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ANIMAL PRODUCTION

The effects of dietary hot pepper *Capsicum annuum* waste powder supplementation on egg production traits of Japanese quail layers

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ABSTRACT: *This study was conducted to evaluate the effects of different levels of dietary hot pepper (Capsicum annuum L.) waste supplementation on egg production performance and quality in Japanese quail hens (Coturnix coturnix japonica). A total of 192, seven-week-old Japanese quail layers were divided into 4 treatment groups of similar mean weight (304±0.5 g), each comprising 12 subgroups (including 1 male and 3 females). Treatment birds were fed on experimental basal layer diet supplemented with 1, 2 or 4 g hot pepper waste powder (HPWP) per kg diet. The laying performance was determined by recording feed intake, egg weight, daily egg production, and biweekly egg quality. Results showed that HPWP supplementation to layer diet did not have any significant (P>0.05) effects on body weight and feed conversion ratio, while it had significant effects on feed intake (P<0.01), and laying egg weight, average egg weight, total egg yield (P<0.05). A 2 g HPWP supplementation resulted in the highest total egg yield (P<0.05) with quadratic effects on egg shape index (P<0.05) and albumen pH (P<0.01). According to the values in the study, the egg shape index of 2 g HPWP group was circular and; therefore, attractive to consumers. We highly recommend the non-economic value of 2 g/kg supplementation of hot pepper waste powder, especially for egg production. To conclude, HPWP can be used for quail layer diets due to its beneficial effects on egg quality since it is an economic and easy agricultural by-product obtained from red pepper paste industry waste.*

Key words: *albumen pH, by-product, egg quality, hot pepper, Japanese quail layers.*

Efeitos da suplementação de pó de *Capsicum annuum* na dieta sobre a produção de ovos de codornas japonesas

RESUMO: *Este estudo foi conduzido para avaliar os efeitos de diferentes níveis de suplementação de resíduos de pimenta na dieta (Capsicum annuum L.), no desempenho e na qualidade da produção de ovos em codornas japonesas (Coturnix coturnix japonica). Um total de 192 codornas japonesas com sete semanas de idade foram alocadas em 4 grupos com peso médio semelhante (304 ± 0.5 g), cada um compreendendo 12 subgrupos (incluindo 1 macho e 3 fêmeas). As aves tratadas foram alimentadas com dieta experimental basal suplementada com 1, 2 ou 4 g de pimenta em pó (HPWP) por kg de dieta. O desempenho da postura foi determinado pelo registro da ingestão de ração, peso dos ovos, produção diária de ovos e qualidade quinzenal dos ovos. Os resultados mostraram que a suplementação de HPWP à dieta não teve efeitos significativos (P> 0,05) no peso corporal e na taxa de conversão alimentar, enquanto teve efeitos significativos no consumo de ração (P <0,01) e no peso do ovo em postura, peso médio do ovo, produção total de ovos (P <0,05). Uma suplementação de 2 g de HPWP resultou na maior produção total de ovos (P <0,05) com efeitos quadráticos no índice de forma dos ovos (P <0,05) e no pH do albumen (P <0,01). De acordo com os valores do estudo, o índice de forma dos ovos de 2 g do grupo HPWP era circular e, portanto, atraente para os consumidores. É altamente recomendável o valor não-econômico de 2 g / kg de suplementação de pó de pimenta, especialmente para a produção de ovos. Para concluir, a HPWP pode ser usada para dietas de codorna devido aos seus efeitos benéficos na qualidade dos ovos, uma vez que é um subproduto agrícola obtido a partir de resíduos da indústria de pasta de pimenta vermelha de maneira econômica e fácil.*

Palavras-chave: *pH do albume, subproduto, qualidade dos ovos, pimenta, camadas de codornas japonesas.*

INTRODUCTION

Egg is the richest and cheapest animal protein resource for human health or food. For this reason, its production has been increased in worldwide scale. Consumers generally have chicken, turkey, goose, duck and quail eggs. Quail eggs are gaining the attention of consumers because of their rich nutrient value. For example; A 9-gram quail egg has 14.22 kcal ME, 1.17 g protein, 5.76 mg Ca, 5.94 µg folate (dietary folate equivalent), 75.96 mg cholesterol (USDA, 2018). Although, Japanese

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quails play an important part in egg production as layer hens, there have been no documents about the production of quail egg quantities. In 2013, the number of eggs produced in the world increased from 1.285.108.506 to 1.378.152.798 trillion in 2018, an increase of 6.75% (FAO, 2020). Nevertheless, an increasing amount of non-conventional or natural additives have been needed to meet this growing production. This growing demand increases the costs of raw feed materials.

While antibiotics are used for growth regulation and health protection in poultry nutrition, there is increasing human resistance to antibiotics (Castanon, 2007). For this reason, the feed industry has not used antibiotics anymore, especially in the EU, in feed formulations for poultry and researchers are searching for new additives in feed formulation materials as an alternative to antibiotics. Consequently, researchers have focused on using antibiotic growth promoters (AGPs): probiotics, prebiotics, synbiotics, organic acids, enzymes, phytogenics, hyperimmune egg yolk antibodies, antimicrobial peptides, bacteriophages, clay and metals (Gadde et al. 2017). Phytogenics use a wide range of plants or extracts like thyme, cinnamon, rosemary, cumin and/or expensive spices like garlic, black pepper, red pepper, etc. (CHOWDHURY and SMITH, 2002; RADWAN NADIA et al. 2008; AL-HARTHI et al. 2009; TORKI et al. 2015). Red pepper (*Capsicum annuum* L.) is used for degassing the intestine, accelerating the removal of metabolic products, increasing the body temperature, facilitating digestion, constructive vasoconstriction, reducing meat and blood cholesterol and reducing abdominal fat accumulation (MAOKA et al. 2001, ERDOST et al. 2006, HOLST et al. 2010, EL-DEEK et al. 2012, SHAHVERDI et al. 2013, PUVAĆA et al. 2014, PUVAĆA et al. 2015). However, there has not been any study on the use of red pepper waste (by-products) in poultry nutrition as a feed or feed additive. For this reason, we conducted this study to investigate whether red pepper paste industry waste would affect the laying performance and egg quality of Japanese quail layers.

MATERIALS AND METHOD

In this experiment, healthy and well-performing quail layers (*Coturnix coturnix japonica*) were used. A total of 192, seven-week-old Japanese quail layers were divided into 4 treatment groups of similar mean weight, comprising 12 subgroups with 1 male and 3 females in each. To determine the chemical content of hot pepper waste powder (HPWP) and basal diet, samples were dried in a ventilated drying oven at 65 °C for 48 hours. The dried sample was ground (Ultra-Centrifugal Mill ZM 200- Retsch) in a 1 mm sieve grinder before analysis. The organic matter or moisture (OM: M method 934.01), crude protein (CP: method 984.13), crude fiber (CF: method 978.10), ether extract (EE: method 920.39), ash (ash: method 942.05 (4.1.10)), Acid Detergent Fiber (ADF: BFM62) and Neutral Detergent Fiber (NDF: BFM119) contents of the hot pepper waste powder and animal feeds were determined according to the AOAC procedure (2006) (Tables 1 and 2).

The birds were fed on a standard layer diet each kg containing 170 g crude protein, 38 g calcium and phosphorus, and 2800 Kcal ME. The HPWP was acquired from a private red pepper paste factory in Şanlıurfa, Turkey. Treatment birds were fed on the experimental basal layer diet supplemented with 1, 2 or 4 g HPWP per kg diet. This study lasted for 8 weeks.

Birds housed in cages were subjected to 16:8 hours, light/dark photoperiod. Eggs were collected twice a day, at 9 am and 3 pm. Feed was given *ad libitum* and water was continuously available from nipple drinkers throughout the day. Laying performance was determined daily by measuring the change in body weight (BW), feed intake (FI), egg mass, feed conversion ratio (FCR) (feed intake: egg

Table 1 - Chemical content of the hot pepper waste powder.

Ingredients (Dry matter basis)	(%)
Organic Matter	91.63
Crude Protein	16.35
Crude Fiber	30.82
Ether Extract	11.28
Ash	8.82

Table 2 - Chemical content and nutrient value of the experimental basal diet.

Ingredients[1,2]	Experimental Basal Diet (g/kg)
Yellow corn	537.739
Soy bean meal 46% CP	170.084
Limestone	96.441
Sunflower meal 36% CP	91.110
Barley 11% CP	30.000
Soy bean Oil	28.312
Wheat bran 14% CP	10.000
D-L Methionine 99%	8.139
MCP 22,7 (TIMAB)	5.792
DDGS 27% CP	5.000
FFS 34	5.000
Chicken feather flour 56	5.000
Salt	2.756
Ekomix VM egg	2.000
Sodium bicarbonat	1.025
Rovabio MAX	1.000
Calsporin	0.600
Chemical Composition[3]	(g)
ME, kcal/kg	2800.000
Crude Protein 15.3	170.00
Calcium 3.8	38.00
Available Phosphorus 0.35	38.00
Lysine 0.75	6.85
Methionine 0.35	10.54
Threonine 0.58	5.27
Tryptophan 0.19	2.00

[1]Provided per kilogram of diet: retinyl acetate, 9012 IU; cholecalciferol, 1500 IU; DL-α-tocopheryl acetate, 7.5 IU; thiamin, 0.6 mg; riboflavin, 4.8 mg; pyridoxine hydrochloride, 1.5 mg; cyanocobalamin, 0.009 mg; calcium-D-pantothenate, 7.5 mg; folic acid, 0.15 mg; niacin, 20 mg.

[2]Provided per kilogram of diet: copper ($CuSO_4*5H_2O$), 6 mg; iron ($FeSO_4{}^*H_2O$), 60 mg; zinc ($ZnSO_4{}^*H_2O$), 80 mg; manganese ($MnSO_4{}^*H_2O$), 60 mg; selenium ($NaSeO_3$), 0.3 mg; iodine (KI), 0.35 mg.

[3]The value of crude protein was analyzed and the value of metabolizable energy (ME) and other values were calculated.

mass) and egg production (hen per day). Egg quality was estimated by measuring the egg-shape index (width*100/length), shell weight, shell thickness, egg weight, yolk weight, albumen weight, albumen height, yolk index (weight/height), Haugh unit, breaking resistance (using a Brookfield, CT3 Texture Analyzer, Probe No: TA24) and yolk color (L^*, a^*, b^*, *chroma* and h^o; using a Konica Minolta, CR-410 Chroma Meter) of each egg obtained on the third day of every week. Shell samples from the top, middle, and bottom of the egg were measured for thickness using a micrometer and the mean was calculated before statistical analysis. The data obtained in the experiment were analyzed using General Linear Models (GLM) and SAS orthogonal polynomials (1997). Linear, quadratic and cubic effects were determined by orthogonal polynomial contrast (DÜZGÜNEŞ et al. 1987).

RESULTS AND DISCUSSION

The performance parameters: change in body weight, daily feed intake (g per bird) and feed conversion ratio (g feed / g egg) results are given in table 3. Results for the change in body weight effects of HPWP supplementation showed that the group subjected to 2 g/kg of HPWP supplementation recorded the least change in BW. Although, FI was affected (P=0.000), but the feed conversion ratio was not affected by supplementation

Table 3 - The effects of different levels of hot pepper waste powder supplementation on body weight gain, feed intake and feed conversion ratio of Japanese quail layers.

Parameters	--------------------------HPWP (g/kg)-------------------------				SED	P	------------Effects[¥]------------		
	0	1	2	4			L	Q	C
Initial BW (g/ bird)	310.3	310.3	310.5	310.1	2.28	1.000	0.975	0.968	0.966
Final BW (g/ bird)	334.8	333.0	331.1	334.6	2.67	0.957	0.924	0.62	0.8203
Change in BW (g/ bird)	24.5	21.9	20.6	24.6	3.33	0.966	0.969	0.623	0.899
FI (g/ day per bird)	34.2[a]	32.5[b]	35.2[a]	34.5[a]	0.17	0.000	0.029	0.143	0.000
FCR	3.2	3.4	3.1	3.4	0.07	0.305	0.895	0.600	0.071

[a-b]Means in the same row bearing different superscripts differ significantly. BW: Body Weight, BWG: Body Weight Gain, FI: Feed Intake, FCR: Feed Conversion Ratio (g feed /g egg).
SED: Standard Error of the Difference between the means.
[¥]:Effects; L: linear, Q: quadratic, C: cubic.

with HPWP (P>0.05). As a result, FI was affected by HPWP supplementation (P=0.000) but the change in BW was not (P>0.05). Conversely, dietary supplemental HPWP did not affect the health status of animals negatively.

The egg production results are given in table 4. The total egg weight and average egg weight (g/ 3 birds/ 56 days) were increased (P<0.05) in 2 g/kg HPWP supplemented group. Hot pepper (*Capsicum annuum* L.) reduced blood and meat cholesterol, and the accumulation of abdominal fat (EL-DEEK et al. 2012; SHAHVERDI et al. 2013; PUVAĆA et al. 2014). At the same time, the carcass fat concentration showed a synergistic relationship between calcium, phosphorus and Vitamin D. The 2 g/kg HPWP supplementation hens used their body reserves to lay (reproduce) more eggs (P<0.05). For this reason, 2 g hot pepper waste powder decreased body weight since the body reserves, most likely, have been used for egg production compared to other experimental groups.

Egg quality results are given in table 5. Results, for egg quality revealed that HPWP supplementation affected egg shape index. It is well known that the egg shape index value is one of the significant quality criteria in egg marketing. The commercial poultry egg shape index should be between 72 and 76. If it is lower than 72, this means that the egg shape is sharp, while a value higher than 76 means it is round (ŞENKÖYLÜ, 2001). However, the quail egg shape index should be between 76 and 79 (ÇOPUR et al. 2010). Generally, customers prefer to buy big or heavy eggs, but the results from the egg shape index showed that HPWP supplementation had a significant quadratic effect (P<0.05). According to the values in this study, the egg shape index indicated round eggs that are preferable for consumers.

Breaking resistance showed a linear increase for the HPWP supplementation groups (P>0.05), but the control group was better than supplemental groups. We can conclude that the reason for the decline of the shell resistance in the HPWP supplementation group, compared to the control group, was fat burning in the body. Vitamin D, Ca and P are vital for the development of the eggshell and they are transferred by fat in the body. As the HPWP supplementation accelerates fat burning, this prevents the transference of the required quantities of Vitamin D, calcium and phosphorus that are so essential for the development of the eggshell in the body. Breaking resistance in the treatment groups decreased in magnitude compared to the control groups.

Average egg thickness was changed by HPWP supplementation; because of this, results showed that the egg shape index is better at 2 g/kg of HPWP compared to other groups. The HPWP supplementation improved the a^* (P<0.05) and b^* values so that the yolk's yellow and red color was increased by HPWP supplementation doses. These findings agree with those of Li et al. (2012) that a^* and b^* values showed a "reddish" yolk color.

Hot pepper waste powder supplementation has antimicrobial activity and improves reproduction performance in laying hens. Zeweil et al. (2011) observed that a 1.5% hot pepper supplementation in the diet had inhibitory

Table 4 - The effects of different levels of hot pepper waste powder on egg weight and yields of Japanese quail layers.

Parameters	------------------HPWP (g/kg)----------------				SED	P	-------------Effects$^{¥}$------------		
Average of egg weight (g)	0	1	2	4			L	Q	C
Week 1	11.3^{a}	11.3^{a}	11.2^{a}	6.4^{b}	0.07	0.000	0.000	0.000	0.000
Week 2	13.00	12.6	12.5	12.7	0.10	0.366	0.342	0.135	0.923
Week 3	13.7^{a}	13.1^{ab}	13.1^{ab}	12.9^{b}	0.11	0.085	0.026	0.289	0.455
Week 4	13.6^{a}	13.1^{ab}	13.2^{ab}	12.9^{b}	0.11	0.179	0.052	0.595	0.363
Week 5	13.7	13.3	13.3	13.2	0.10	0.337	0.125	0.426	0.537
Week 6	12.4^{b}	13.5^{a}	13.5^{a}	13.4^{a}	0.14	0.018	0.014	0.027	0.469
Week 7	13.8	13.6	13.7	13.8	0.11	0.814	0.876	0.347	0.885
Week 8	13.8	13.5	13.6	13.6	0.10	0.891	0.806	0.551	0.656
----------Total egg weight (g)----------									
Week 1	175.4^{ab}	181.4^{a}	190.4^{a}	154.3^{b}	4.53	0.044	0.181	0.023	0.237
Week 2	225.1^{ab}	209.6^{ab}	246.7^{a}	200.7^{b}	6.58	0.081	0.538	0.248	0.025
Week 3	232.5^{ab}	201.1^{b}	244.3^{a}	212.7^{ab}	6.11	0.066	0.764	0.995	0.008
Week 4	242.7^{ab}	212.8^{b}	261.2^{a}	218.3^{b}	6.17	0.027	0.654	0.597	0.003
Week 5	246.0^{a}	207.9^{b}	258.1^{a}	226.7^{ab}	5.74	0.018	0.878	0.771	0.002
Week 6	236.4^{ab}	216.3^{b}	279.6^{a}	250.3^{ab}	7.44	0.030	0.119	0.757	0.011
Week 7	245.25	220.67	260.75	240.5	6.92	0.274	0.688	0.880	0.057
Week 8	232.8^{ab}	208.8^{b}	232.6^{ab}	252.9^{a}	7.04	0.187	0.183	0.120	0.417
Morning (9 AM)	1381.4^{ab}	1202.8^{b}	1425.6^{a}	1300.7^{ab}	31.93	0.080	0.945	0.673	0.011
Afternoon (3 PM)	450.6	518.4	509.7	463.3	21.28	0.605	0.876	0.189	0.837
Laying egg weight (g)	10.9^{ab}	9.9^{b}	11.8^{a}	10.5^{ab}	0.23	0.040	0.827	0.796	0.005
Egg production (%)	82.7^{ab}	76.0^{b}	90.2^{a}	86.5^{a}	1.66	0.028	0.093	0.656	0.012
Average egg weight (g/3 birds 56 days)	32.8^{ab}	29.6^{b}	35.2^{a}	31.4^{ab}	0.69	0.040	0.827	0.796	0.005
Total egg yield (g/3 birds/56 days)	1836.1^{ab}	1658.6^{b}	1973.5^{a}	1756.3^{ab}	38.27	0.040	0.827	0.796	0.005
----------Total egg----------									
Morning (9AM)	105.4^{ab}	93.8^{b}	110.8^{a}	110.7^{a}	2.34	0.041	0.121	0.217	0.032
Afternoon (3PM)	34.0	38.6	37.7	35.5	1.53	0.716	0.794	0.275	0.753
Pieces (3 birds/56 days)	139.0^{ab}	127.8^{b}	151.6^{a}	145.3^{a}	2.79	0.028	0.093	0.656	0.012

$^{a-b}$Means in the same row bearing different superscripts differ significantly.
SED: Standard Error of the Difference between the means.
$^{¥}$:Effects; L: linear, Q: quadratic, C: cubic.

effects on the *E. coli*, *Salmonella* and *Streptococci* count. Normally, the albumen pH value is between 7.6 and 9.5 in avian eggs and negatively affected by the microorganism population (GUYOT et al. 2013). In this study, albumen pH showed that HPWP supplementation had a significant linear effect (P<0.01). The reason for the increase in the pH of the hot pepper waste powder supplementation groups compared to the control group was due to the antimicrobial activity of hot pepper. Supplementation of the basal layer diet with hot pepper waste powder doses significantly improved albumen pH. The rise in CO_2 within the egg air space can be explained by the increased microorganism activities; however, high pH values inhibited and limited CO_2 production in the egg. The pH value is a very important parameter in the shelf life of eggs during storage and transporting for retailers.

CONCLUSION

The HPWP used in this study increased egg yield and egg weight at 2 g/kg level without affecting the health status of birds, their feed intake and body weight changes. However, this level tended to increase egg quality for shape index, albumen weight, albumen pH and color. Above all, HPWP can be obtained easily and economically from red pepper paste industry waste. Also, this usage in poultry nutrition can contribute to the prevention of environmental pollution. For this reason, HPWP can be used for quail layer diets due to its beneficial effects on egg quality and albumen pH.

Table 5 - The effects of different levels of hot pepper waste powder on the egg quality parameters of Japanese quail layers.

Parameters	--------------------------HPWP (g/kg)---------------------				SED	P	------------Effects$^{¥}$-----------		
	0	1	2	4			L	Q	C
Egg weight (g)	13.1	13.1	13.0	13.2	0.07	0.849	0.805	0.460	0.669
Shell weight (g)	1.5^{ab}	1.4^{b}	1.4^{b}	1.5^{a}	0.01	0.055	0.507	0.009	0.532
Shell weight (%)	11.1^{ab}	10.8^{b}	10.7^{b}	11.2^{a}	0.07	0.032	0.522	0.004	0.603
Shape index (%)	79.1	80.1	80.3	79.6	0.20	0.145	0.316	0.037	0.942
Width (mm)	26.6	26.7	26.7	26.7	0.06	0.829	0.463	0.586	0.850
Length (mm)	33.6	33.3	33.3	33.6	0.09	0.461	0.716	0.122	0.933
Egg shell top (mm)	0.2	0.2	0.2	0.2	0.01	0.194	0.670	0.421	0.050
Egg shell mid (mm)	0.2^{a}	0.2^{ab}	0.2^{ab}	0.2^{b}	0.01	0.104	0.042	0.259	0.362
Egg shell bottom (mm)	0.2	0.2	0.2	0.2	0.00	0.354	0.223	1.000	0.185
Average thickness (mm)	0.2^{a}	0.2^{b}	0.2^{ab}	0.2^{b}	0.00	0.091	0.110	0.426	0.070
Albumen weight (g)	7.7	7.7	7.7	7.8	0.05	0.973	0.757	0.803	0.801
Albumen weight (%)	58.7	58.9	59.1	58.8	0.16	0.823	0.805	0.379	0.803
Albumen height (mm)	5.5	5.6	5.4	5.4	0.05	0.562	0.409	0.548	0.319
Albumen width (mm)	40.7	40.5	40.1	39.4	0.26	0.353	0.091	0.548	0.922
Albumen length (mm)	51.9	51.6	51.2	51.7	0.34	0.882	0.733	0.553	0.668
Albumen pH	8.8^{b}	9.1^{a}	9.2^{a}	9.2^{a}	0.04	0.003	0.001	0.048	0.511
Albumen index (%)	7.6	7.8	7.6	7.6	0.08	0.766	0.743	0.455	0.494
Yolk weight (g)	4.00	4.00	3.9	4.00	0.03	0.968	0.834	0.791	0.709
Yolk weight (%)	30.3	30.3	30.2	30.0	0.16	0.920	0.588	0.659	0.986
Yolk height (mm)	12.4^{b}	12.9^{a}	12.8^{ab}	12.8^{ab}	0.07	0.122	0.220	0.094	0.210
Yolk width (mm)	24.6	25.2	24.9	24.8	0.13	0.417	0.832	0.218	0.262
Yolk index (%)	50.6	51.2	51.3	51.4	0.26	0.646	0.266	0.561	0.808
L^*	69.7	69.6	70.8	70.7	0.33	0.374	0.115	0.919	0.443
a^*	11.0^{b}	11.8^{ab}	11.8^{ab}	11.9^{a}	0.14	0.100	0.038	0.197	0.503
b^*	17.3^{b}	17.7^{ab}	18.5^{a}	18.6^{a}	0.20	0.045	0.007	0.669	0.457
Chroma	344.6^{b}	377.9^{ab}	389.7^{ab}	414.2^{a}	10.52	0.137	0.022	0.835	0.715
h^o	8.6	8.8	8.6	8.6	0.05	0.545	0.387	0.463	0.361
Haugh unit	93.6	94.2	93.4	93.2	0.26	0.553	0.445	0.424	0.353
Breaking resistance (kg/cm^2)	1166.2	1056.9	1115.0	1118.2	23.10	0.421	0.676	0.224	0.283

$^{a-b}$Means in the same row bearing different superscripts differ significantly.
SED: Standard Error of the Difference between the means.
$^{¥}$: Effects; L: linear, Q: quadratic, C: cubic.

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BIOETHICS AND BIOSECURITY COMMITTEE APPROVAL

The study followed an ethics document taken from the Animal Experiments Local Ethics Committee of Kırşehir Ahi Evran University dated and numbered 07/11/2018-21-2.

DECLARATION OF CONFLICT OF INTERESTS

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

GF and AGF conceived and designed experiments. GF, AGF and AA performed the experiments, AGF carried out the lab analyses. GF supervised and coordinated the animal experiments and provided clinical data. GF performed statistical analyses of experimental data. GF and AGF prepared the draft of the manuscript. All authors critically revised the manuscript and approved of the final version.

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