Dietary supplementation of total flavonoids from *Rhizoma Drynariae* improves bone health in older caged laying hens

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ABSTRACT Caged layer osteoporosis (**CLO**) is a common bone metabolism diseases and poses a great threat to the production of laying hens. So far, there is no effective nutrition intervention to prevent CLO. The objective of this study was to evaluate the effects of dietary total flavonoids from *Rhizoma Drynariae* (**TFRD**), a Chinese herbal, on bone health, egg quality, and serum antioxidant capacity of caged laying hens. A total of two hundred sixteen, 54-wk-old Lohmann Pink-shell laying hens at were allocated to 3 groups with 6 replicates of 12 hens per replicate. The control group was fed a basal diet (**BD**) and 2 treatment groups additionally supplied with 0.5 or 2.0 g/kg TFRD, respectively. Results showed that supplying 2.0 g/kg TFRD enhanced the activities of serum total antioxidant capacity ($P < 0.01$) and glutathione peroxidase ($P < 0.05$) and had higher femur and tibia bone mineral density (both $P < 0.05$) compared with the control group. Dietary 2.0 g/kg TFRD also reduced the activities of serum alkaline phosphatase ($P < 0.01$), tartrate resistant acid phosphatase ($P < 0.01$), and the contents of osteocalcin ($P < 0.01$). Furthermore, tibia histomorphology observation showed that the microstructure of bone tissue was improved after TFRD treatment. Egg quality was not affected by TFRD while the egg weight significantly increased ($P < 0.01$). These findings suggested that TFRD has beneficial effects on bone health in older caged laying hens.

Key words: osteoporosis, bone metabolism, laying hen, total flavonoids of *Rhizoma Drynariae*

2020 Poultry Science 99:5047–5054
https://doi.org/10.1016/j.psj.2020.06.057

INTRODUCTION

Bone health has always been the focal point of laying hen breeding industry (Tarlton et al., 2013; Nie et al., 2018). Hens have a unique bone physiology because of the demands placed on them through egg production (Whitehead, 2004). Laying hens have 3 distinctive kinds of bones related to egg formation: cortical, cancellous, and medullary bones (Kim et al., 2012). Under the pressure of constant egg production, the cortical bone of the laying hen is progressively lost during the laying period, resulting in skeletal fragility and great susceptibility to fractures which was descripted as caged layer osteoporosis (**CLO**) (Jiang et al., 2019). With the breeding density of laying hens increasing, the limitation of sport environment has also increased the loss of bone mass (Aguado et al., 2015; Rodriguez-Navarro et al., 2018). What worse, laying hens with skeletal problems usually have a poor egg laying performance (Rufener et al., 2018). Caged layer osteoporosis has caused huge economic losses and involved in animal welfare issues (Riber et al., 2018; Hardin et al., 2019). So far, there is no effective nutrition intervention to prevent CLO.

Rhizoma Drynariae (Gu-Sui-Bu in Chinese) is one of the most frequently used traditional Chinese medicines which can replenish the kidney, strengthen the bones, promote the healing fracture, and relieve pain (Liu et al., 2012a,b). Studies have demonstrated that *Rhizoma Drynariae* contains flavonoids, triterpenes, and phenylpropanoids (Yuan et al., 2019). Among them, the study on the active components of *Rhizoma Drynariae* mainly focused on total flavonoids. Total flavonoids from *Rhizoma Drynariae* (**TFRD**) is a Chinese herbal product extracted from the dried root of *Rhizoma Drynariae* (Liu et al., 2012a,b), which has been developed into a postmarketing Chinese medicine called Qianggu

Received October 25, 2019.
Accepted June 11, 2020.

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capsule. Total flavonoids from *Rhizoma Drynariae* is widely used for the treatment of bone fractures or related diseases in Asian countries. It has been proven that TFRD has antiosteoporosis activity by inhibiting the bone resorption and stimulating bone formation (Wang et al., 2011) and finally achieves the goal of prevention and treatment of osteoporosis in humans and rat model (Song et al., 2016). Recently, studies found that TFRD has therapeutic effect on tibial dyschondroplasia in broilers (Yao et al., 2018). However, it is unclear whether TFRD has beneficial effects on older caged laying hens.

Therefore, this study was conducted to determine the effects of dietary TFRD supplementation on laying performance, egg quality, antioxidant capacity, and bone health in older caged laying hens.

MATERIALS AND METHODS

Birds, Diets, and Management

All procedures involving animals was approved by the Institutional Animal Care and Use Committee of Huazhong Agricultural University, China. A total of 216, 54-wk-old Lohmann Pink-shell laying hens with an average laying rate of 91% were obtained from a commercial farm in the Hubei province of China. The hens were randomly allocated to 3 groups (CON, TFRD1, and TFRD2) with 6 replicates of 12 hens per replicate. The CON group was fed a corn–soybean basal diet (**BD**) (Table 1) according to National Research Council (Dale, 1994), and the 2 experimental groups were fed BD supplemented with 0.5 and 2.0 g/kg TFRD. The TFRD used in this study was an extract from *Rhizoma Drynariae* and was purchased from Xi'an Kailai Biological Engineering Co., Ltd (Xi'an, China), and the total flavonoid content is 90.25% by HPLC analysis. The hens were randomly assigned to cages (80 cm-width × 80 cm-length × 40 cm-height) of 6 hens per cage based on completely randomized design. The hens were kept in an environmentally controlled room with ad libitum feeding and watering and with the temperature controlled at 22°C and 16 h/D of illumination throughout the entire experimental period. The experiment lasted 13 wk, included a 1-wk acclimation period and a 12-wk experimental period.

Sample Collections

The number of eggs and egg weight was recorded everyday (at 13:00) throughout experiment on a replication basis, and hen-day laying rate was calculated. Average egg weight was calculated as the mean weight of all eggs from each replicate. Feed consumption was measured weekly on a replication basis. Average daily feed intake **(ADFI)** was calculated using the following equation: ADFI = feed consumption (g)/(hen number × time (D)), and feed conversion ratio was calculated as (feed intake/(egg weight × egg production)). At 5, 9, and 13 wk, 2 eggs were randomly selected from each replicate for egg quality determination. At the end of the experiment (13 wk), 1 hen from each replicate was randomly selected, and blood samples were individually collected from the wing vein and then were centrifuged at 3,000 × *g* for 10 min at 4°C to obtain serum. In addition, hens were slaughtered to collect femurs and tibias.

Egg Quality Determination

Egg shape index was calculated by height/width. The eggs were weighed before being cracked. The eggshell strength was evaluated using an egg shell force gauge model II (Robotmation Co., Ltd., Tokyo, Japan). Eggshell thickness was measured on the large end, equatorial region, and small end, respectively, using an eggshell thickness gauge (Robotmation Co., Ltd). Egg weight, albumen height, yolk color, and Haugh unit were measured by using a digital egg tester (DET-6000, Nabel Co., Ltd, Kyoto, Japan).

Antioxidant Capacity Determination

The activities of total antioxidant capacity contents, total superoxide dismutase, glutathione peroxidase, and the contents of malondialdehyde in serum were analyzed using analysis kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Bone Mineral Density Measurement

The bone mineral density (**BMD**) was measured with dual energy X-ray absorptiometry (InAlyzer; MEDIKORS Inc., Gyeonggi-Do, Korea). The detection sensitivity of the instrument was 0.001 g/cm^2. A standard calibration block was used to calibrate the device before measurements were made, according to the operator's manual.

Bone Histomorphometry Analysis

The left tibias were removed from hens and fixed overnight at 4°C in 4% paraformaldehyde (Luo et al., 2019), then decalcified in 10% EDTA at room temperature for 2 wk and embedded into paraffin. Serial sections (5 μm) were cut and stained with Goldner Trichrome stain. In addition, the static parameters of bone histomorphometry were analyzed.

Bone Metabolism Biomarkers Analysis

The activities of alkaline phosphatase (**ALP**), tartrate-resistant acid phosphatase (**TRACP**), and the contents of calcium in serum were measured by specific assay kits from the Nanjing Jiancheng Bioengineering Institute of China. The concentrations of osteocalcin (**OCN**) in serum were measured with the use of ELISA kits (CH50029; Bio-Swamp, China) according to the manufacturer's instructions.

Table 1. Basal diet formulation and nutrient levels.

Dietary ingredient	Content (%)[1]
Corn	62.7
Soybean meal	26.3
Limestone	8.5
DL-Methionine	0.1
Calcium hydrogen phosphate	1.0
Choline chloride (50%)	0.1
NaCl	0.3
Vitamin and trace mineral premix[2]	1.0
Total	100
Nutrient levels (calculated)	
Metabolizable energy, MJ/kg	11.09
Crude protein, %	16.61
Calcium, %	3.5
Available phosphorus, %	0.35
Methionine, %	0.35
Lysine, %	0.85

[1]Values are expressed on as-fed basis.

[2]Premix provided per kilogram of diet: vitamin A (retinyl palmitate), 7,715 IU; vitamin D_3 (cholecalciferol), 2,755 IU; vitamin E (dl-a-tocopheryl acetate), 8.8 IU; vitamin K (menadione sodium bisulfate complex), 2.2 mg; vitamin B_{12} (cobalamin), 0.01 mg; menadione (menadione sodium bisulfate complex), 0.18 mg; riboflavin, 4.41 mg; pantothenic acid (d-calcium pantothenate), 5.51 mg; niacin, 19.8 mg; folic acid, 0.28 mg; pyridoxine (pyridoxine hydrochloride), 0.55 mg; manganese (manganese sulfate), 50 mg; iron (ferrous sulfate), 25 mg; copper (copper sulfate), 2.5 mg; zinc (zinc sulfate), 50 mg; iodine (calcium iodate), 1.0 mg; and selenium (sodium selenite), 0.15 mg.

Total RNA Extraction and Real-Time Quantitative PCR

According to the manufacturer's protocol, the total RNA was extracted from the tissues of the femur by adding TRIzol Reagent (Invitrogen, Waltham, MA). After extraction with chloroform, isopropanol was added to make the RNA precipitate. After washing with 75% ethanol, the RNA was eluted in ribonuclease-free water. The cDNA was synthesized using ABScript II RT Master Mix (ABclonal Technology, Wuhan, China). Runt-related transcription factor 2 (**RUNX2**) is a multifunctional transcription factor that controls skeletal development by regulating the differentiation of mesenchymal stem cells into osteoblasts and the expression of other related genes during osteoblast differentiation (Vimalraj et al., 2015). Osteoprotegerin (**OPG**), a soluble receptor that binds to receptor activator of nuclear factor kappa-B ligand (**RANKL**), is the natural inhibitor of RANKL and protects bone from excessive resorption (Tanaka et al., 2011). The forward and reverse primer sequences of RUNX2, OPG, and RANKL were designed based on available sequences on the NCBI GenBank and are listed in Table 2. β-actin was chosen as an internal standard to control for normalization purposes. Quantitation of the mRNA level by QPCR was performed on a real-time PCR system using iTaq Universal SYBR Green Supermix (Bio-Rad, Richmond, CA). The threshold cycle indicated the fractional cycle number at which the amount of amplified target reached a fixed threshold, so we can obtain the relative gene expression level by the $2^{-\Delta\Delta CT}$ method for fold induction. All PCR operations were performed in triplicate.

Statistical Analysis

Data were analyzed using the SPSS statistical software (SPSS version 22.0, for windows, SPSS Inc., Chicago, IL). One-way ANOVA was used for the analysis of group differences. For measures that presented heterogeneous variances, a Welch-ANOVA was employed. Post hoc analyses were performed by means of Bonferroni test or Games–Howell post hoc test (following the Welch-ANOVA) to characterize the significant effects. Differences were considered statistically significant at $P < 0.05$. The results are presented as the means and SEMs.

RESULTS

Performance of Laying Hens

As shown in Table 3, laying rate, ADFI, and feed conversion ratio did not differ among groups ($P > 0.05$). Dietary TFRD supplementation significantly increased the average egg weight ($P < 0.01$) compared with the CON group.

Egg Quality

No significant difference was found in egg quality parameters ($P > 0.05$) (Table 4).

Serum Antioxidant Capacity

Table 5 shows the effects of dietary TFRD on serum redox indicators in caged laying hens. The contents of malondialdehyde and the activities of total superoxide dismutase did not differ among groups ($P > 0.05$). Dietary TFRD supplementation significantly increased the levels of total antioxidant capacity ($P < 0.01$).

Table 2. Primers used for the quantitative polymerase chain reaction.

Genes	GenBank ID	Primers sequence (5′ to 3′)	Products (bp)
RUNX2	NM_204128.1	F: GATTACAGACCCCAGGCAGG R: TGGCTCAAGTAGGACGGGTA	75
OPG	NM_001033641.1	F: GTTCCTACTCGTTCCACACC R: GCTCTTGTGAACTGTGCCTTTG	115
RANKL	NM_001083361.1	F: AGGAGAAATAAGCCCGAGAA R: TTTGTTATGATGCCAGGATGTA	108
β-actin	NM_205518.1	F: CACGATCATGTTTGAGACCTT R: CATCACAATACCAGTGGTACG	100

Abbreviations: OPG, osteoprotegerin; RUNX2, runt-related transcription factor 2; RANKL, receptor activator of nuclear factor kappa-B ligand.

Table 3. Effects of dietary TFRD on laying performance in caged laying hens.

Items[1]	CON	TFRD1	TFRD2	SEM	*P*-value
Laying rate, %	86.90	86.86	87.78	1.23	0.949
AEW, g	60.97[b]	62.92[a]	63.07[a]	0.29	0.001
ADFI, g	121.86	121.83	121.79	0.04	0.844
FCR	2.31	2.23	2.22	0.02	0.099

Means within a row lacking a common superscript differ ($P < 0.05$), n = 6 per group.

[1]TFRD, total flavonoids of *Rhizoma Drynariae*; AEW, average egg weight; ADFI, average daily feed intake; FCR, feed conversion ratio; CON, basal diet; TFRD1, basal diet supplemented with 0.5 g/kg TFRD; TFRD2, basal diet supplemented with 2.0 g/kg TFRD.

Supplying 2.0 g/kg TFRD in diet had a higher activities of glutathione peroxidase compared with the control group ($P < 0.05$).

Femur and Tibia BMD

Figure 1 shows the effects of dietary TFRD on BMD of femur and tibia in caged laying hens. Dietary 0.5 g/kg TFRD supplementation did not induce an increase in BMD, whereas supplying 2.0 g/kg TFRD significantly increased the femur BMD by 19.4% and tibia BMD by 13.9% (both $P < 0.05$).

Tibia Histomorphometry

Figure 2 shows that, in the control group, many large absorption cavities appeared on the cortical bone, and the cortical bone area and width were reduced. The trabecular bone became loose or broken with decreased area. When TFRD was supplied, the absorption cavity was reduced, and the trabecular bone structure was more complete. In addition, the static parameters of bone histomorphometry were analyzed (Table 6). Compared with the control group, a significantly higher ($P < 0.05$) percent trabecular area and trabecular thickness was observed in all TFRD treated groups. Dietary supplemented with 2.0 g/kg TFRD enhanced cortical area ration and cortical width ($P < 0.05$) than that of control hens. Furthermore, a lower ($P < 0.05$) trabecular separation was observed with TFRD treatment. Trabecular number among groups did not differ in the study ($P > 0.05$).

Table 4. Effects of dietary TFRD on egg quality in caged laying hens.

Items[1]	CON	TFRD1	TFRD2	SEM	*P*-value
Egg shape index					
5 wk	1.30	1.31	1.31	0.01	0.908
9 wk	1.30	1.32	1.30	0.01	0.630
13 wk	1.31	1.32	1.31	0.01	0.958
Eggshell strength, N					
5 wk	44.75	48.02	46.02	0.70	0.156
9 wk	39.65	44.95	42.63	1.05	0.118
13 wk	34.74	39.38	36.92	0.87	0.088
Eggshell thickness, mm					
5 wk	0.36	0.38	0.38	0.01	0.165
9 wk	0.34	0.35	0.35	0.004	0.157
13 wk	0.30	0.32	0.32	0.006	0.234
Yolk color					
5 wk	13.30	13.10	13.64	0.14	0.311
9 wk	14.01	13.86	13.84	0.12	0.813
13 wk	13.98	13.66	14.01	0.11	0.402
Albumen height, mm					
5 wk	7.41	7.85	7.33	0.15	0.309
9 wk	7.40	6.93	6.99	0.13	0.292
13 wk	7.54	8.29	7.98	0.18	0.260
Haugh unit					
5 wk	85.17	87.46	83.49	1.04	0.308
9 wk	85.35	83.11	82.96	0.87	0.477
13 wk	83.45	91.74	87.96	1.50	0.077

Means within a row lacking a common superscript differ ($P < 0.05$), n = 12 per group.

[1]TFRD, total flavonoids of *Rhizoma Drynariae*; CON, basal diet; TFRD1, basal diet supplemented with 0.5 g/kg TFRD; TFRD2, basal diet supplemented with 2.0 g/kg TFRD.

Table 5. Effects of dietary TFRD on serum antioxidant indicators in caged laying hens.

Items[1]	CON	TFRD1	TFRD2	SEM	*P*-value
MDA, nmol/mL	3.90	3.80	3.69	0.12	0.787
T-AOC, U/mL	6.40[b]	8.58[a]	8.92[a]	0.34	0.001
T-SOD, U/mL	207	224	232	4.82	0.090
GSH-Px, U/mL	994[b]	1060[a,b]	1247[a]	44.8	0.045

Means within a row lacking a common superscript differ ($P < 0.05$), n = 6 per group.

[1]TFRD, total flavonoids of *Rhizoma Drynariae*; MDA, malondialdehyde; T-AOC, total antioxidant capacity; T-SOD, total superoxide dismutase; GSH-Px, glutathione peroxidase; CON, basal diet; TFRD1, basal diet supplemented with 0.5 g/kg TFRD; TFRD2, basal diet supplemented with 2.0 g/kg TFRD.

Serum Biomarkers of Bone Metabolism

Dietary TFRD supplementation decreased the activities of TRACP ($P < 0.01$) in serum (Table 7). Compared with the control group, supplying 2.0 g/kg TFRD significantly reduced the levels of ALP and OCN (both $P < 0.05$). No significant difference was observed in serum calcium ($P > 0.05$).

Real-Time Quantitative PCR

As shown in Figure 3, dietary 0.5 g/kg TFRD supplementation increased the mRNA expression of RUNX2 ($P < 0.05$) and OPG ($P < 0.05$) and decreased RANKL ($P < 0.01$). Compared with the control group, supplying 2.0 g/kg TFRD had a higher mRNA expression of RUNX2 ($P < 0.01$) and OPG ($P < 0.01$) and a lower RANKL ($P < 0.01$).

DISCUSSION

At present, there is no research directly indicating the effect of TFRD on the performance and egg quality of laying hens, but some of the previous studies involved the effects of the main active ingredients of TFRD (e.g., naringin and naringenin) on laying hens. In a study, naringenin (0.5 g/kg) for 30-wk-old Leghorn laying hens found that egg production and eggshell quality did not differ significantly among groups (Lien et al., 2008). In other similar study, dietary supplementation of

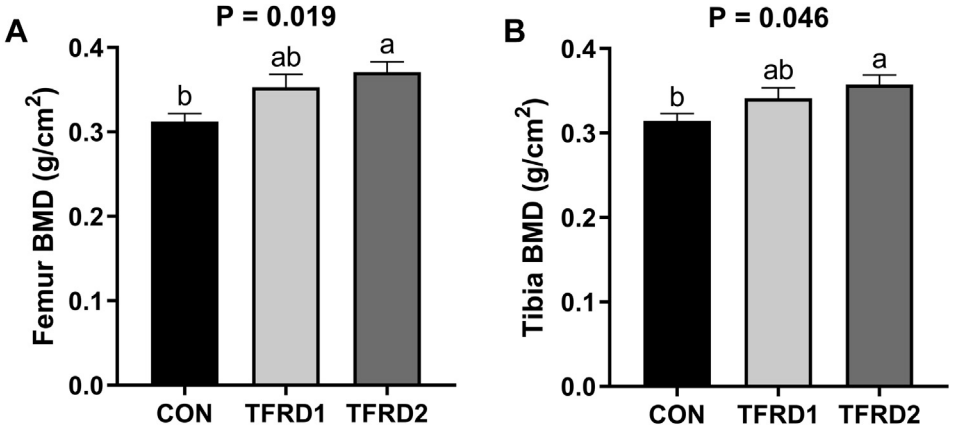

Figure 1. Effects of dietary TFRD on BMD in caged laying hens. (A) Femur BMD and (B) tibia BMD. Values are means ± SEM (n = 6 per group). The a, b, and c means every bars without same letter differ significantly ($P < 0.05$). Abbreviations: BMD, bone mineral density; TFRD, total flavonoids of *Rhizoma Drynariae*; CON, basal diet; TFRD1, basal diet supplemented with 0.5 g/kg TFRD; TFRD2, basal diet supplemented with 2.0 g/kg TFRD.

naringin (0.5, 0.75, or 1.5 g/kg) also did not affect performance and eggshell quality (Iskender et al., 2017; Goliomytis et al., 2019). In the present study, consistent with previous results, dietary supplementation of TFRD did not affect laying rate and egg quality. However, TFRD significantly increased average egg weight, which may be related to the estrogen-like effects of TFRD (Wong et al., 2013). It has been proved that estrogen is important in controlling egg weight, and mean plasma estradiol concentrations were very highly correlated with the changes in egg weights (Whitehead et al., 1993).

In addition, the results of serum antioxidant indicators showed that TFRD enhanced the antioxidant capacity of caged laying hens. Recently, there has been a tendency toward using antioxidants, especially from natural sources, for the protection of animal health (Xu et al., 2017). Total flavonoids from *Rhizoma Drynariae* have received enormous attention because they are plant-derived antioxidants and have the ability to suppress the formation of reactive oxygen species (**ROS**) (Matkowski et al., 2013). Many studies have indicated that ROS might have a role in osteoporosis development, which involves bone resorption activation and bone formation suppression (Huang et al., 2014). It is worth noting that in addition to TFRD, flavonoids from other sources also have shown a regulatory effect on ROS generation not only through gene expression and cell signaling pathways to activate enzymes that eliminate oxygen radicals but can also sequester potential oxidants (Weaver et al., 2012).

Traditionally, TFRD have long been considered an ideal drug for the treatment of bone-related disease (Song et al., 2016). Osteoporosis in caged laying hens is a common bone metabolic disease which leads to bone fragility, and the incidence rates of fracture up to 30% during the laying period (Whitehead and Fleming, 2000; Johnsson et al., 2015). Our results showed that supplying TFRD in diets increased the femur and tibia BMD in caged laying hens, which can reduce the risk of osteoporosis. Bone mineral density is the gold standard for the osteoporosis diagnosis and is useful in evaluating the risk of fracture (Richards et al., 2008). Previous study demonstrated that treatment with TFRD increased BMD and prevented bone loss in ovariectomized rats, thereby exerting antiosteoporotic effects (Yu et al., 2019). In vivo research also indicated that administration of TFRD significantly increased the BMD and mechanical strength and prevented bone loss induced by hindlimb unloading in rats (Song et al., 2017). Furthermore, clinical practice have proved that TFRD has therapeutic effect on osteoporotic fractures by improving BMD (Zhang et al., 2017).

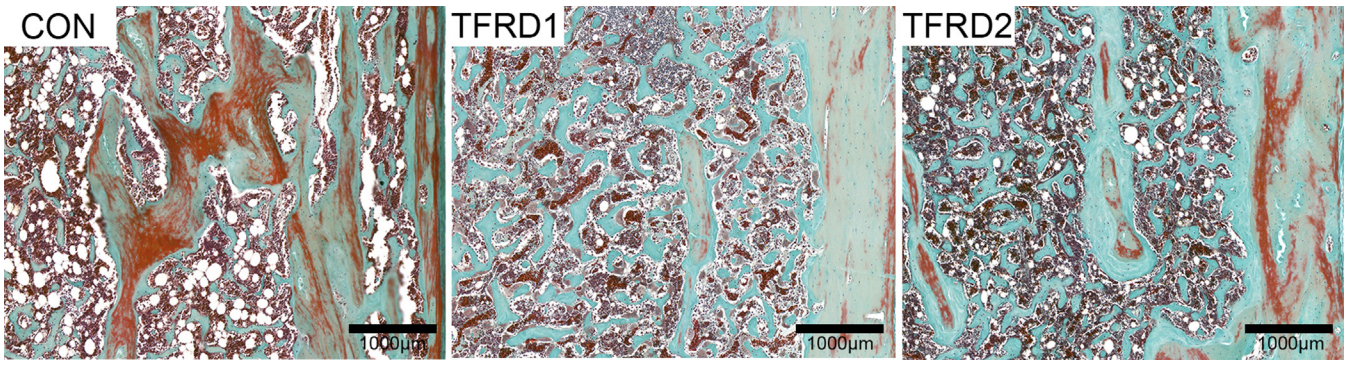

Figure 2. Effects of dietary TFRD on the microstructure of tibia tissue in caged laying hens. Goldner's trichrome staining (magnification, 10×). Abbreviations: CON, basal diet; TFRD1, basal diet supplemented with 0.5 g/kg TFRD; TFRD2, basal diet supplemented with 2.0 g/kg TFRD; TFRD, total flavonoids from *Rhizoma Drynariae*.

Table 6. Effects of dietary TFRD on bone histomorphometry parameters in caged laying hens.

Items[1]	CON	TFRD1	TFRD2	SEM	*P*-value
Ct.Ar, %	11.42^{b}	15.42^{a,b}	17.82^{a}	0.90	0.010
CW, mm	0.29^{b}	0.38^{a,b}	0.46^{a}	0.02	0.005
Tb.Ar, %	24.82^{b}	41.07^{a}	34.15^{a}	2.47	0.005
Tb.Th, um	16.27^{b}	25.58^{a}	22.93^{a}	2.11	0.014
Tb.Sp, um	49.33^{a}	40.61^{b}	43.21^{b}	1.15	0.001
Tb.N, #/mm	15.28	14.61	15.29	0.50	0.927

Means within a row lacking a common superscript differ ($P < 0.05$), n = 6 per group.

[1]TFRD, total flavonoids of *Rhizoma Drynariae*; Ct.Ar, cortical area ration; CW, cortical width; Tb.Ar, percent trabecular area; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; Tb.N, trabecular number; CON, basal diet; TFRD1, basal diet supplemented with 0.5 g/kg TFRD; TFRD2, basal diet supplemented with 2.0 g/kg TFRD.

Through bone histomorphometry observation, it was found that TFRD improved the microstructure of bone tissue, which was manifested in raised cortical area, cortical width, trabecular area, and trabecular thickness. Cortical bone is a structural bone that plays a critical role in bone strength (Seeman and Delmas, 2006). Preservation of the trabecular bone architecture significantly promotes bone strength and may be more important in decreasing fracture risk than improving BMD (Turner, 2002). Therefore, bone microarchitecture determinants are necessary to evaluate the true impact of a treatment on bone quality.

Caged laying hens are in a state of imbalance of bone metabolism during laying period, which ultimately reduces the bone strength of the laying hens and causes fractures (Kim et al., 2012). Some markers of bone metabolism may be conveniently classified either as indicators of bone formation and bone resorption (Christenson, 1997). Markers of bone formation assess either osteoblastic synthetic activity or postrelease metabolism of procollagen (Thiel et al., 2018). Resorption markers reflect osteoclast activity and collagen degradation (Soysa and Alles, 2016). Serum levels of total ALP provide a good identification of the extent of new bone formation and osteoblast activity (Naylor and Eastell, 2012). Osteocalcin, a hydroxyapatite-binding protein, which is considered as a specific marker of osteoblast function and bone metabolism (Chapurlat and Confavreux, 2016). Tartrate-resistant acid phosphatase is a potent enzyme that plays an important role in the

Table 7. Effects of dietary TFRD on serum bone metabolism biomarkers in caged laying hens.

Items[1]	CON	TFRD1	TFRD2	SEM	*P*-value
ALP, U/L	750^{a}	677^{a,b}	589^{b}	23.4	0.009
OCN, ng/mL	27.80^{a}	25.04^{a,b}	20.83^{b}	0.95	0.003
TRACP, U/L	78.42^{a}	61.56^{b}	56.45^{b}	2.80	<0.001
Ca, mmol/L	2.54	2.57	2.64	0.04	0.585

Means within a row lacking a common superscript differ ($P < 0.05$), n = 6 per group.

[1]TFRD, total flavonoids of *Rhizoma Drynariae*; ALP, alkaline phosphatase; OCN, osteocalcin; TRACP, tartrate-resistant acid phosphatase; CON, basal diet; TFRD1, basal diet supplemented with 0.5 g/kg TFRD; TFRD2, basal diet supplemented with 2.0 g/kg TFRD.

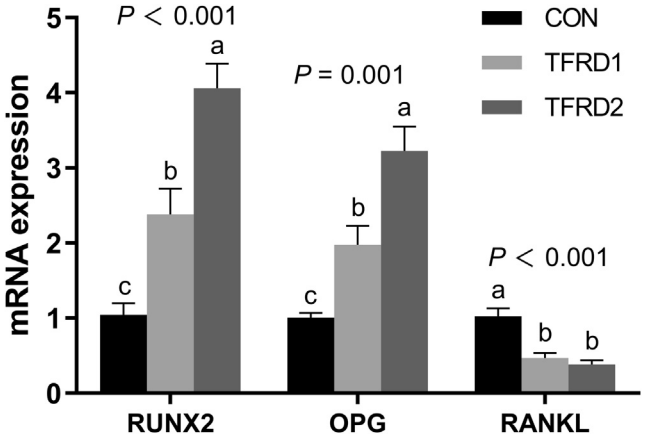

Figure 3. Effects of dietary TFRD on RUNX2, OPG, and RANKL mRNA expressions in caged laying hens. Values are means ± SEM (n = 6 per group). The a, b, and c means every bars without same letter differ significantly ($P < 0.05$). Abbreviations: TFRD, total flavonoids from *Rhizoma Drynariae*; RUNX2, runt-related transcription factor 2; OPG, TNF receptor super family member 11b; RANKL, tumor necrosis factor super family member 11; CON, basal diet; TFRD1, basal diet supplemented with 0.5 g/kg TFRD; TFRD2, basal diet supplemented with 2.0 g/kg TFRD.

bone resorption process (Linder et al., 2017). In our study, dietary TFRD supplementation decreased the levels of serum ALP, OCN, and TRACP, which indicated TFRD could affect osteoblast and osteoclast activities, thereby improving bone metabolism in older caged laying hens.

Runt-related transcription factor 2 is a multifunctional transcription factor that controls skeletal development by regulating the differentiation of osteoblasts and the expression of many extracellular matrix protein genes during osteoblast differentiation (Komori, 2010). Receptor activator of nuclear factor kappa-B ligand promotes the differentiation and activation of osteoclasts and stimulates and maintains their resorption activity (Martin and Sims, 2015). Previous studies revealed that the administration of soluble RANKL results in an increase in the formation and activation of osteoclasts that lead to osteoporosis in mice (Piemontese et al., 2016). On the contrary, OPG is a potent inhibitor of osteoclast formation and acts as a decoy receptor for RANKL (Behera et al., 2018). In vivo experiments showed that OPG knockout mice developed severe osteoporosis (Zhao et al., 2019). Dietary TFRD enhanced the mRNA expressions of RUNX2 and OPG/RANKL in older caged laying hens. It was suggested that TFRD could protect the bone health via regulating osteoblast and osteoclasts activity.

CONCLUSION

In summary, dietary TFRD supplementation did not affect egg quality, whereas significantly increased the egg weight. Supplying 2.0 g/kg TFRD to diet significantly enhanced serum antioxidant capacity and the BMD of femur and tibia of older caged laying hens. Furthermore, bone tissue microstructure and bone metabolism were improved with TFRD treatment. These

findings suggested that TFRD has beneficial effects on bone health in older caged laying hens.

ACKNOWLEDGMENTS

This work was supported by the National Key Research and Development Program of China (No. 2017YFD0502200, No. 2016YFD0501210) and Fundamental Research Funds for the Central Universities (No. 2662014BQ023).

Conflict of Interest Statement: The authors did not provide a conflict of interest statement.

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