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## Article

# Cuban Policosanol (Raydel®) Exhibited Potent Anti-Inflammatory Activity in the Liver and Protection of the Ovary and Testis with an Improvement of Blood Lipid Profile by 12 Weeks Supplementation in Hyperlipidemic Zebrafish: Comparison Study of Efficacy with Three Chinese Policosanols

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**Abstract:** Although many policosanols from different sources, such as sugar cane, rice bran, and insects, have been marketed worldwide, there has been no comparative study of the *in vivo* efficacy using a hyperlipidemic animal model. This study compared the efficacy of treating dyslipidemia with four different policosanols, including one sugar cane wax alcohol from Cuba (Raydel®) and three policosanols from China (Xi'an Natural sugar cane, Xi'an Realin sugar cane, and Shaanxi rice bran) in hyperlipidemic zebrafish. After 12 weeks of consumption of each policosanol (final 0.1% in diet, wt/wt) and high cholesterol diet (HCD, final 4%, wt/wt) in a tetrabit, the Raydel policosanol group and Xi'an Natural policosanol group showed the highest survivability of approximately 81%, whereas the Xi'an Realin policosanol group and Shaanxi policosanol group showed 57% and 67% survivability, respectively. Among five HCD groups, the Raydel policosanol group showed the lowest serum total cholesterol (TC,  $p < 0.001$  versus HCD control) and triglyceride (TG,  $p < 0.001$  versus HCD control) with the highest percentage of high-density lipoproteins (HDL)-C in TC. The Raydel policosanol group also showed the lowest serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels with the least infiltration of inflammatory cells and interleukin-6 production in hepatocytes. In the ovary, the Raydel policosanol group also showed the highest contents of mature vitellogenic oocytes with the lowest production of reactive oxygen species and cellular apoptosis in ovarian cells. In the testis, the Raydel policosanol group also showed the healthiest morphology for spermatogenesis with the least interstitial area and reactive oxygen species (ROS) production in testicular cells. In conclusion, 12 weeks of Raydel policosanol consumption resulted in remarkable improvement of the blood lipid profile, amelioration of inflammation in the liver, and enhancement of the cell morphology in the ovary and testis with the highest survivability under the HCD.

**Keywords:** Policosanol; high cholesterol diet; high-density lipoproteins; apoA-I; inflammation; interleukin-6; liver; testis; ovary

## 1. Introduction

A higher HDL-C quantity and improved HDL quality have been associated with healthy human longevity [1,2] through enhanced antioxidant and anti-inflammatory activities in the blood [3,4]. Dyslipidemia, however, particularly low levels of serum high-density lipoproteins cholesterol (HDL-C), is a major risk factor for metabolic syndrome [5], combination of hypertension [6] and diabetes [7], and dementia [8]. Dyslipidemia and diabetes are also frequently linked with oxidative stress and

glycation stress [9], chronic inflammation [10], and sexual dysfunction [11]. In particular, erectile dysfunction [12] and unexplained female infertility [13] have shown strong associations with dyslipidemia and diabetes, such as low HDL-C.

Many pharmaceuticals, such as CETP inhibitors [14], and functional foods [15], such as policosanols, have been developed and marketed to increase the HDL-C levels [16]. Policosanols are a mixture of aliphatic alcohols, ranging from 24–34 carbon atoms [17], such as octacosanol, triacontanol, dotriacontanol, hexacosanol, and tetratriacontanol as the major components, which were purified from sugar cane (*Saccharum officinarum* L.) wax [18] or various plants, such as oats [19] and barley [20], insects [21], and bees wax [22]. Many policosanols have been purified from various plant sources, such as sugar cane, rice bran [23,24], wheat germ [20], and barley sprouts [20]. Despite the diversity of policosanols, there are no reports comparing the efficacy in treating dyslipidemia and anti-inflammatory activities among the various sources and origins of policosanols.

Cuban policosanol (Raydel®) has been used to treat blood dyslipidemia, hypercholesterolemia [25,26], hypertension [27,28], and Alzheimer's disease [29] by increasing the HDL-C and lowering the LDL-C with the removal of amyloid plaque in the brain [29]. In the preceding paper [30], a reconstituted HDL containing Cuban policosanols exhibited the largest particle size and the highest antioxidant, anti-glycation, and anti-inflammatory activity. In contrast, three Chinese policosanols did not exhibit beneficial activity [30]. Except for Cuban policosanol, there is insufficient information on the physiological effects of policosanols on the lipid profile and the extent of inflammation in hyperlipidemic animal models. Furthermore, no information is available on the hidden toxicities and influences on the hepato-endocrinological system and reproduction system by policosanol consumption under hyperlipidemia.

A zebrafish (*Danio rerio*) model displaying induction of hypercholesterolemia by a high-cholesterol diet (HCD) supplementation were used to mimic dyslipidemia and hepatic inflammation, as suggested by a previous report, and compare the physiological efficacy [31,32]. Adult zebrafish consuming a HCD for several weeks showed the induction of atherosclerosis symptoms, including hypercholesterolemia, lipoprotein oxidation, and fatty streak formation [33]. Hypercholesterolemic zebrafish is a useful experimental model to display liver damage [34], infiltration of neutrophils in hepatocytes, and fatty liver change, which can be ameliorated by consuming Cuban policosanols. Interestingly, hyperlipidemia and hepatic damage were linked with the impairment of the reproduction system with abrupt changes in sperm motility [35] and an increase in immature oocytes [36].

It is very interesting that there has been no study to compare the *in vivo* efficacy of various policosanols from different sources and origins regarding improving dyslipidemia, hepatic inflammation, and the reproduction system. Therefore, the current study was designed to compare the *in vivo* physiological effects of various policosanols under hyperlipidemia because there has been no sufficient information on policosanol efficacy on the lipid profile, antioxidant, and anti-inflammatory activities in the liver and other organs, such as the ovary and testis.

## 2. Results

### 2.1. Change of Survivability and Body Weight.

After 12 weeks of consumption, the normal diet (ND) group showed 97% survivability, whereas the HCD group, with high cholesterol in the diet (final 4%, wt./wt.), showed 61% survivability, suggesting that 4% cholesterol supplementation induced more acute death via progression of hyperlipidemia and hyperinflammation (Table 1). On the other hand, the PCO1 (Raydel) group and PCO2 (Xi'an Natural) group showed the highest survivability of approximately 81%, while the PCO3 (Xi'an Realin) group and PCO4 (Shaanxi) group showed lower survivability of 57% and 67%, respectively.

**Table 1.** Diet composition and change of body weight and survivability during 12 weeks consumption of policosanols under high cholesterol diet (HCD).

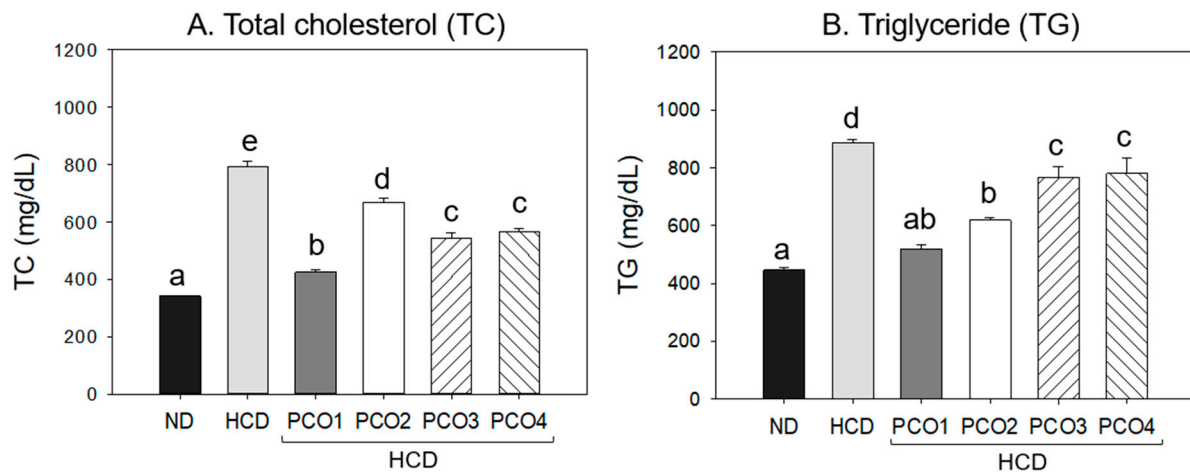
		HCD					
	Groups	ND control	Control	PCO1 Raydel Sugarcane wax alcohol	PCO2 Xi'an Natural Sugarcane	PCO3 Xi'an Realin Sugarcane	PCO4 Shaanxi Rice bran
	Tetrabits	100	96	95.9	95.9	95.9	95.9
Diet compo-sition (%)	Cholesterol (% wt./wt.)	-	4	4	4	4	4
	PCO (% wt./wt.)	-	-	0.1	0.1	0.1	0.1
Body weight (mg)	0	276 ±13	275±9	278±12	265±8	262 ±7	271 ±5
	12	507±14 <sup>a</sup>	635 ±31 <sup>c</sup>	547±17 <sup>ab</sup>	605±23 <sup>bc</sup>	602±24 <sup>bc</sup>	509±13 <sup>a</sup>
	<i>p</i> <sup>†</sup>	-	-	< 0.001	ns	ns	< 0.001
Survi-vability (%)	0	100 (n=70)	100 (n=70)	100 (n=70)	100 (n=70)	100 (n=70)	100 (n=70)
	12	97 (n=68)	61 (n=43)	81 (n=53)	81 (n=53)	57 (n=40)	67 (n=47)

Data of body weight are expressed as mean±SEM. The *p*<sup>†</sup> value indicates whether the ANCOVA is statistically significant between the HCD control group at week 0 and each group. ns, not significant. The letters in superscript indicate a statistical difference between the different groups (*p*<0.05). ND, normal diet; HCD, high cholesterol diet; PCO, policosanols.

As shown in Table 1, the ND and HCD alone group showed a 1.8-fold and 2.3-fold increase in body weight (BW), respectively, after the 12 weeks of consumption from the baseline (week 0). Under HCD, the PCO1 group and PCO4 group showed a 1.9-fold (*p*<0.001) and 1.8-fold (*p*<0.001) increase in BW, while the PCO2 and PCO3 groups showed a 2.3-fold increase in BW. These results suggest that the Raydel policosanols group showed the highest survivability and the second lowest increase in BW during the 12 weeks. Interestingly, the PCO4 group showed the lowest increase in BW with lower survivability than the PCO1 group, suggesting that lower body weight was not associated with higher survivability.

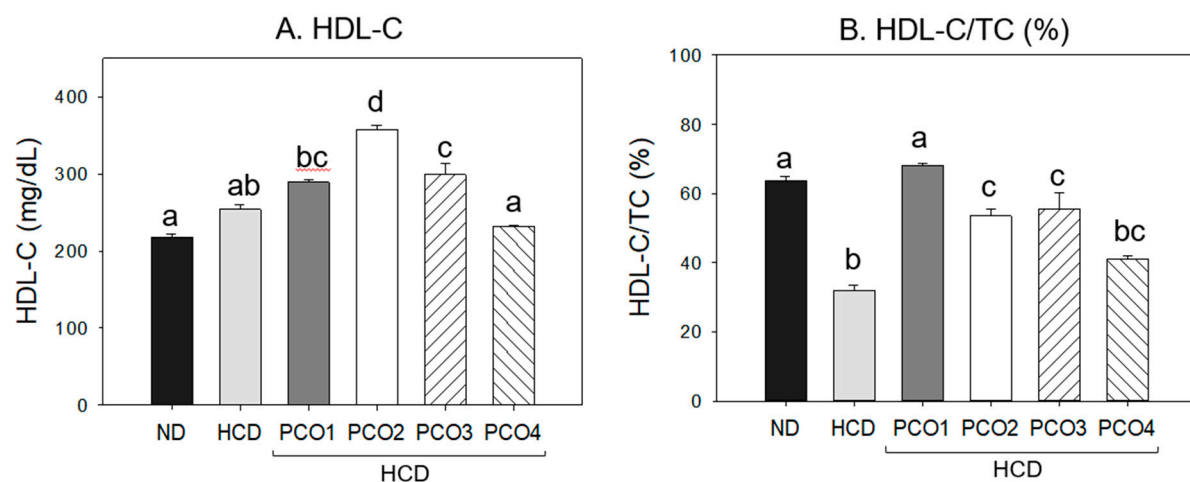
2.2. Change in Blood Lipid Levels

After 12 weeks, the HCD control group showed the highest total cholesterol (TC, Figure 1A) and triglyceride (TG, Figure 1B), 794±18 mg/dL and 889±46 mg/dL, respectively, while the ND group showed 341±12 mg/dL and 244±10 mg/dL, respectively, suggesting that 4% cholesterol supplementation induced a 2.3-fold and 3.6-fold increase in blood TC and TG levels. On the other hand, the PCO1 group showed the lowest TC (425±7 mg/dL) and TG (518±15 mg/dL) levels up to 47% and 32% lower than the HCD alone group, respectively, while other policosanols groups showed up to 32% lower TC (PCO3 group) and 30% lower TG levels (PCO2 group). The Chinese policosanols groups showed weaker blood lipid lowering profiles in TC and TG than in the Cuban policosanols (PCO1) group.



**Figure 1.** Total cholesterol (TC) and triglyceride (TG) contents in the blood after 12 weeks of supplementation of each policosanol (PCO) under high-cholesterol diet consumption. HCD, high cholesterol diet; ND, normal diet; PCO1, Raydel policosanol; PCO2, Xi'an Natural policosanol; PCO3, Xi'an Realin policosanol; PCO4, Shaanxi policosanol. The different letters on each bar indicate the significant differences between the groups ( $p<0.05$ ). .

The HCD group and ND group showed similar HDL-C levels of  $217\pm4$  mg/dL and  $253\pm6$  mg/dL, respectively (Figure 2A). On the other hand, the HCD group showed 50% lower HDL-C/TC (%) than the ND group (Figure 2B), because the ND group and HCD group showed 64% and 32% of HDL-C in the TC, respectively. Although the PCO1 group showed the 2<sup>nd</sup> highest serum HDL-C level ( $299\pm6$  mg/dL, Figure 2A), it showed the highest percentage of HDL-C/TC of approximately 68% (Figure 2B). The PCO2 and PCO3 groups showed an adequate increase ratio of HDL-C/TC (%) of approximately 53% and 55%, respectively, while the PCO4 group showed the lowest HDL-C/TC (%) of 41%.

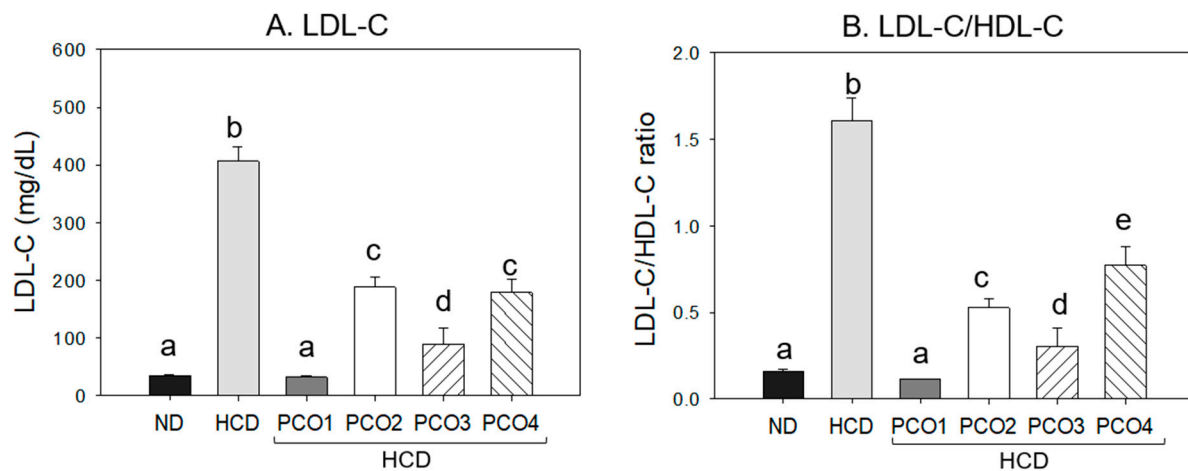


**Figure 2.** High-density lipoproteins cholesterol (HDL-C) and HDL-C in the total cholesterol (HDL-C/TC, %) contents in the blood after 12 weeks supplementation of each policosanol (PCO) under high-cholesterol diet consumption. HCD, high cholesterol diet; ND, normal diet; PCO1, Raydel policosanol; PCO2, Xi'an Natural policosanol; PCO3, Xi'an Realin policosanol; PCO4, Shaanxi policosanol. The different letters on each bar indicate significant differences between the groups ( $p<0.05$ ).

As shown in Figure 3A, the LDL-C level was elevated remarkably in the HCD group at approximately  $406\pm24$  mg/dL, while the ND group showed  $34\pm3$  mg/dL, suggesting that 4%

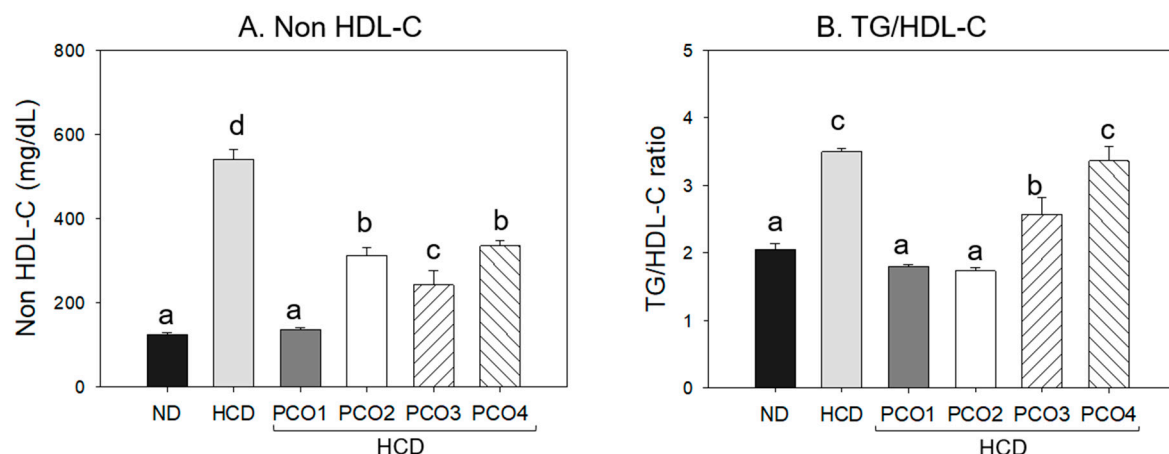


cholesterol supplementation elevated the LDL-C level. Interestingly, the PCO1 group showed a remarkable decrease in LDL-C of approximately  $32 \pm 2$  mg/dL. This level is similar to the ND group, which showed the lowest level among the HCD-consumed groups. The other policosanols groups showed adequate activity to lower the LDL-C levels to around 90–187 mg/dL. The LDL-C:HDL-C (L:H) ratio was elevated in the HCD control group by approximately 1.6, while the ND group showed approximately 0.2 (Figure 3B), suggesting that HDL-C was the dominant lipid profile in zebrafish. On the other hand, the cholesterol supplementation caused an eight-fold increase in the L:H ratio, which was the LDL-C dominant lipid profile in zebrafish similar to the human lipid profile. After 12 weeks of consumption, the PCO1 group showed the lowest L:H of approximately 0.1, while the PCO2, PCO3, and PCO4 groups showed around 0.5, 0.3, and 0.8, respectively (Figure 3B).



**Figure 3.** Low-density lipoproteins cholesterol (LDL-C) contents and LDL-C/HDL-C ratio in blood after 12 weeks supplementation of each policosanol (PCO) under high cholesterol diet consumption. HCD, high cholesterol diet; ND, normal diet; PCO1, Raydel policosanol; PCO2, Xi'an Natural policosanol; PCO3, Xi'an Realin policosanol; PCO4, Shaanxi policosanol. The different letters on each bar indicate a significant differences between the groups ( $p < 0.05$ ).

A calculation of the total amount of non-HDL-C showed that the HCD control group had a 4.4-fold higher level than the ND control group, suggesting that the cholesterol supplementation caused the elevation of non-HDL-C (Figure 4A). Interestingly, the PCO1 group showed the lowest level of non-HDL-C around  $136 \pm 5$  mg/dL. In contrast, the PCO4 group showed the highest non-HDL-C of approximately  $335 \pm 13$  mg/dL. The PCO2 and PCO3 group showed a higher non-HDL-C level than the PCO1 group, around  $312 \pm 18$  mg/dL and  $242 \pm 34$  mg/dL, respectively. These results suggest that only the Cuban policosanol group showed the most remarkable reduction of non-HDL-C (~75% lower than the HCD group). The other Chinese policosanol groups showed an adequate reduction of HDL-C, approximately 38–56% lower than that of the HCD control.

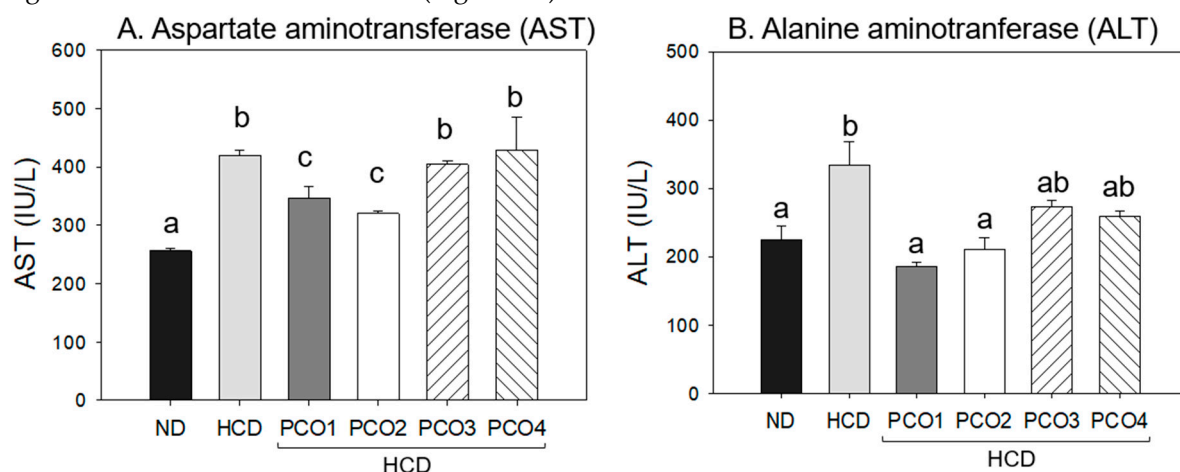


**Figure 4.** Change in the non-HDL-C and the triglyceride (TG)/HDL-C ratio in the blood after 12 weeks of supplementation of each policosanols (PCO) under high cholesterol diet consumption. The different letters on each bar indicate a significant differences between the groups ( $p < 0.05$ ). (A) Non-high-density lipoproteins cholesterol (non-HDL-C). (B) TG/HDL-C ratio. HCD, high cholesterol diet; ND, normal diet; PCO1, Raydel policosanols; PCO2, Xi'an Natural policosanols; PCO3, Xi'an Realin policosanols; PCO4, Shaanxi policosanols.

Calculations of the TG/HDL-C level showed that the PCO4 policosanols group showed the highest ratio of TG/HDL-C, around 3.4. In contrast, the HCD control group showed a similar level of approximately 3.5 (Figure 4B). The PCO1 and PCO2 groups showed the lowest TG/HDL-C level (~1.7–1.8), whereas the ND control group showed a TG/HDL-C ratio of approximately 2.1. The PCO3 group showed an approximately 2.6 lower TG/HDL-C ratio than the HCD control group, but the PCO4 group showed a similar level to the HCD control (~3.4). These results suggest that Cuban policosanols (Raydel®) consumption resulted in the highest HDL-C content in TC with the lowest L:H ratio and TG:HDL-C ratio. In contrast, the other Chinese policosanols showed weaker effects in increasing the HDL-C and HDL-C/TC (%).

### 2.3. Change of Hepatic Function Parameters

After 12 weeks consumption, the HCD control group showed 1.6-fold ( $p = 0.024$ ) and 1.5-fold ( $p = 0.044$ ) higher blood AST and ALT levels than those of the ND control group (Figure 5), suggesting that cholesterol (final 4%) supplementation-associated damage occurred in the liver and muscle. In the AST level, the three sugar cane policosanols groups (PCO1, PCO2, and PCO3) showed similar levels, which were lower than the rice bran policosanols group. The PCO1 and PCO2 policosanols groups showed 18% and 24% lower AST levels than the HCD control, and the PCO4 group showed higher levels than the HCD control (Figure 5A).



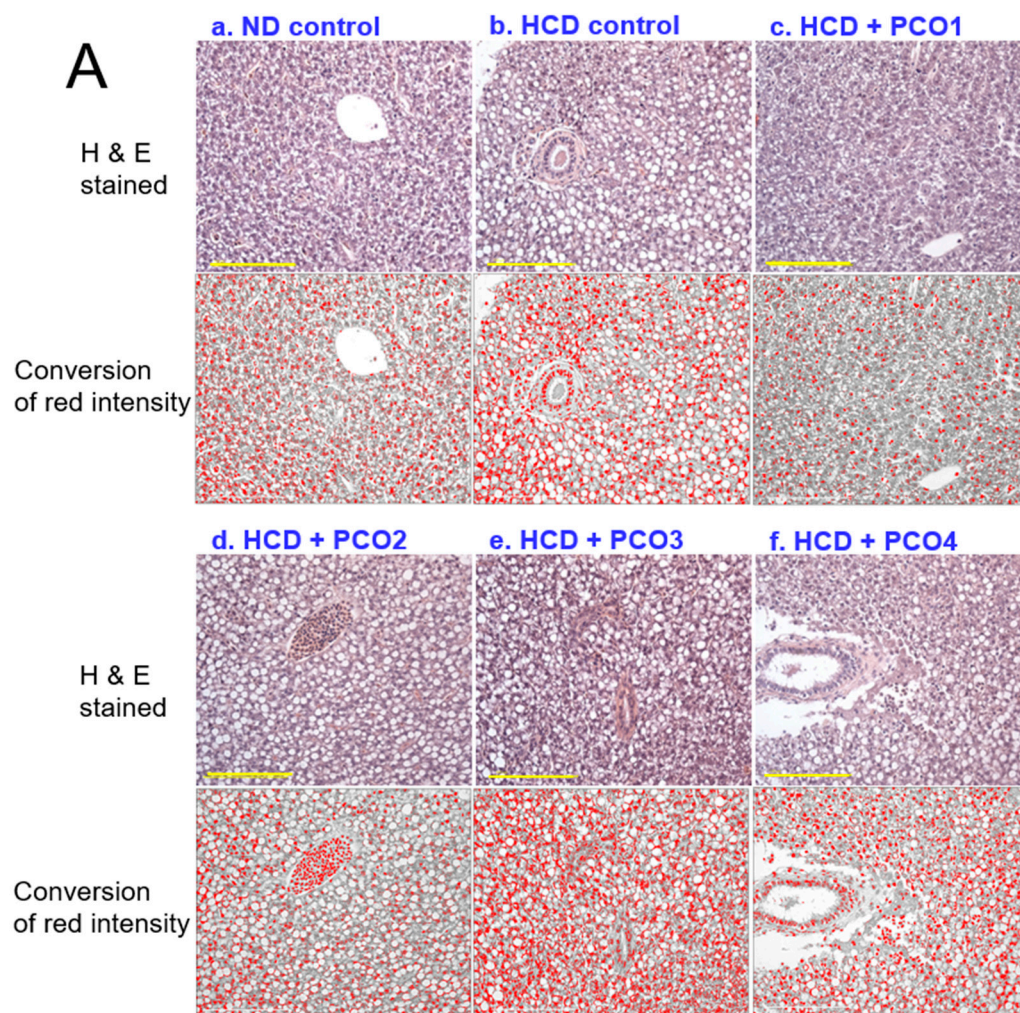
**Figure 5.** Comparison of the blood hepatic damage parameters of aspartate aminotransferase (AST) and alanine aminotransferase (AST) after 12 weeks supplementation of each policosanols under high-cholesterol diet consumption. HCD, high cholesterol diet; ND, normal diet; PCO1, Raydel policosanols; PCO2, Xi'an Natural policosanols; PCO3, Xi'an Realin policosanols; PCO4, Shaanxi policosanols. The different letters on each bar indicate a significant difference between the groups ( $p < 0.05$ ).

Although the HCD control group showed the highest blood ALT level, the PCO1 group showed the lowest level: 45% lower ( $p = 0.010$ ) than the HCD control (Figure 5B). The PCO2, PCO3, and PCO4 groups showed 27% ( $p = 0.026$ ), 18%, and 23% lower levels than the HCD control, respectively. Although all policosanols groups showed lower ALT levels than the HCD control group, the extent of the serum ALT levels differed according to the origins, suggesting that the hepatic protection activity of policosanols may vary depending on the composition of long-chain aliphatic alcohols. Cuban policosanols showed remarkably higher protection activity against hepatic damage from high

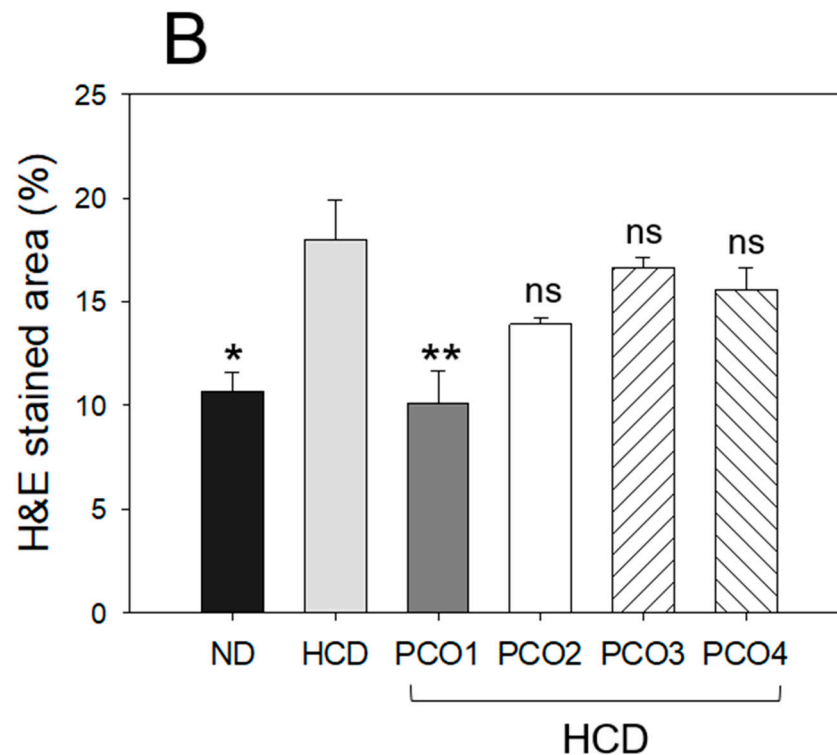
cholesterol consumption, whereas Chinese policosanols, particularly Xi'an Realin and Shaanxi, did not show the protection activity.

#### 2.4. Histopathological Analysis with Liver

Histological analysis with H&E staining showed that the HCD control group (photograph b) exhibited the strongest H&E stained area and red intensity (Figure 6A), 1.7-fold higher ( $p=0.008$ ) than the ND control group (photograph a), indicating the highest number of infiltrated neutrophils in the HCD control group. In contrast, the PCO1 group (photograph c) showed the lowest number of neutrophils, ~48% lower than the HCD control group ( $p=0.005$ ), as shown in Figure 6B. The other policosanols groups showed a similar H&E-stained pattern: approximately 23% ( $p=0.018$ ), 8% ( $p=0.022$ ), and 14% ( $p=0.034$ ) lower than the HCD control group, respectively. The PCO1 group showed the smallest H&E-stained area ( $\sim 10.2 \pm 1.5\%$ ), whereas the PCO2, PCO3, and PCO4 groups showed a  $13.9 \pm 0.3\%$ ,  $16.6 \pm 0.5\%$ , and  $15.9 \pm 1.0\%$  stained area, respectively (Figure 6B). The larger H & E stained area in the PCO3 and PCO4 group correlated well with the higher AST and ALT level than the PCO1 group.



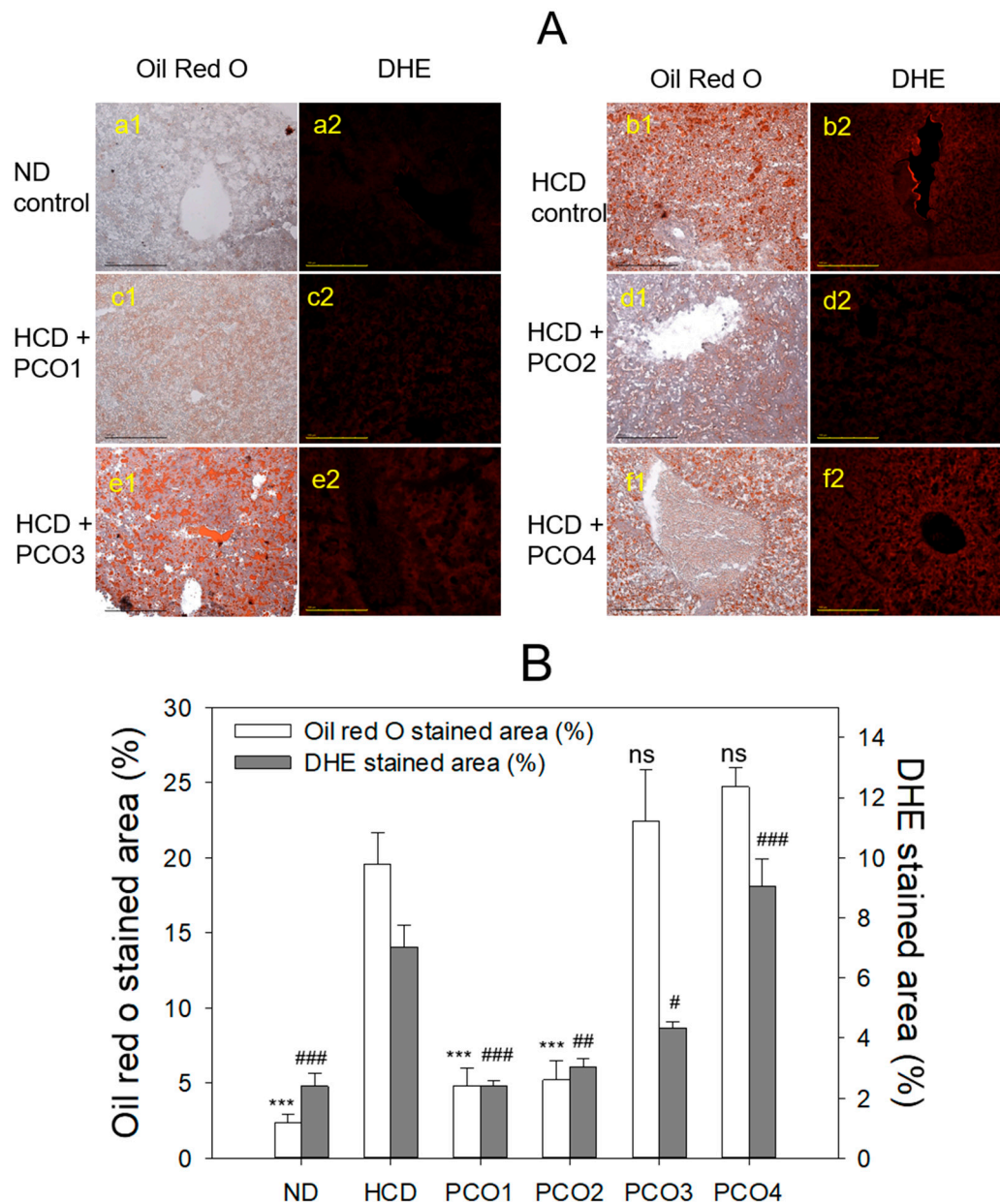




**Figure 6.** Histology analysis of the hepatic tissue from zebrafish after 12 weeks of supplementation of each policosanol (PCO) under high cholesterol diet consumption. HCD, high cholesterol diet; ND, normal diet; PCO1, Raydel policosanol; PCO2, Xi'an Natural policosanol; PCO3, Xi'an Realin policosanol; PCO4, Shaanxi policosanol. (A) Photographs showing the infiltration of neutrophils by Hematoxylin & Eosin (H&E) staining after converting the Hematoxylin stained area into a red intensity. The yellow scale bar indicates 100  $\mu$ m. (B) Quantification of the nucleus area from the H&E staining using Image J software (<http://rsb.info.nih.gov/ij/> accessed on 16 May 2023). The statistical significance of the groups was indicated as *p* values at the top of the graph. \*, *p* < 0.05 versus HCD control; \*\*, *p* < 0.01 versus HCD control; ns, not significant.

Oil red O staining with hepatic section showed that the HCD control group (photo b1) had an 8.1-fold higher red intensity and area than the ND group (photo a1), as shown in Figure 7A, suggesting that severe fatty liver change occurred by the cholesterol supplementation. Under HCD consumption, the PCO1 group (photo c1) and PCO2 (photo d1) had the lowest oil red O-stained area, up to 75% and 73% lower, respectively, than the HCD control group (Figure 7B). On the other hand, the PCO3 group (photo e1) and PCO4 group (photo f1) showed 14% and 26% higher stained area than the HCD control group.

DHE staining revealed the HCD control group (photo b2) to have a 3.0-fold stronger red intensity than the ND group (photo a2), suggesting that cholesterol consumption was associated with ROS production. Among the HCD groups, the PCO1 group (photo c2), PCO2 (photo d2), and PCO3 (photo e2) groups showed a 68%, 58%, and 39% smaller DHE stained area, respectively, than the HCD control group (Figure 7B). On the other hand, the PCO4 group (photo f1) showed the highest stained area, approximately 28% higher than the HCD control group, indicating the most severe fatty liver change and ROS production.

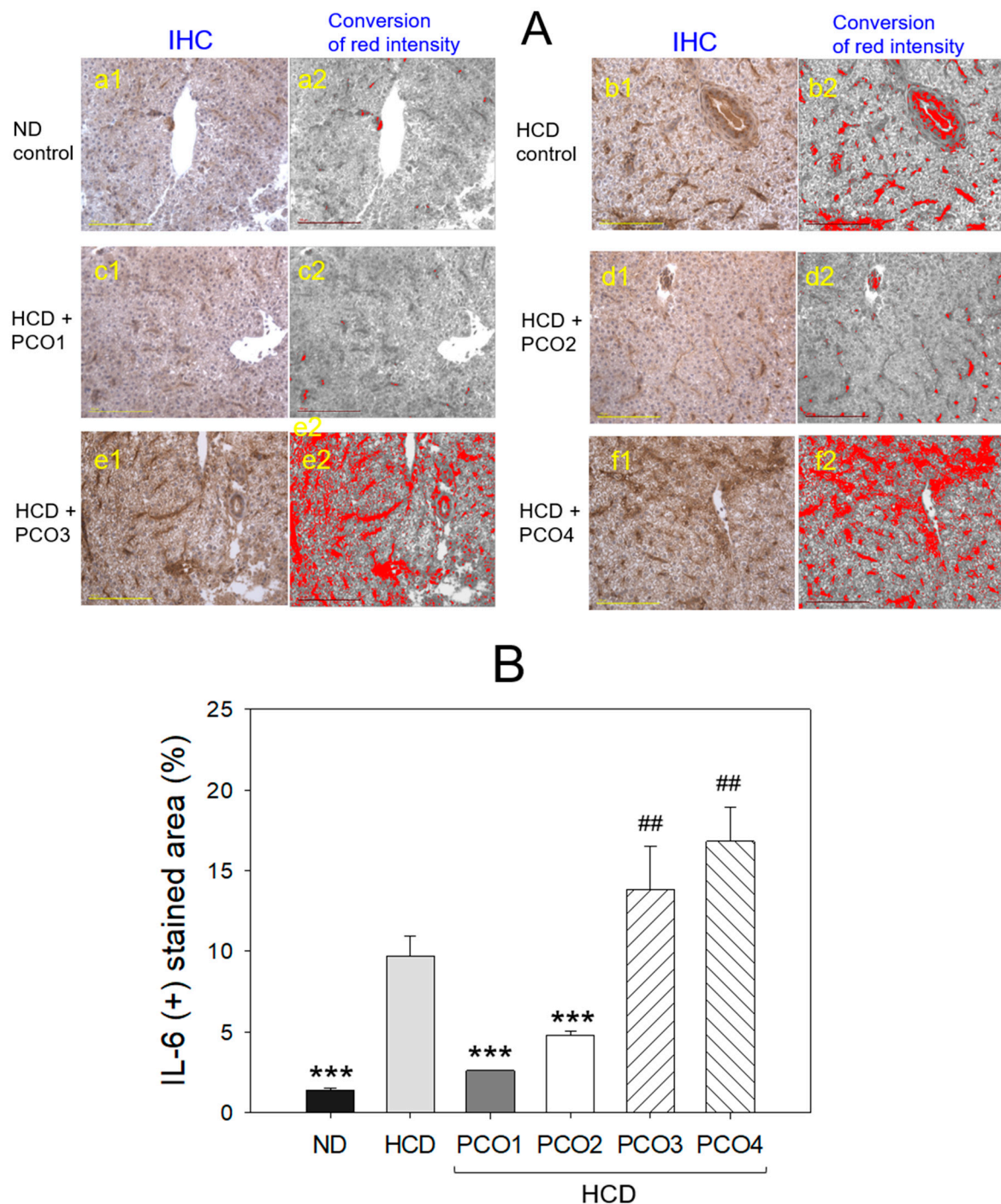


**Figure 7.** Comparisons of the fatty liver changes and reactive oxygen species (ROS) by Oil red O staining and dihydroethidium (DHE) staining, respectively, in zebrafish after 12 weeks supplementation of each policosanols (PCO) under high cholesterol diet consumption. HCD, high cholesterol diet; ND, normal diet; PCO1, Raydel policosanols; PCO2, Xi'an Natural policosanols; PCO3, Xi'an Realin policosanols; PCO4, Shaanxi policosanols. (A) Representative image of oil red O stained and DHE stained hepatic tissue at 180 min post-injection. The scale bar indicates 100  $\mu$ m. (B) Quantification of the oil red O intensity and DHE fluorescence (Ex=585 nm, Em=615 nm) intensity using Image J software (<http://rsb.info.nih.gov/ij/> accessed on 16 May 2023). \*\*\*,  $p < 0.001$  versus HCD from the DHE-stained area; #,  $p < 0.05$  versus HCD from the AO-stained area; ##,  $p < 0.01$  versus HCD from the AO-stained area; ###,  $p < 0.001$  versus HCD from the AO-stained area; ns, not significant.

### 2.5. Immunohistochemical Analysis with Liver Tissue

As shown in Figure 8A, immunohistochemistry detection of IL-6 with hepatic tissue revealed the HCD control group to have a larger stained area (photo b, around  $9.7 \pm 1.2\%$ ), which was 6.8 times higher ( $p = 0.008$ ) than that of the ND control group (photo a). Hence, these results indicate that cholesterol supplementation for 12 weeks caused severe IL-6 accumulation in hepatic tissue. The PCO1 group (photo c) showed the least IL-6 stained area around  $2.6 \pm 0.1\%$ , which was 74% ( $p = 0.002$ )

of that of the HCD control, whereas the PCO2 group (photo d) showed the 2<sup>nd</sup> lowest IL-6 stained area, approximately  $4.8 \pm 0.3\%$ , 50% lower ( $p=0.008$ ) than that of the HCD control. In contrast, the PCO3 and PCO4 groups showed the largest IL-6 stained area of  $13.8 \pm 3.1\%$  and  $16.8 \pm 2.9\%$ , respectively, which was 1.4-fold ( $p<0.001$ ) and 1.7-fold higher ( $p<0.001$ ) than the HCD control (Figure 8B). These results suggest that the anti-inflammatory activity of policosanols might vary according to the sources and country of origin: PCO1 and PCO2 policosanols showed potent anti-inflammatory activity to inhibit IL-6 production, whereas PCO3 and PCO4 did not (Figure 8B). These differences in the IL-6 stained area showed good agreement with the different serum AST/ALT levels, fatty liver changes, and ROS production in the liver.



**Figure 8.** Comparison of the interleukin (IL)-6 stained area of immunohistochemistry (IHC) with hepatic tissue from zebrafish after 12 weeks supplementation of each policosanol (PCO) under high cholesterol diet consumption. HCD, high cholesterol diet; ND, normal diet; PCO1, Raydel

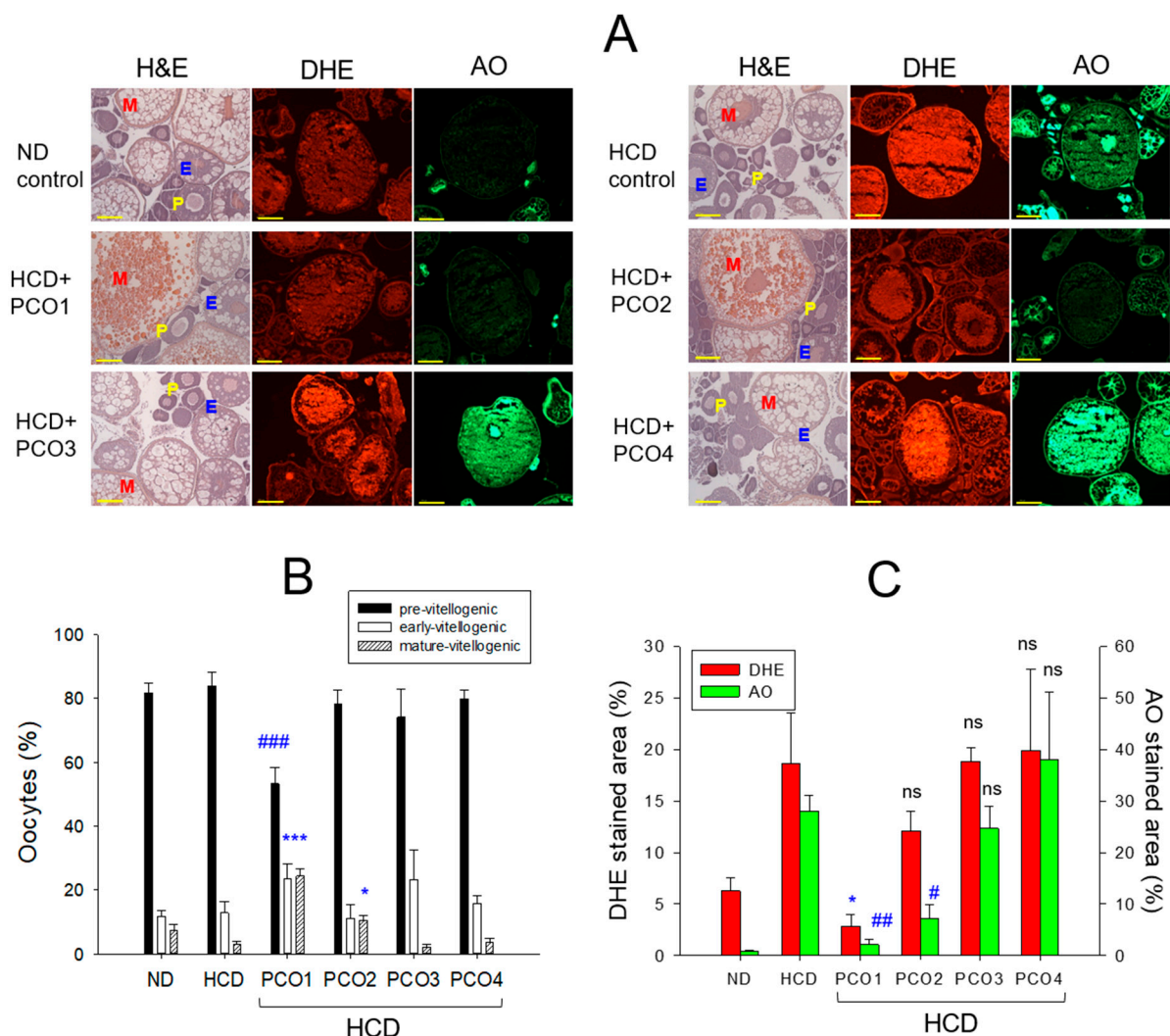


policosanols; PCO2, Xi'an Natural policosanols; PCO3, Xi'an Realin policosanols; PCO4, Shaanxi policosanols. (A) Representative image of IL-6 stained hepatic tissue at 180 min post-injection. The scale bar indicates 100  $\mu$ m. (B) Quantification of the oil red O intensity and DHE fluorescence (Ex=585 nm, Em=615 nm) intensity using Image J software (<http://rsb.info.nih.gov/ij/> accessed on 16 May 2023). \*\*\*,  $p < 0.001$  versus HCD; ##,  $p < 0.01$  versus HCD

## 2.6. Histological Analysis with Ovary

H&E staining (Figure 9A,B) showed that the HCD group had the highest previtellogenic oocyte content (~83%) and the lowest content of early and mature vitellogenic oocytes (~13% and 3%, respectively), with a smaller size than the ND group. On the other hand, the PCO1 group showed the highest ratio of early and mature vitellogenic oocytes, approximately 47%, with the lowest ratio of premature oocytes (~53%), whereas the three Chinese policosanols showed a much larger ratio of premature oocytes (~74–80%) with a lower content of early and mature vitellogenic oocyte (~20–26%).

DHE staining showed that the HCD group had a 2.9-fold stronger red intensity than the ND group, indicating that higher ROS production in oocytes was associated with HCD consumption (Figure 9A,C). The PCO1 group showed the weakest red intensity, 85% lower than the HCD group. Although the PCO2 group showed a 33% lower DHE-stained area than the HCD control group, the PCO3 and PCO4 groups showed similar and 7% higher DHE-stained areas, respectively, than the HCD control group (Figure 9C).



**Figure 9.** Comparisons of the ovarian cell morphology according to Hematoxylin and Eosin (H & E) staining, extent of reactive oxygen species (ROS) by dihydroethidium (DHE) staining, and extent of apoptosis by acridine orange (AO) staining in zebrafish after 12 weeks supplementation of each

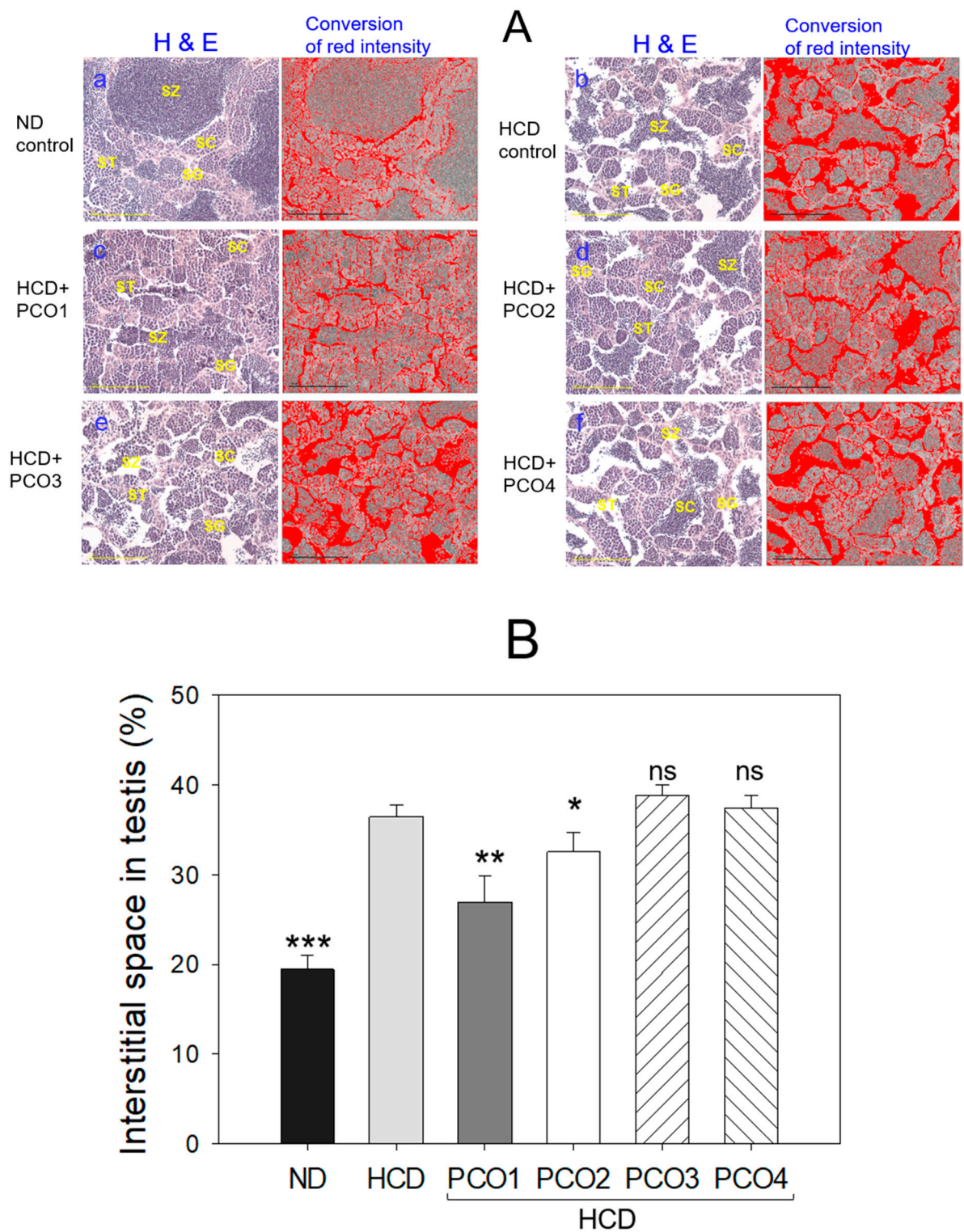


policosanol (PCO) under high cholesterol diet consumption. HCD, high cholesterol diet; ND, normal diet; PCO1, Raydel policosanol; PCO2, Xi'an Natural policosanol; PCO3, Xi'an Realin policosanol; PCO4, Shaanxi policosanol. (A) Representative image of H & E stained, DHE stained, and AO stained image of ovarian cells. The scale bar indicates 100  $\mu$ m. E, early vitellogenic oocytes; M, mature vitellogenic oocytes; P, pre-vitellogenic oocytes. (B) Percentage distribution of the stages in oocytes depends on the developmental stage. \*\*\*,  $p < 0.001$  versus HCD in early and mature vitellogenic; ###,  $p < 0.01$  versus HCD in pre-vitellogenic; \*,  $p < 0.05$  versus HCD in mature-vitellogenic. (C) Quantification of the DHE fluorescence (Ex=585 nm, Em=615 nm) intensity and acridine orange fluorescence in oocytes (Ex=505 nm, Em=535 nm) using Image J software (<http://rsb.info.nih.gov/ij/> accessed on 16 May 2023). \*,  $p < 0.05$  versus HCD from the DHE-stained area; ##,  $p < 0.01$  versus HCD from AO-stained area; #,  $p < 0.05$  versus HCD from the AO-stained area; ns, not significant.

AO staining revealed the HCD group to have a 23-fold higher green intensity than the ND group (Figure 9A,C), suggesting that high cholesterol consumption is also linked with increased cellular apoptosis. The PCO1 and PCO2 groups showed 93% and 75% lower green intensity, respectively, than the HCD control group. In contrast, the PCO3 and PCO4 groups showed a similar or 35% higher AO-stained area, respectively, than the HCD control group, suggesting that the greatest extent of cellular apoptosis in oocytes occurred in the PCO4 group. These results suggest that PCO1 consumption resulted in the highest content of mature oocytes with the least extent of cellular apoptosis and ROS production in ovarian cells.

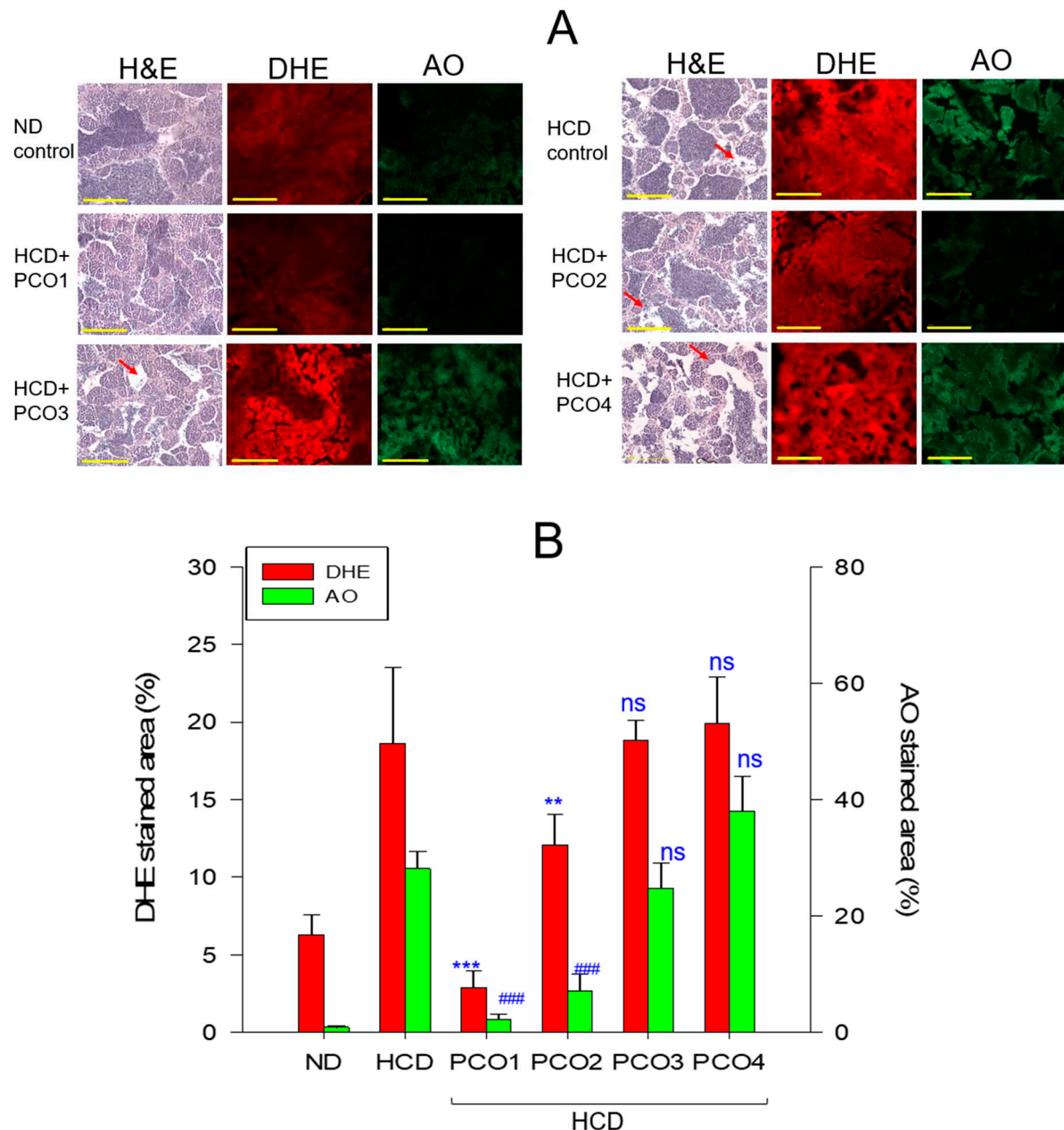
### 2.9. Histological Analysis with Testis

H&E staining (Figure 10A) analysis with a testis section showed that the ND group (photo a) had healthy seminiferous tubules with a full cell population adherent to the basal membranes without any notable gaps between the membranes and interstitium, approximately 19% of interstitial area (Figure 10B). On the other hand, the HCD control group displayed irregularly outlined seminiferous tubules with disarranged cellular layers as well as a broken lamina basal membrane with the largest interstitial area of approximately 36%, indicating impaired ability of spermatogenesis. The morphometric results of spermatogenesis in the HCD group (photo b) showed that spermatids and sperm (mature forms) were smaller than the ND group (photo a). In contrast, the PCO1 group showed the largest increase in the area of spermatids, as shown in Figure 10A (photo c), with the smallest interstitial area among HCD groups. The average interstitial areas of the PCO1 and PCO2 groups were approximately 26% and 32% smaller than the HCD control group, respectively ( $p < 0.001$ ). In contrast, the PCO3 and PCO4 groups showed 39% and 37% of the average interstitial areas, respectively, which was higher than that of the HCD control group (Figure 10B).



**Figure 10.** Comparison of the testicular histology in zebrafish after 12 weeks supplementation of each policosanols (PCO) under high cholesterol diet consumption. HCD, high cholesterol diet; ND, normal diet; PCO1, Raydel policosanols; PCO2, Xi'an Natural policosanols; PCO3, Xi'an Realin policosanols; PCO4, Shaanxi policosanols. (A) Representative image of a H & E stained testicular section and red conversion image to show empty intra/inter spaces in seminiferous tubules. The red area indicates empty space. The scale bar indicates 100  $\mu$ m. SC, spermatocytes; SG, spermatogonia; ST, spermatid; SZ, spermatozoa. (B) Quantification of the red area to compare the interstitial space in the testis using Image J software (<http://rsb.info.nih.gov/ij/> accessed on 16 May 2023). \*,  $p < 0.05$  versus HCD; \*\*,  $p < 0.01$  versus HCD; \*\*\*,  $p < 0.001$  versus HCD; ns, not significant.

DHE staining showed that the HCD control group had 19% red-stained area, indicating three times higher ROS production than the ND control group (~6% red-stained area) (Figure 11A). The PCO1 group showed the lowest ROS production (~3% red-stained area), whereas the PCO2 policosan group showed the 2<sup>nd</sup> lowest ROS production (~12% red-stained area) (Figure 11B). On the other hand, the PCO3 and PCO4 groups showed similarly higher ROS production than the HCD group (~18% and 20% red stained area, respectively).



**Figure 11.** Comparison of the testicular cell morphology by Hematoxylin and Eosin (H & E) staining, the extent of reactive oxygen species (ROS) by dihydroethidium (DHE) staining, and the extent of apoptosis by acridine orange (AO) staining in zebrafish after 12 weeks supplementation of each policosan (PCO) under a high cholesterol diet. HCD, high cholesterol diet; ND, normal diet; PCO1, Raydel policosan; PCO2, Xi'an Natural policosan; PCO3, Xi'an Realin policosan; PCO4, Shaanxi policosan. (A) Representative image of H & E stained, DHE stained, and AO stained image of a testicular cell. The red arrow indicates increased interstitial area. The scale bar indicates 100  $\mu$ m. (B) Quantification of the DHE fluorescence intensity (Ex=585 nm, Em=615 nm) and AO fluorescence intensity (Ex=505 nm, Em=535 nm) using Image J software (<http://rsb.info.nih.gov/ij/> accessed on 16



May 2023). \*\*,  $p < 0.01$  versus HCD from DHE-stained area; \*\*\*,  $p < 0.001$  versus HCD from DHE-stained area; ###,  $p < 0.001$  versus HCD from the AO-stained area; ns, not significant.

AO staining showed that the HCD group had 33-fold higher green intensity than the ND group (Figure 11), suggesting that high cholesterol consumption caused a remarkable increase in cellular apoptosis in the testis. The PCO1 and PCO2 policosanols groups showed 93% and 75% lower green intensity, respectively, than the HCD control group. In contrast, the PCO3 and PCO4 groups showed 15% lower or 35% higher AO stained area, respectively, than the HCD control group, suggesting that the highest extent of cellular apoptosis in the testis occurred in the PCO4 group. These results suggest that the PCO1 group had the highest protective activity with the least extent of cellular apoptosis and ROS production in spermatogenesis.

Spermatogenesis and testicular abnormalities were examined to determine the effects of the HCD consumption on male fertility, as shown in Figures 10 and 11. The histological examination of the testes from zebrafish fed an ND revealed a normal morphology of seminiferous tubules with a full cell population adherent to the basal membrane with a few gaps between them and the interstitium (photo a, Figure 10A). The testis section of the ND group showed well-developed spermatogonia (SG), spermatocytes (SC), spermatids (ST), and spermatozoa (SZ) with a normal size and distributions. On the other hand, however, zebrafish fed HCD (Photo b, Figure 10A) displayed irregularly outlined seminiferous tubules with disarranged cellular layers and a broken lamina basal membrane, indicating reduced spermatogenesis ability. The morphometric spermatogenesis results of zebrafish fed a HCD showed that the spermatids, spermatozoa, and spermatocytes were smaller in area than those of the ND group. On the other hand, co-supplementation of PCO1 resulted in a remarkably improved morphology (photo c, Figure 10A) with a smaller interstitial area up to 27% lower than the HCD control (Figure 10B). The other Chinese policosanols did not show notable improvement in testicular cell morphology (photo d, e, f of Figure 10A); PCO2 group showed an 11% smaller interstitial area, whereas the PCO3 and PCO4 group showed 6% and 2% larger interstitial area, respectively. The average size of the seminiferous tubules decreased significantly.

The degeneration of the seminiferous tubules with empty spaces was observed in the inter- and intra-seminiferous tubules (red arrows, Figure 11A). Areas of these empty spaces increased significantly in the three Chinese policosanols groups as indicated by red arrowhead. Tissue tearing occurred during H&E staining in the cryosection slides for tissues treated with HCD because of the fragile testicular interstitium. Interestingly, the interstitial space of the PCO3 and PCO4 group with HCD was larger than that of the HCD-alone group (Figure 10B). These significant impairments of the ovary and testis in the PCO3 and PCO4 groups correlated well with the higher inflammation and fatty liver change in the liver (Figures 6–8), elevation of AST and ALT (Figure 5), and lower HDL-C and HDL-C/TC (%) (Figure 2).

### 3. Discussion

The current study was designed to compare the *in vivo* efficacy of various policosanols on the blood lipid-lowering effect, anti-inflammatory activity, and enhancement of reproduction system, such as ovarian and testicular tissue in hyperlipidemic zebrafish. The current results showed that 12 weeks of Cuban policosanols (Raydel®) consumption resulted in the highest survivability (Table 1) and potent efficacy to lower the blood TC, LDL-C, and TG with higher HDL-C/TC (%) under HCD supplementation (Figures 1–4). In the liver function, the Cuban policosanols group (PCO1) showed the lowest blood AST and ALT level (Figure 5) with the least infiltration of neutrophils in hepatocytes (Figure 6). Histology analysis of the liver revealed the PCO1 group to have the least fatty liver change and ROS production (Figure 7) with the smallest IL-6 production (Figure 8). In ovarian tissue, the PCO1 group showed the highest percentage of mature vitellogenic stage oocytes with the least ROS production and cellular apoptosis (Figure 9). In the testis tissue, PCO1 group showed the least damage to the spermatogenesis cell morphology with the smallest interstitial area (Figure 10), ROS production, and apoptosis (Figure 11).



The current results showed that hyperlipidemia, which was induced by a high cholesterol diet (final 4%), was associated with damage to the hepatic function, and the elevation of ROS production and inflammation in the liver (Figures 5–8), and impairment of male and female reproduction system, such as the testis and ovary (Figures 9–11). The HCD control group showed the highest serum AST and ALT levels with severe fatty liver changes and ROS production after 8 weeks of consumption, which was also reported in a previous study [34]. Interestingly, the HCD control group showed a remarkable increase in IL-6 production in the liver, a severe decrease in mature vitellogenic oocytes, and severe enlargement of the interstitial area in the testis. These results suggest that dyslipidemia is associated with hepatic inflammation and infertility, which is in good agreement with previous reports showing that high cholesterol-consuming mice for 12 weeks showed impairment of sperm maturation and capacitation [37].

In contrast, the co-supplementation of Cuban policosanols in the HCD (final 0.1%, wt./wt.) improved the lipid profile and liver functions. These results concur with previous clinical studies of healthy Japanese participants [38,39]. Twelve weeks of Cuban policosanols (Raydel®) consumption offered some protection of the liver functions via lowered the AST, ALT,  $\gamma$ -GTP, and alkaline phosphatase (ALP) levels. In addition, the serum antioxidant abilities were elevated by policosanols consumption at week 12; 37% increase in ferric ion reduction ability (FRA) and 29% increase in paraoxonase (PON) activity [38]. Interestingly, the purified HDL<sub>2</sub> and HDL<sub>3</sub> from the policosanols group also showed enhanced FRA and PON ability, approximately 1.3–1.6-fold higher than the placebo group [39]. Similarly, 12 weeks of Cuban policosanols consumption (5 mg and 10 mg per day) by hypercholesterolemic patients resulted in a significant decrease in ALT and  $\gamma$ -GTP, and a decrease in TC, LDL-C, and an increase in HDL-C [40]. Furthermore, the *in vitro* comparison of antioxidant ability showed that Cuban policosanols had the highest potent inhibition ability of LDL oxidation in the presence of cupric ions [30]. Under the *in vitro* treatment of fructose, Cuban policosanols showed the highest inhibition activity of the extent of glycation with prevention of apoA-I degradation [30]. Furthermore, the clinical study with healthy Japanese participants showed that the policosanols group had 4% lower glycated hemoglobin (HbA<sub>1c</sub>) levels than at week 0 and the placebo group at week 12 [38,39]. These enhanced serum antioxidant abilities and hepatic function parameters might help improve the hepatic function and reproductive organs, testis, and ovary.

It has been well established that oxidative stress promotes inflammation in the ovaries and causes ovarian aging and infertility [41]. The consumption of a high-cholesterol diet for 21 weeks in zebrafish caused a remarkable increase in AST and ALT with an impairment of the testicular morphology; the HCD group showed 1.7-fold greater enlargement of the interstitial area than the ND group [42]. To the best of the authors' knowledge, the current study is the first to show that Cuban policosanols (Raydel®) consumption could ameliorate damage to the liver and reproduction system, ovary and testis, under high cholesterol consumption. The alleviation of hepatic inflammation and ROS production is based on the increase in HDL-C and the enhancement of HDL functionality, as shown in previous reports with hyperlipidemic zebrafish [34] and spontaneously hypertensive rats (SHR) [43]. In hyperlipidemic zebrafish, eight weeks of policosanols consumption ameliorated the elevated infiltration of neutrophils, ROS production, and fatty liver changes with a significant reduction of AST and ALT [34]. In the SHR, elevated oxidized species in hepatic tissue and C-reactive protein in the blood were reduced by eight weeks of policosanols consumption in a dosage-dependent manner [43].

A previous study reported that policosanols did not impair the male reproduction system without genetic defects from long-term supplementation (5, 50, and 500 mg/kg of body weight/day) throughout three successive generations [44]. In the female reproduction ability, there were no teratogenic effects in the rats and rabbits given 500–1000 mg/kg of body weight/day two weeks before mating and throughout mating and pregnancy to day 21 of lactation [45]. Although no reports describe the improvement in the reproduction ability by policosanols consumption, these results showed that treatment of dyslipidemia with Cuban policosanols is associated with an improved ovarian cell and testicular cell morphology and functions.

Increased oxidative stress in hyperlipidemia has been associated with fatty liver change and pro-inflammatory response in hepatic tissue [46]. On the other hand, the current results showed that hyperlipidemia and fatty liver change are also linked with severe impairments of the reproduction system (Figures 9–11). HCD supplementation caused a decrease in the mature vitellogenic stage of approximately 2.7%, while the ND group showed 6.5%, indicating that elevated oxidative stress by hyperlipidemia inhibited oocyte maturation, as reported elsewhere [47,48]. In the current study, however, co-supplementation of Cuban policosanols resulted in the largest increase in early-vitellogenic and mature-vitellogenic oocytes, indicating enhanced oocyte maturation and folliculogenesis.

In conclusion, 12 weeks of Cuban policosanols consumption under HCD resulted in remarkable improvements in the blood lipid profile, amelioration of liver inflammation, and enhanced cell morphology in the ovary and testis. These results showed how Cuban policosanols differs from Chinese policosanols regarding the *in vivo* efficacy in treating hyperlipidemia, hepatic inflammation, and impairments of the reproduction system.

## 4. Materials and Methods

### 4.1. Materials

Policosanols (PCO) 1, Raydel® sugar cane wax alcohol, was obtained from National Center for Scientific Research (CNIC), Habana, Cuba. PCO2 and PCO3 were acquired from sugar cane and supplied by Xi'an Natural Field Biotechnology Co, Ltd. (Xi'an, China) and Xi'an Realin Biotechnology Co, Ltd. (Xi'an, China), respectively. PCO4, from rice bran, was purchased from Shaanxi Pioneer Biotech (Xi'an, China). All raw materials of each policosanols were analyzed using the same gas chromatography (GC, HP-5890A GC, Agilent, Palo Alto, CA, USA) procedure with a GC-flame ionization detector and a Zebron ZB-5 column (30 m × 0.53 mm × 1.50 µm) from Phenomenex (Torrance, CA, USA) at the Korea Advanced Food Research Institute (Uiwang-si, Republic of Korea), as described elsewhere [30].

### 4.2. Zebrafish and Policosanols Supplementation

Zebrafish and embryos were maintained using the standard protocols [49] according to the *Guide for the Care and Use of Laboratory Animals* [50]. The procedures using zebrafish were approved by the Committee of Animal Care and the Use of Raydel Research Institute (approval code RRI-20-003, Daegu, Republic of Korea). The fish were maintained in a system cage at 28°C during treatment under a 14:10 hr. light cycle with the consumption of normal tetrabit (TetrabitGmbH D49304, 47.5% crude protein, 6.5% crude fat, 2.0% crude fiber, 10.5% crude ash, containing vitamin A [29770 IU/kg], vitamin D3 [1860 IU/kg], vitamin E [200 mg/kg], and vitamin C [137 mg/kg]; Melle, Germany).

Zebrafish around 10 weeks old were randomly divided into six groups, as shown in Table 1; each group had 70 zebrafish and were supplemented with one of each policosanols (final 0.1%, wt./wt.) with HCD (final 4% cholesterol, wt./wt.) in the zebrafish diet (Tetrabit, GmbH D49304, Melle, Germany) for 12 weeks. The groups were as follows: exposed to normal diet group (ND, control, n=70), exposed to 4% high-cholesterol diet group (HCD, n=70), PCO1 (exposed to Cuban policosanols, Raydel®, with HCD, n=70), PCO2 (exposed to Xi'an Natural policosanols with HCD, n=70), PCO3 (exposed to Xi'an Realin policosanols with HCD, n=70), and PCO4 (exposed to Shaanxi policosanols with HCD, n=70). Before feeding each policosanols, all groups were acclimated to HCD for four weeks except the ND control group. Subsequently, the zebrafish were supplemented each 0.01 mg of policosanols in 10 mg of Tetrabit, fed twice daily at 9 am and 6 pm with 20 mg of the designated diet, containing 0.02 mg of policosanols/day, per zebrafish.

### 4.3. Blood Collection and Analysis

After 12 weeks of supplementation, blood (2 L) was drawn from the hearts of the adult zebrafish, combined with 3 mL of phosphate-buffered saline (PBS)-ethylenediaminetetraacetic acid (EDTA, final concentration, 1 mM) and collected in EDTA-treated tubes as reported elsewhere [51].

The plasma of each group was collected after centrifugation (5,000 xg) for 15 min. The total cholesterol (TC) and triglyceride (TG) levels in the plasma were determined using a commercial assay kit (cholesterol, T-CHO, and TGs, Cleantech TS-S; Wako Pure Chemical, Osaka, Japan). HDL-C (AM-202), aspartate transaminase (AST), and alanine transaminase (ALT) were measured using a commercially available assay kit using AM-202, AM-103K, and AM102-K (Asan Pharmaceutical, Hwasung, Korea), correspondingly.

#### 4.4. Histopathology Analysis

For the morphological tissue observations using Hematoxylin and Eosin (H & E) staining, some hepatic tissues were fixed with a 10% formaldehyde solution for 24 hr. The samples were then exchanged twice with the same solution, dehydrated with double ethanol, and formatted in paraffin, producing a 5 µm thick tissue slice treated with poly-L-lysine. For morphological analysis, the prepared tissue sections were stained with H & E for the liver tissue at ×400 and ×1,000 magnification with an optical microscope (Motic microscopy PA53MET, Hong Kong, China). The nucleus area was quantified using Image J software (<http://rsb.info.nih.gov/ij/> accessed on 15 September, 2022) to convert the red intensity from H&E staining.

For oil red O staining, the stained liver samples were entrenched in Tissue-Tek OCT compound (Thermo, Walldorf, Germany) and frozen. Subsequently, 7 µm sections of these tissues were mounted on 3-aminopropyltriethoxysilane (3-APS)-coated slides and viewed under a Leica microcryotome (model CM1510s, Heidelberg, Germany). Seven consecutive sectioned slides of each zebrafish were first stained with Oil Red O (Cat # O0625, Sigma, St. Louis, MO) and then counterstained with hematoxylin, highlighting the fatty streak lesions. The extent of oxidative stress in these tissues was compared by observing the totality of reactive oxygen species (ROS) with dihydroethidium (DHE, cat # 37291; Sigma, St. Louis, MO) using a Nikon Eclipse TE2000 microscope (Tokyo, Japan), as described elsewhere [52]. The section fluorescence was quantified using Image J software (<http://rsb.info.nih.gov/ij/> accessed on 16 September 2022).

Immunohistochemistry analysis was carried out with the anti-human IL-6 antibody (ab9324, Abcam, London, UK), as the primary antibody and horseradish peroxidase conjugated-anti mouse immunoglobulin G antibody, as the secondary antibody using an Envision+ system (K4001, Dako, Denmark), as in a previous report [53].

#### 4.5. Histological Analysis with Ovarian Cell

The pre-vitellogenic, early-vitellogenic, and mature-vitellogenic stages of the ovary were differentiated manually, as described previously [54,55]. The classification criteria were as follows. For the pre-vitellogenic stage, follicles measuring 250 µm diameter or smaller were categorized as such, including the smallest pre-vitellogenic follicles and those in the perinucleolar stage. Follicles, 250–500 µm in diameter, were classified as being in the early-vitellogenic stage. This encompassed the largest pre-vitellogenic follicles and those at the peripheral cortical alveolar stage. Follicles, 500 µm in diameter or larger, were assigned to the mature-vitellogenic stage. This stage included follicles characterized by yolk-filled alveoli distributed throughout the ooplasm and vitellogenic follicles. The ovary follicles were effectively categorized into their respective stages of development using these classification criteria.

#### 4.6. Histological Analysis with Testicular Cell

The testes were collected from zebrafish fed a HCD (4%) for 16 weeks or HCD + policosanol for 12 weeks. The tissues were fixed in Bouin solution for two days and then dehydrated in 30% sucrose solution for two days. Frozen testes in OCT cryo-embedding compound were freshly sectioned to a thickness of 6 µm and stained using the standard H&E staining protocol. H&E staining was also performed on formalin-fixed, paraffin-embedded 3 µm sections after deparaffinization, as described previously [42,56]. Testicular abnormalities were examined by measuring spermatogenesis, the size of seminiferous tubules, and the size of the interstitial space in the testis slides from at least three fish

per group. Spermatogenic defects were examined by measuring the area of each spermatogenic cell, as defined elsewhere [42,57].

#### 4.7. Statistical Analysis

The data are expressed as the mean $\pm$ SEM from at least three independent experiments with duplicate samples. For the blood cholesterol determination and histopathology study, multiple treatments were compared using a one-way analysis of variance (ANOVA) using a Tukey's test as a post-hoc analysis. Statistical analysis was carried out using the SPSS software program (version 28.0; SPSS, Inc., Chicago, IL, USA). A  $p$ -value  $< 0.05$  was considered significant.

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**Institutional Review Board Statement:** The animal study protocol was approved by the Committee of Animal Care and Use of Raydel Research Institute (approval code RRI-20-003, approval date 3 January 2020). The protocol of human blood donation was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board of Yeungnam University (approval code 7002016-A-2016-021, approval date 4 July 2016).

**Informed Consent Statement:** Informed consent was obtained from all subjects involved in the study.

**Data Availability Statement:** The data used to support the findings of this study are available from the corresponding author upon reasonable request.

**Conflicts of Interest:** The author declares no conflict of interest.

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