Research Note: Effect of *Rubia cordifolia* L. processed *Terminalia chebula* Retz polysaccharide on the histological structure and apoptosis in the spleen in immunosuppressed Chinese yellow quail

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ABSTRACT It is generally accepted herbal polysaccharide and is a bioactive compound of herbal medicines with immunomodulatory activities. It has a wide range of pharmacological effects. It can be used as a green substitute for antibiotics or as a feed additive in quail breeding. Therefore, the herbal polysaccharide has a broader and safer application prospect. The immunosuppressive disease of quail is one of the most important infectious diseases. It seriously affects the growth, development, and production performance of quail, causing huge economic losses to quail industry. However, there is no report on the effective alleviation of spleen injury in immunosuppressed animals by herbal polysaccharide. Therefore, we established a pathological model of immunosuppressive Chinese yellow quail for the first time, with the *Terminalia chebula* Retz polysaccharide (**TCP**) as the control, and histological observation, TUNEL staining were used to study the effects of *Rubia cordifolia* L. processed *Terminalia chebula* Retz polysaccharide (**RTCP**) on splenic tissue structure and apoptosis of immunosuppressed Chinese yellow quail. The experimental results showed that spleen organ index of the cyclophosphamide (**CTX**) group was significantly lower than these of blank control group, the TCP group and the RTCP group ($P < 0.05$). And the number of splenic nodules in the CTX group was significantly lower than that in the blank control group ($P < 0.01$). Compared with the CTX group, the spleen volume of the TCP group and the RTCP group increased, and the number and area of spleen nodules increased. Among them, the spleen nodules in the RTCP group were significantly more higher than that in the CTX group ($P < 0.01$). Meanwhile, TUNEL staining showed that the TUNEL positive cells in the CTX group were the most significantly higher than those in the blank control group ($P < 0.01$). TCP group and RTCP group were significantly higher than the blank control group ($P < 0.01$), but significantly lower than CTX group ($P < 0.05$). All these results suggested that RTCP could effectively improve CTX-induced spleen damage in immunosuppressed Chinese yellow quails by promoting the recovery of spleen organ index, repairing the spleen tissue structure, and diminishing the apoptosis. Moreover, RTCP is more effective than TCP. The results prove that the efficacy of RTCP in protecting spleen from CTX induced injury was enhanced after processing with *Rubia cordifolia* L. Therefore, our findings will provide more possibilities to promote the clinical application and development of processed traditional Chinese medicine in the further.

Key words: *Rubia cordifolia* L. processed *Terminalia chebula* Retz polysaccharide, spleen, immunosuppression, Chinese yellow quail

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INTRODUCTION

The quail have high economic value for meat and eggs, and an experimental model for the basic and applied research. At present, with the expansion of quail culture scale and the continuous occurrence of immunosuppressive diseases in China, the growth, development, and production performance of quail are seriously affected. This caused huge economic losses to the quail breeding industry. It is urgent to develop a kind of drugs or feed additives that can treat quail immunosuppressive diseases and improve its autoimmune function.

In 1997, the use of antibiotics in livestock was first addressed in Denmark with avoparcin as an antibiotic growth promoter (**AGP**). The trend continued and led to the widespread use of antibiotics in animals. Then it has led to drug residues, bacterial resistance, and its potential spread to threaten humans. So a European

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Union (**EU**)-wide ban on AGPs in animal feed (poultry) took effect in 2006 (EC Regulation No 1831/2003) (Sjofjan et al., 2021). Since then, this type of regulation has spread to countries. And China also gradually banned to use antibiotics in feed. It marks the global has entered the era of "ban-antibiotics in feed," thus the development of safe alternatives becomes a focus. Herbal polysaccharide is a herbal bioactive compound of medicines with immunomodulatory activities. It can be used as an ideal safe substitute for antibiotics. A large number of experiments have proved that plant polysaccharides contained in Polygonatum sibiricum, *Terminalia chebula* Retz (**TC**), Ganoderma atrum, etc. have the effect of enhancing immunity (Li et al., 2017; Jeong et al., 2019; Shu et al., 2021). Among them, TC is known as the "king of Tibetan medicine" due its various effects (He et al., 2021). And it is one of the most representative medicines in Tibetan medicine. TC are used for diverse symptoms, and they are known to involve many bioactivities, including immunomodulatory activities (Jeong et al., 2019). At present, our research group has experimentally confirmed that the polysaccharide content of TC will increase after processing with *Rubia cordifolia* L. (Jingjing et al., 2019). However, whether its immune regulation function is also enhanced with processing, it needs to be proved by further experiments.

Spleen is the largest immune organ in animals. And it is an important immune response site for animals. It not only has specific immune function, but also has nonspecific immune function. Therefore, the state of spleen largely determines the immune state and immune performance of the body. However, the spleen is extremely sensitive to changes in internal and external environment, and its state determines the strength of spleen immunity (Dunaievska, 2018). At present, our research group has studied the basic morphological structure of the spleen of Chinese Yellow quails (Piao et al., 2018). But the effects of RTCP on splenic histological structure in immunosuppressed quail have not been studied. And no relevant reports were found by consulting the literature. Therefore, our experiment is the first to establish a pathological model of Chinese yellow quail with immunosuppression induced by CTX, with TCP as the control, and intend to study the effects of RTCP on splenic histological structure in immunosuppressed Chinese yellow quail, so as to explore whether the immune regulation function of TC is enhanced after processing with *Rubia cordifolia* L. we are hope that our findings will provide a reference for the research and development of RTCP as a medicine or a feed additive to treat immunosuppressive diseases and improve autoimmune function in quail.

MATERIAL AND METHODS

Plant Materials

TC and *Rubia cordifolia* L. were obtained from a local supermarket in Sichuan Chengdu (China) and authenticated by associate professor Fan QJ of the Department of Pharmacy at Sichuan Agricultural University, Sichuan, China (He et al., 2021). Based on the existing research foundation of our research group, we processed RTC, extracted TCP and RTCP (Jingjing et al., 2019; He et al., 2021).

Preparation of Processed Products

Preparation of RTC (He et al., 2021): *Rubia cordifolia* L. was burnt to ash. And *Rubia cordifolia* L. was mixed in a 1:3 ratio with wheat wine. The mixture was heated in a pot until it boils. Then, TC was mixed in a 1:3 ratio with the mixture and boiled for 10 min and cool to 25°C. Next, boil again and cool to 25°C. And then TC was soaked in the pot at 25°C for 1 d. On the second day, TC was removed and kneaded.And TC meat was obtained. Then, TC meat was washed with distilled water 3 times and dried at 70°C for 30 h.

Preparation of Polysaccharide

Preparation of RTCP: According to the existing research basis of our research group (Jingjing et al., 2019), the best extraction process for crude polysaccharides from RTC was extracted by enzymatic extraction. Methods: take RTC 5 g, put it into a 250 mL beaker, and add 1.8% cellulase. Then extract it was carried out according to the solid−liquid ratio of 1:40, 40℃ extraction temperature, and 90 min extraction time. After extraction, it was inactivated in boiling water bath for 10 min. Then the mixed filtrate was concentrated to 100 mL, then adding 3 times 95% ethanol. Let it stand overnight and take the precipitate. The precipitate is washed successively with ethanol, acetone, and ether, filtered and dried to obtain crude polysaccharide.

Preparation of TCP: The extraction method is the same as that of RTCP (Jingjing et al., 2019), but the extraction conditions are slightly changed: 1.8% cellulase enzyme dosage, 1:50 solid−liquid ratio, 50℃ extraction temperature, and 60 min extraction time.

Experimental Animals

A total of sixty 21-day-old Chinese yellow quail (obtained from Chengdu Da'an quail farm, China) were randomly divided into 4 groups: the blank control, CTX, TCP, and RTCP groups (n = 15 in each group). All the quails were treated in accordance with the Guidelines for Care and Use of Laboratory Animals, and the study was approved by the Animal Ethics Committee of Sichuan Agriculture University. The quail were housed in a SPF animal room at 23°C to 25°C and provided a standard diet and water throughout the 10-d experimental period. From day 1 to day 3, CTX was intramuscularly injected at 80 mg/kg to 3 groups except the blank control group. The blank control group was injected with an equal volume of saline. For 4 to 10 d of experiment, the experimental group was intragastrically administered with TCP or RTCP at the dose of 0.5 g/kg

(Yu-ning et al., 2021; Jingjing et al., 2019). And the blank control group and CTX group were administered an equal volume of saline by gastric perfusion, once a day for 7 consecutive days. The above cyclophosphamide injection dose and drug intragastric dose were from the previous research results of our research group (Jingjing et al., 2019; Yu-ning et al., 2021).

Main Reagents

A box of CTX (product code: S30563) was purchased from Shanghai yuanye Bio-Technology Co., Ltd. (Shanghai, China.). An H&E staining kit (product code: G1120) was purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). A TUNEL staining cell apoptosis detection kit-POD (product code: MK1025) was purchased from Wuhan Boster Biological Technology Co., Ltd. (Wuhan, China).

Spleen Organ Index Measurement

The quails 24 h after the last gavage were anesthetized with an intraperitoneal injection of 10% chloral hydrate (2 mL/kg) and placed on an electronic balance to obtain the body weight, which was then recorded (Piao et al., 2018). The quail sternum was then incised at the midline to expose and collect the spleen. After cleaning, the spleen was weighed with an electronic balance. The spleen organ index is calculated according to the following formula (Li et al., 2017):

$$\text{Spleenorganindex} = \text{spleenweight(mg)}/\text{quailweight(g)}$$

H&E Staining

Five spleen samples were extracted from each group and fixed immediately in 4% paraformaldehyde for 24 h. Fixed tissue was dehydrated in ethanol, transparented in xylene, and embedded in paraffin. Serial sections of 5 μm were obtained using a conventional method, and stained using hematoxylin and eosin (Piao et al., 2018).

TUNEL Staining

Spleen samples were the collected and fixed paraffin embedding of the method as the same as that of H&E staining. The apoptosis of spleen cells was detected by a TUNEL staining cell apoptosis detection kit-POD (product code: MK1025). And the measurement method was as the same as H&E staining.

Statistical Analysis

The stained slices were observed under an Olympus BH-2 microscope. A total of 10 fields (200×) from each tissue section were randomly selected for number of splenic nodules, the area of splenic nodules and the splenic TUNEL positive cells. Above indicators in each field (200×) were measured with an Image-Pro Plus 6.0 analysis software (He et al., 2021).

All data are expressed as the mean ± standard deviation ($\overline{X}$± s). Duncan's multiple comparison method and t tests were performed in this study ($P < 0.05$ indicates a significant difference). SPSS 20.0 software was used for statistical analysis of biological data (He et al., 2021).

RESULTS AND DISCUSSION

The Effect of RTCP on the Spleen Organ Index in Chinese Yellow Quail

Spleen is an important immune organ, and its organ index can reflect the strength of immune function to a certain extent. Studies have shown that Ganoderma atrum polysaccharide enhanced the ratio of spleen weight to body weight in CTX-mediated immunosuppressed mice (Li et al., 2017). Polygonatum sibiricum polysaccharide could significantly promote the recovery of organs index and maintain its structure and function in immunosuppressed chickens by CTX induced (Shu et al., 2021). These were consistent with our experimental results. As shown in Table 1, the spleen weight and the spleen organ index of the CTX group were significantly lower than those of the blank control group, the TCP group, and the RTCP group ($P < 0.05$), indicating that CTX damaged the spleen. And there were no significant difference in spleen weight and spleen organ index among the blank control group, the TCP group, and the RTCP group ($P > 0.05$). It showed that both RTCP and TCP could promote the recovery of spleen organs index in CTX-induced immunosuppressed Chinese yellow quails. And RTCP is more effective than TCP. It is speculated that the mechanism may be that RTC can improve the digestive function, promote organ growth, and improve the immune regulation and antioxidant

Table 1. The effect of RTCP on the spleen organ index, splenic histological structure, and apoptosis in Chinese yellow quail.

Group	Spleen weight (mg)	Spleen organ index (mg/g)	The number of splenic nodules (number/mm^2)	The mean value of splenic nodule area (μm^2)	TUNEL positive cells (number/mm^2)
CTX	49.90 ± 18.43	0.44 ± 0.15	1.5 ± 0.55	898.96 ± 299.8	81.12 ± 20.28
TCP	81.50 ± 12.82*	0.77 ± 0.16*	3.54 ± 0.65*	3852.93 ± 395.35**	35.88 ± 3.12*
RTCP	117.72 ± 37.61*	1.00 ± 0.27*	4.58 ± 0.72*	3963.62 ± 250.18**	29.64 ± 4.68*
Blank	87.88 ± 10.38*	0.87 ± 0.03*	5.52 ± 0.83**	4132.93 ± 281.55**	14.04 ± 2.12**

The data are presented as the means ± standard deviations (n = 10).
*indicates a significant difference between CTX group and the experimental group ($P < 0.05$);
**indicates an extremely significant difference between CTX group and the experimental group ($P < 0.01$).

capacity of animals better than TC (Jingjing et al., 2019). Bo et al. (2021) reported that the selenylation modification of garlic polysaccharides could improve its immune activity in chickens. This indicated that the polysaccharide efficacy of plants was significantly enhanced after processing (Bo et al., 2021). Our experimental results also showed that the efficacy of RTCP was enhanced by processing. This might be related to the synergistic effect of *Rubia cordifolia* L. on TC (He et al., 2021). However, the specific mechanism of action is still unclear, which needs further experiments to prove.

The Effect of RTCP on the Histological Structure in the Spleen in Chinese Yellow Quail

Plant polysaccharide is a bioactive compound with immunomodulatory activities. It can protect animals against CTX-mediated immunosuppression, as evidenced by enhancing the immune organ index, promoting the lymphocytes survival, and recovering and maintaining the structure and function of animal organs (Li et al., 2017; Shu et al., 2021). The animal model of CTX-induced immunosuppressed Chinese Yellow quail was successfully established in our experiment. We found that the number of splenic nodules in the CTX group was significantly less than that in other groups (Figures 1A1−1A4). And the area of splenic nodules in the CTX group was small than other groups. Moreover, compared with other groups, the blood storage of red pulp splenic sinus in the CTX group decreased significantly (Figures 1A1−1A4). As shown in Figures 1A1 and 1A2, compared with the CTX group, the spleen tissue structure of the TCP group was improved or reversed. As can be seen from Figure 1A2, the number of splenic nodules increased and the area of splenic nodules also increased. But it was lower than that in the RTCP group and significantly lower than that in the blank control group (Figures 1A3 and 1A4). Meanwhile, Figures 1A1−1A4 showed that the blood storage of red pulp splenic sinus increased gradually. The splenic blood storage in the RTCP group was more than that in the TCP group, but less than that in blank control group (Figures 1A2−1A4). The research results on the spleen white pulp of immunosuppressed quail are basically consistent with the report of Li et al. on immunosuppressed mice (Li et al., 2017). However, there were differences in the histological observation of red pulp. We speculated that the animal species selected in the experiment are different, and the immunosuppressive symptoms of the CTX-induced animal model may not be exactly the same.

Meanwhile, as shown in Table 1, the number of splenic nodules in the CTX group was lower than that in the TCP group and the RTCP group ($P < 0.05$). And then it was significantly lower than that in the blank control group ($P < 0.01$). And the mean value of splenic nodule area in the CTX group was significantly lower than those of the blank control group, the TCP group, and the RTCP group ($P < 0.01$). These results are consistent with Shu et al. (2021). These results showed that TCP and RTCP could greatly promote the proliferation of the spleen lymphocyte and effectively repair the spleen structure when the spleen tissue structure of Chinese yellow quails damaged by CTX (Shu et al., 2021). Meanwhile, our experimental results also showed that the effect of RTCP to repair the spleen tissue structure of quails would be more significantly than that of TCP. The reason may be that the polysaccharide structure of TC may be changed after *Rubia cordifolia* L. processing, which leads to the enhancement of the efficacy of RTCP (Jingjing et al., 2019). Although the specific mechanism of action needs to be further studied, it will provide more possibilities for the development and application of processed traditional Chinese medicine processing.

The Effect of RTCP on the TUNEL Positive Cells in the Spleen in Chinese Yellow Quail

TUNEL staining made apoptotic cells in splenic tissue sections positive. Apoptosis, also known as programmed cell death, is a course involved in genetical regulation and lead to a variety of changes in morphology and metabolic activity, which cause cell death ultimately (Liu et al., 2021). Studies have shown that Ganoderma atrum polysaccharide protected immune organs against CTX-induced immune dysfunction in mice by ameliorating reactive oxygen species generation and apoptosis in spleen and thymus (Li et al., 2017). Hericium erinaceus polysaccharide can reduce the immunosuppression of Muscovy duck reovirus-infected ducklings by reducing organ damage, improving antioxidant capacity and diminishing the apoptosis (Liu et al., 2021). These reports are consistent with our experimental results. Our results showed that the TUNEL positive cells were brownish yellow, which were distributed in the white pulp and red pulp of the spleen of each group. And these positive cells were mainly distributed in the white pulp, especially in the splenic nodules (Figures 1B1−1B4). Meanwhile, from Figure 1B1 to Figure 1B4, we could observe that the number of positive cells was gradually decreased. It also showed that the number of positive cells in the CTX group was significantly higher than that of other groups (Figures 1B1−1B4). Meanwhile, as shown in Table 1, the number of the TUNEL positive cells in the CTX group was higher than that in the TCP group and the RTCP group ($P < 0.05$). And then it was significantly higher than that in the blank control group ($P < 0.01$). It showed that RTCP could more diminish the apoptosis and effectively improve CTX-induced spleen damage than that of TCP group. And it also showed that the efficiency of RTCP is enhanced with processing, which protected the spleen against CTX-induced apoptosis in quails. Haibin et al. (2019) reported that Lycium barbarum polysaccharide could promote the expression of apoptosis factor Bcl-2 and inhibit the expression of apoptosis promoting factor Bax, so as to reduce apoptosis. Whether the mechanism of RCTP

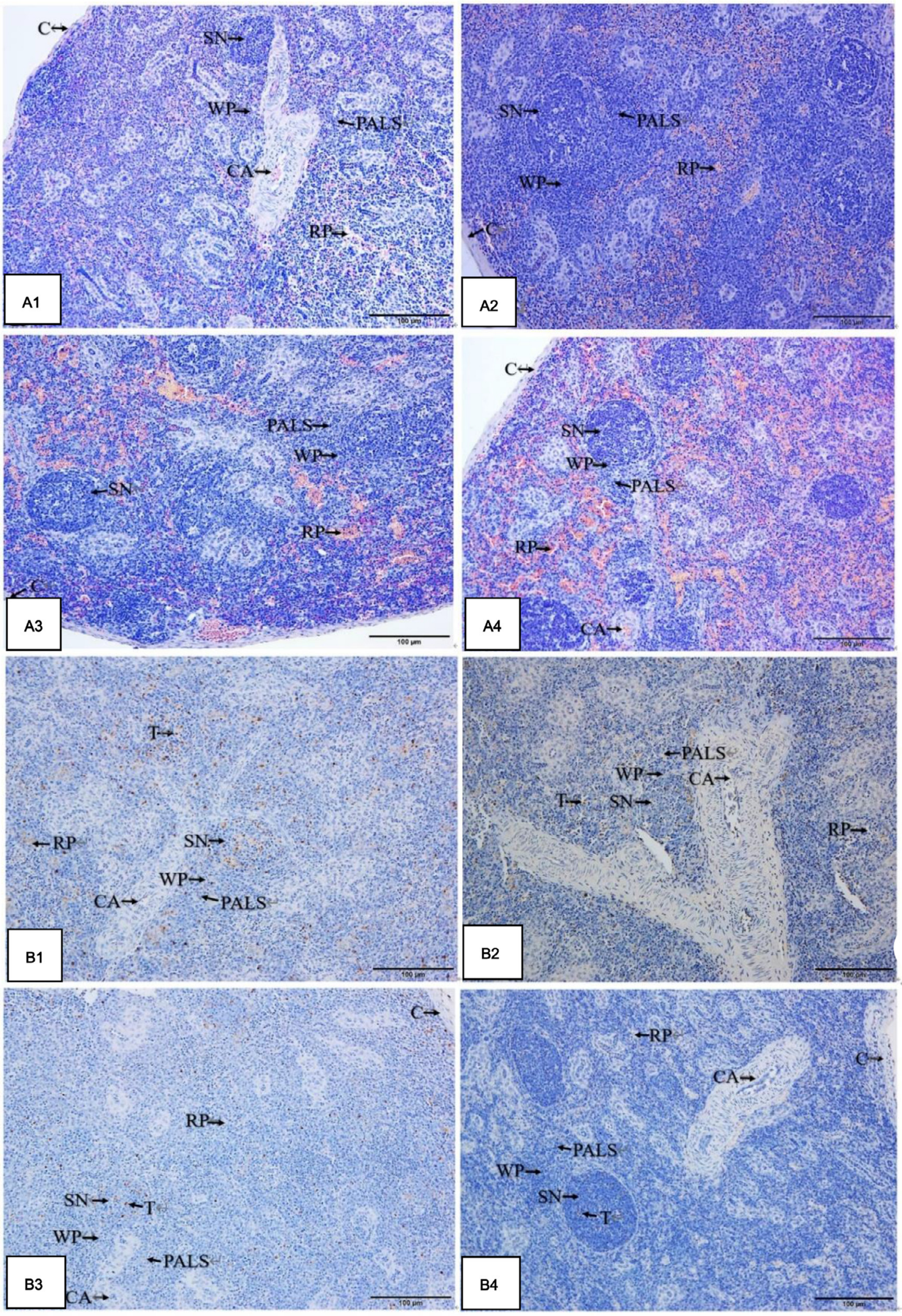

Figure 1. (A) Histological observation of the spleen of Chinese yellow quail in different groups (H&E, 200×). (A1) CTX group; (A2) TCP group; (A3) RTCP group; (A4) blank control group. C indicates capsule (arrow); RP indicates red pulp (arrow); WP indicates white pulp (arrow); SN indicates splenic nodule (arrow); CA indicates central artery (arrow); PALS indicates periarterial lymphatic sheath (arrow). (B) The apoptosis of the spleen of Chinese yellow quail in different groups (TUNEL, 200×). (B1) CTX group; (B2) TCP group; (B3) RTCP group; (B4) blank control group. C indicates capsule (arrow); T indicates TUNEL positive cells (arrow); RP indicates red pulp (arrow); WP indicates white pulp (arrow); SN indicates splenic nodule (arrow); CA indicates central artery (arrow); PALS indicates periarterial lymphatic sheath (arrow).

effectively reducing apoptosis in immunosuppressive quails is the same as that of Lycium barbarum polysaccharide remains to be confirmed by further experiments.

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Data Availability Statement: The data used to support the findings of this study are included within the article.

DISCLOSURES

The authors declare no conflict of interest.

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