested under controlled condition. The resulting product was dehydrated and sterilized to produce the final product. The nutrient composition of the purified amino acid is shown in Table 1.

Laying Hens and Experimental Design

This experiment was designed to investigate the effects of four different levels of purified amino acid supplementation (Control: 0%; T1: 0.05% of PAA supplementation; T2: 0.1% of PAA supplementation; T3: 0.5% of PAA supplementation) on laying hens. All the laying hens were reared on corn soyabean based basal diet (2800 kcal/kg Metabolizable energy, 17% Crude Protein). The PAA were supplemented as a top dressing. A total of 144 laying hens were divided into 4 treatment groups based on completely randomized design in a research farm facility in Anseong, South Korea. Each treatment group had 4 replicate pens, with 9 laying hens in each replicate pen. Each pen was provided with 2 nesting boxes (10″deep× 12″ wide and 10″ high). The hens were reared on deep litter system; on floor made of concrete but covered with saw dust. The laying hens used in this experiment were 56 weeks old commercial laying hens (Isa Brown) and the experiment lasted for 60 days. The laying hens were maintained on 16 h of light: 8h dark cycle. The handling protocol of Hankyong National University Animal Care and Use Committee was followed in this study. The protocol ensures proper care and treatment of the experimental laying hens. Laying hens were given a week to adapt before the start of the experimental period. They were fed once a day with basal ration based on the Korean Nutrient Requirements Standards and water was provided ad-libitum through nipple drinking system. The composition of the basal diet used in the experiment is presented in Table 2. Feed supply was supplied at the rate of 110 g per birds per day and leftover was recorded every day in order to determine the average daily feed intake and average daily gain. Body weight was determined at the beginning and at the end of experiment. The overall mortality rate (Number of laying hens died/Total number of laying hens×100) for the laying hens was recorded to be 3.4% for the experimental period of 60 days.

Egg Production and Egg Quality

Eggs were collected from the nesting boxes and their total number and weight were recorded daily. The eggs were labeled according to the treatment groups. The experiment was carried out for 60 days. Every week, 12 eggs from each treatment were weighed (Ohaus EPG214C with 210 gm weighing capacity and 0.1 mg readability) and analyzed for both internal and exter-

Table 1. Nutrient compositions of the purified amino acid (PAA) used in the experiment

Nutrients	Concentration
Moisture, (%)	3.79
Crude protein, (%)	93.37
Ether extract, (%)	0.84
Crude fiber, (%)	0.39
Gross energy (kcal/kg)	5374.00
Calcium (mg/kg)	406.63
Phosphorous (mg/kg)	1676.12
Cysteine, (%)	1.041
Methionine, (%)	0.458
Aspartate, (%)	10.576
Threonine, (%)	3.407
Serine, (%)	4.565
Glutamine, (%)	8.875
Glycine, (%)	3.787
Alanine, (%)	6.602
Valine, (%)	6.483
Isoleucine, (%)	0.835
Leucine, (%)	11.654
Tyrosine, (%)	2.100
Phenylalanine, (%)	6.219
Lysine, (%)	7.313
Histamine, (%)	5.703
Arginine, (%)	3.809
Proline, (%)	2.674

Table 2. Nutrient compositions of laying hen basal diets used in the experiment

Ingredients	Concentration (%)
Corn (8.6% CP)	64.63
Soybean meal (48% CP)	23.30
Wheat barn	1.80
Corn gluten meal	3.00
Soybean oil	2.50
Dicalcium phosphate	1.92
Limestone	1.25
Salt	0.25
L-Lysine	1.22
Dl –methionine	0.25
Premix	1.00
Crude protein, %	17.00
Methionine+Cysteine+MHA	0.70
ME, kcal/kg	2800.00

Note: MHA= Methionine Hydroxy Analogue; ME= Metabolizable energy

nal variables including egg shell strength, thickness, albumen ratio, yolk albumen ratio, Haugh unit, etc. Mitutoyo Coolant proof Digital micrometer (0-25mm #293-340-30) was used to measure the shell thickness. An EG-001 egg shell strength tester (FHK NFN388, FHK, Japan) was used to measure shell strength. The minimum force that cracked the shell was recorded in terms of kilogram-force (kgf). The other variables were calculated as follows:

1. Hen day egg production(HDEP)= (Total number of egg produced on the day / Total number of hens present on that day) × 100
2. Hen housed egg production(HHEP)= (Total number of eggs laid on that day / Total number of hens housed at the beginning of laying period) × 100
3. Albumen index= [Albumen height (mm) / Albumen width (mm)] × 100%
4. Albumen ratio= [Albumen weight (g) / Egg weight (g)] × 100%
5. Yolk index= [Yolk height (mm) / Yolk width(mm)] × 100%
6. Yolk ratio= [Yolk weight (g) / Egg weight (g)] × 100%
7. Yolk : Albumen ratio= [Yolk weight (g) / Albumen weight (g)] × 100%
8. Haugh unit= the Haugh Unit (HU) score is calculated using the egg weight and albumen height (Haugh, 1937). The formula used to calculate the Haugh unit is as follows: $HU=100 \log_{10} (H+7.5-1.7W^{0.37})$

Where, H is the observed height of the albumen at the boundary with the yolk (in mm)* and W is weight of egg in grams. *Note that P6085 spherometer (tripod micrometer) with an accuracy of 0.01 mm was used to measure the yolk and albumen height.

Blood Parameters and Plasma Immunoglobulin

At the end of the experiment, blood samples were collected randomly from the 10 laying hens from each treatment group. Immediately after drawing blood, plasma samples were obtained by centrifuging at 3,000 r.p.m. for 10 min at 4 °C and then stored at -70 ºC until analysis. Plasma biochemical analyses (glucose, total cholesterol, total bilirubin, blood urea nitrogen, glutamate oxalate transaminase, and glutamate pyruvate transaminase) was conducted using SPOTCHEM EZ SP-4430. The samples were also analyzed for the concentration of pro-inflammatory markers IgA (CSB-E11232Ch), IL-1 (CSB-E10069), IL-2 (CSB-E06755Ch), TNFα (CSB-E11231Ch), and IFNγ (CSB-EO8550Ch)) with the aid of commercial ELISA Kit (Cusabio, Biotech Co., Ltd., Wuhan, China). Plasma concentrations of IgG ((Competitive EIA)-LS-F4752) were measured using a commercial Chicken IgG ELISA kit (LifeSpan BioSciences, Inc., Seattle, WA98121, USA). All the analyses were performed following the manufacturers' instructions and the absorbance was read with the aid of spectrophotometer (infinite F50 TECAN).

Statistical Analysis

All the experiments were replicated according to the requirement and data are expressed as the mean ± SD (standard deviation). The differences between groups were analyzed by one-way analysis of variance (ANOVA), followed by Duncan's multiple range tests. The level of statistical significance was set at $P<0.05$. The statistical software package SPSS 15.0 (SPSS, Inc., Chicago, IL, USA) was used for all data analyses.

RESULTS

Egg production and live body weight gain of laying hens were influenced by supplementation with PAA (Table 3). Laying hens in both T2 and T3 had significantly higher HDEP, HHEP, and live body weight gain than laying hens in the control group (P<0.05). The egg weight in treatment groups T1 and T2 were not significantly different from egg weights in the control group. Physical appearance of the laying birds in treatment groups was better than that of laying hens in the control group, as they were heavier and densely covered with feathers. The results suggest that supplementing commercial corn soybean based diet (17% CP) with PAA improves the egg production and live body weight gain of laying hens. The laying hens in the control group and all the treatment groups consumed all the feed provided at the rate of 110 g per hen per day. On the basis of this finding, it can be concluded that dietary supplementation of PAA upto 0.5% did not have adverse effects on daily feed intake.

Dietary supplementation with PAA had positive effects on the egg quality of eggs produced by the laying hens (Table 4). The external parameters including shell strength and shell thickness improved in the treatment groups as compared to the control group. The laying hens fed a basal diet with 0.05% PAA (T1) produced eggs with the highest shell strength and shell thickness. Eggs produced by laying hens in the T3 treatment had the highest (P<0.05) albumen ratio, whereas eggs

produced by laying hens in the T1 treatment had the highest Haugh unit. However, increasing the PAA level above 0.05% did not improve the Haugh unit which indicated that PAA supplementation at 0.05% was adequate to improve egg quality.

Blood samples were collected at the end of the experiment. The concentrations of the all inflammatory marker were significantly lower in laying hens in PAA treatment groups than in laying hens in the control group, or there were no significant differences (Table 5), which indicated that PAA was not source of infectious agents. However, extensive studies are needed to clarify the specific effects of purified amino acid supplementation on intestine morphology and microflora of laying hens.

To evaluate the overall health condition of laying hens, plasma biochemical analyses were conducted. Plasma glucose, total cholesterol and total bilirubin concentrations of laying hens differed significantly between laying hens in the PAA treatments and laying hens in the control treatments. The laying hens in control treatment had highest blood glucose level whereas laying hens in T2 treatment had highest total cholesterol and total bilirubin levels. As shown in Table 6, all parameters were within the normal ranges, indicating that purified amino acid supplementation had no negative effect on the health of laying hens.

DISCUSSION

Blood, an unavoidable by product of slaughter houses is a good source of protein (Bah *et al*., 2013). The use of blood derived proteins as feed ingredients has significant economic and environmental benefits. Tons

Table 3. Production performance of laying hen fed diets supplemented with different percentages of purified amino acid (PAA)

Variables	Control (0.00% PAA)	Treatments		
		T1 (0.05% PAA)	T2 (0.1% PAA)	T3 (0.5% PAA)
Average daily egg production	15.91±3.6^{a}	16.02±3.4^{a}	17.66±2.7^{b}	17.71±3.1^{b}
Total egg production during the 60-days experimental period	891.00±3.6^{a}	897.00±3.4^{a}	989.00±2.7^{b}	992.00±3.1^{b}
Hen day egg production (HDEP), %	48.69±8.4^{a}	49.80±8.7^{a}	53.63±6.9^{b}	54.86±8.5^{b}
Hen house egg production (HHEP), %	45.46±8.4^{a}	47.49±9.8^{a}	51.56±6.3^{b}	53.14±7.7^{b}
Egg weight, g/hen/day	63.48±1.7^{a}	63.49±1.2^{a}	63.63±1.6^{a}	64.03±1.0^{b}
Live body weight gain (kg)	0.02±0.01^{a}	0.04±0.01^{a}	0.06±0.03^{b}	0.08±0.02^{b}

Note: All data are presented as means ± SD (n= 34). Means in the same row with different superscripts differ significantly (P<0.05).

Table 4. Internal and external egg characteristics of egg produced by laying hen fed diets supplemented with different percentages of purified amino acids (PAA)

Variables	Control (0.00% PAA)	Treatments		
		T1 (0.05% PAA)	T2 (0.1% PAA)	T3 (0.5% PAA)
Shell strength, kgf	3.36±0.3^{a}	3.62±0.2^{b}	3.65±0.3^{b}	3.60±0.3^{b}
Shell thickness, mm	0.66±0.02^{a}	0.71±0.04^{b}	0.70±0.04^{ab}	0.69±0.01^{ab}
Shell color	9.84±0.6^{a}	9.84±0.5^{a}	10.71±0.5^{b}	9.59±0.5^{a}
Yolk ratio, %	44.00±0.02	43.00±0.02	45.00±0.03	44.00±0.03
Albumen height, mm	6.17±0.5	6.50±0.9	5.99±1.1	5.84±1.0
Albumen width, mm	65.86±2.6^{a}	67.12±4.1^{b}	66.41±3.8^{ab}	66.89±3.6^{ab}
Albumen ratio, %	53.00±0.64^{a}	56.00±0.07^{ab}	55.00±0.07^{a}	59.00±0.05^{b}
Yolk albumen ratio, %	82.00±0.1^{b}	78.00±0.2^{ab}	85.00±0.2^{b}	74.00±0.1^{a}
Haugh unit	72.53±2.8^{a}	81.28±4.6^{b}	77.17±6.1^{ab}	72.56±5.6^{ab}

Note: All data are presented as means ± SD (n= 12). Means in the same row with different superscripts differ significantly (P<0.05).

Table 5. Plasma concentrations variables associated with the immune response of laying hens fed diets supplemented with different percentages of purified amino acid (PAA)

Variables	Control (0.00% PAA)	Treatments		
		T1 (0.05% PAA)	T2 (0.1% PAA)	T3 (0.5% PAA)
IgA, ng/mL	0.10±0.0	0.10±0.0	0.13±0.0	0.10±0.0
IgG, μg/mL	0.81±0.0	0.81±0.0	0.80±0.0	0.81±0.0
IL-1, pg/mL	0.18±0.0	0.18±0.0	0.18±0.0	0.18±0.0
IL-2, pg/mL	0.08±0.0	0.08±0.0	0.08±0.0	0.08±0.0
IFNγ, pg/mL	0.42±0.1^{b}	0.44±0.02^{b}	0.59±0.2^{c}	0.30±0.99^{a}
TNFα, pg/mL	0.74±0.4^{d}	0.44±0.2^{b}	0.28±0.3^{a}	0.66±0.2^{c}

Note: All data are presented as means ± SD (n= 10). Means in the same row with different superscripts differ significantly (P<0.05).

Table 6. Results of plasma biochemical analyses of laying hens fed diets supplemented with different percentages of purified amino acid (PAA)

Variables	Control (0.00% PAA)	Treatments		
		T1 (0.05% PAA)	T2 (0.1% PAA)	T3 (0.5% PAA)
Glucose, mg/dl	200.00±21.40^{c}	177.40±19.00^{a}	181.60±36.80^{b}	178.70±20.60^{a}
Total cholesterol , mg/dl	140.60±21.50^{a}	157.40±22.00^{a}	187.20±33.90^{c}	175.90±36.50^{b}
Blood urea nitrogen, mg/dl	5.00± 0.00	5.00± 0.00	5.00± 0.00	5.00± 0.00
Total bilirubin, mg/dl	0.17± 0.10^{a}	0.38± 0.20^{b}	0.86± 0.60^{c}	0.34± 0.20^{b}
GOT, IU/L	169.30±39.00	187.80± 6.70	177.70±37.20	201.90±29.90

Note: Normal range for plasma biochemical parameters is referenced from The Merck Veterinary Manual, 10th edition, 2010; Glucose: 180-400mg/dl; Total cholesterol: 129-297 mg/dl; Total bilirubin: 0.01mg/dl; GOT: 10-400 IU/L.
All data are presented as means ± SD (n= 10). Means in the same row with different superscripts differ significantly (P<0.05).

of blood are generated in abattoirs each year which can be used in animal feed to boost its protein or amino acid content (Adhikari, 2018). The purified amino acids (PAA) used in the present study were prepared from the blood generated in a slaughterhouse. Blood collected from the slaughterhouses was processed to isolate the protein from other constituents. The processing of the blood increased the crude protein concentration to 93.37%. According to Li *et al.* (2011), glutamine and leucine are the most abundant amino acids in blood meal. The glutamine (8.87%) and leucine (11.64%) contents were relatively higher in PAA. Utilization of blood and blood proteins in the feed chain not only improves feed quality but also largely solves environmental problems related to blood waste from slaughterhouses (Jayathilakan *et al.*, 2012; Ofori & Hsieh, 2014). Supplementing laying hens with different concentrations of PAA increased their egg productions and live body weight gains. This result agrees with the findings of Bunchasak *et al*. (2005) who found that higher dietary protein contents improved egg production of laying hens. According to Alagawany *et al*. (2016), the live body weight of laying hens improved in the hens supplemented with high levels (18% and 20 % CP) as to low protein diets (12%, 14%, and 16%). Similarly, Adeyemo *et al*. (2012) reported that higher levels of dietary protein lead to increases in egg production and egg weight, which resulted significant improvement in the feed to egg mass ratio. However, the result is contrary to the findings of Kingori *et al*. (2010) who reported the body weight of laying hens was not affected by dietary protein level.

In addition to overall protein availability, egg production is also affected by the availability of specific essential amino acids (Houston *et al*., 1995). The qualitative composition of the protein source is of immense importance as the quality of protein depends not only on its nitrogen content, but also on its composition and bioavailability of specific amino acids (Ravindran & Bryden, 1999). Moreover, there should be perfect balance between the amino acids, as high intake of one amino acid and deficiency of another might have detrimental effects (Novak *et al*., 2004).

According to Novak *et al*. (2004), supplementation laying hens with amino acids, particularly lysine, increases albumen content, which is the major cause for increased egg weight. Similarly, Shafer *et al*. (1996) reported that increasing dietary supplementation of methionine resulted in increased egg weight, yolk and albumen weight and total solids in albumen and yolk. There is a thin line between meeting the amino acid requirements of laying hens and providing excess of certain amino acids, such as isoleucine (Peganova & Eder, 2002). Excess dietary isoleucine results in a marked reduction in feed consumption in laying hens (Peganova & Eder, 2003). Similarly, high dietary L-cysteine content (7 folds higher than required) results in acute metabolic acidosis (Baker, 2009).

The external as well as internal characteristics of egg such as shell strength, shell colour, shell thickness etc. are crucial as they influence consumer's acceptability and price of the egg (Tabeekh, 2011).The egg shell strength is related to the amount and thickness of the egg shell (Roberts, 2004). The plasma mineral profiles of laying hens are considered as one of the contributing factors for the egg shell quality (Palvik *et al*., 2009). Egg shell strength and thickness is an economically important trait and has a marked effect on the marketability of eggs (Melesse *et al*., 2013). However, egg shell color was not affected by dietary PAA supplementation. The PAA contains a considerable amount of calcium and phosphorous which might be the underlying cause for increased calcium availability. Dietary supplementation of calcium can improve egg shell quality (Kebreab *et al*., 2009). With increase in demand for liquid egg, egg powder yolk oil, the importance for interior characteristics such as yolk Index and Haugh unit is gaining more importance (Hanusova *et al*., 2015). Albumen is an important determining factor for internal egg quality. A higher albumen height and Haugh unit indicates better egg quality (Şekeroğlu & Altuntaş, 2009; Oke *et al*., 2014). The albumen height and Haugh unit determines the viscosity of the thick albumen (Hanusova *et al*., 2015). Dietary protein and amino acid content have a considerable effect on Haugh unit (Roberts, 2004). Inclusion of PAA above 0.05% seems to lower the albumen height and Haugh unit. Exposure to ammonia adversely affects albumen quality (Roberts, 2004). The addition of PAA above 0.05% might have elevated the nitrogen concentration in the feces, and this in turn might have increased the ammonia concentration of the litter.

As the PAA is manufactured from animal blood; there is a possibility that it contains harmful microor-

ganisms, toxins, and toxic metabolites (Ofori & Hsieh, 2014). Thus, it is essential to rule out any possible harm caused by PAA prior to its inclusion in animal feed. Immunoproteins, such as IgM, IgA, IgE, and IgG increase during antigenic stimulation, usually from infectious agents (Bunchasak *et al*., 2005). Pathogenic organisms activate the gastrointestinal immune system, which is characterized by elevation of serum concentrations of TNFα, IL-1β, and IL-6 (Liu, 2015) which diverts large amount of energy that could be used for productive traits (Deng *et al*., 2012; Torrallardona & Polo, 2016). Supplementation of PAA didn't elevate the plasma cytokine level in the laying hens which indicates that PAA doesn't impose any adverse effect on the health of the laying hens.

CONCLUSION

The dietary inclusion of different levels of purified amino acid (PAA) has positive effects in layers as inclusion of 0.5% PAA resulted in increased the egg production and 0.05% PAA improved egg quality traits. Moreover, supplementing laying hens with 0.5% PAA didn't affect the average daily feed intake or the health of the laying hens. Thus, PAA can be considered as a valuable protein supplement for the laying hens. However, further study should be carried out to analyze if PAA can replace the conventional sources of protein for laying hens.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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