Nanoemulsification of Rice Bran Wax Policosanol Enhances Its Cardio-protective Effects via Modulation of Hepatic Peroxisome Proliferator-activated Receptor gamma in Hyperlipidemic Rats

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Abstract: Policosanol, a mixture of long-chain alcohols found in animal and plant waxes, has several biological effects including lipid-lowering that have been extensively studied. However, its bioavailability is low. To investigate the effect of nanoemulsified rice bran wax policosanol (NPOL) on plasma homocysteine, heart and liver histology in hyperlipidemic rats, high-fat diet containing 2.5% cholesterol was used to induce hyperlipidemia in Sprague Dawley rats. The hyperlipidemic rats were treated with NPOL and rice bran wax policosanol (POL) in comparison with normal diet (ND), high-cholesterol diet (HCD) and simvastatin-treated rats. Plasma homocysteine, heart and liver histology, and hepatic mRNA expression of peroxisome proliferator-activated receptor gamma (PPARG) were evaluated. The NPOL group, similar to the simvastatin group, showed reduced plasma homocysteine, preserved heart and liver histology, and down-regulated hepatic PPARG mRNA in comparison to the control group, and was better than the POL group. The results suggest that the modest effect of NPOL on homocysteine and preservation of heart and liver histology could be through the regulation of PPARG expression on a background of increased assimilation of rice bran wax policosanol.

Key words: policosanol, nanoemulsion, homocysteine, heart and liver histology, PPARG

1 Introduction
Pathological consequences of persistent hyperlipidemia are numerous, they include atherosclerosis, cardiovascular disease (CVD)1,2, biliary cirrhosis3, and cardiac hypertrophy4. Elevated blood lipids are well-documented risk factors for the development of CVD. Plasma homocysteine level has also been associated with the risk of CVD5,6 and has been correlated with elevated lipid levels7. Similarly, the peroxisome proliferator-activated receptor gamma (PPARG), an important regulator of adipogenesis, insulin sensitivity, lipid metabolism, and glucose homeostasis has been recognized as a key player in the pathogenesis of many diseases including CVD8-10.

Policosanol a naturally occurring mixture of high molecular weight primary aliphatic alcohols was initially isolated from sugar cane by Cuban researchers11. Several reports, including many clinical trials, have shown that policosanol has lipid-lowering effects12-19. Other reports have, however, demonstrated no lipid-lowering effect for policosanol20-23. Direct comparisons of these divergent findings have been limited by differences in regional dietary patterns and by the unknown variability of the composition of the policosanol products that were tested24. Being lipophilic, policosanol was reported to have bioavailability between 5 to 12%25. In this regard, we have developed and characterized the policosanol nanoemulsion from rice bran wax, which we found to be very stable26. In the present study, we focused on the effects of rice bran wax policosanol (POL) and its nanoemulsion (NPOL) on plasma homocysteine, heart and liver histology, and regulation of hepatic PPARG mRNA in hyperlipidemic Sprague Dawley rats.

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2 Materials and Methods

2.1 Materials

Lipid profile kits for low-density lipoprotein (LDL), high-density lipoprotein (HDL), total cholesterol (TC), and triglycerides (TG) were purchased from Randox Laboratories Ltd (Crumlin, County Antrim, UK). Homocysteine ELISA kit was purchased from Cusabio Biotech Co., Ltd. (Wuhan, China) while the GenomeLab™ GeXP Start Kit was purchased from Beckman Coulter Inc (Miami, FL, USA). Simvastatin was purchased from Pfizer (New York, NY, USA), and RCL2 Solution was purchased from Alphelys (Toulouse, France). All solvents used were of analytical grade and were purchased from Merck (Darmstadt, Germany).

2.2 Preparation of rice bran wax policosanol nanoemulsion

Extraction of rice bran wax policosanol (RBWP), formulation of its nanoemulsion and characterization is as reported previously \(^{26}\). Briefly, the nanoemulsion consisted of 5 g RBWP (30.17% of the wax), 3 g Tween 80, and 92 g deionized water. The emulsion had average particle size of 92.69 nm, potential of \(-47.1\) mV, and pH of 6.83.

2.3 Animal handling, feeding and dosing

The animals were handled per the ethical guidelines of University Putra Malaysia Institutional Animal Care Committee (Approval No. UPM/FPSK/PADS/BR-UUH/00477). Thirty 9-weeks old male Sprague Dawley rats, weighing 150 to 170 g were kept in polypropylene cages with wood shavings bedding and acclimatized for 7 days on a normal chow diet with free access to water; at room temperature \((25 \pm 2)\ degrees\ Celsius\), relative humidity of 65 \pm 5\% and a 12/12-hour light/dark cycle in a well-ventilated room. Three rats were kept per cage and bedding was changed weekly. Six rats were maintained on a normal chow diet (NG group) and the rest were given a high-fat diet containing 40% fat energy from Canola oil, sugar, starch, chow and water supplemented with 2.5% cholesterol for 4 weeks to induce hyperlipidemia. Daily feed was regulated at 35 kcal/100 g body weight/day. Rice bran wax policosanol and NPOL dosing were based on the study by Ng et al. (2005) \(^{27}\). The high cholesterol diet-fed animals were then randomly divided into treatment groups of 6 rats each viz: high cholesterol diet (HCD) received distilled water, rice bran wax policosanol (POL) received 250 mg/kg body weight/day of the policosanol suspended in distilled water, rice bran wax policosanol nanoemulsion (NPOL) received 250 mg/kg body weight/day of the policosanol, and simvastatin (SMV) received 0.18 mg/200 g body weight/day of simvastatin (dissolved in distilled water) \(^{28}\) by oral gavage. The animals were maintained on their diet during the treatment and weight was measured weekly. Blood was collected through cardiac puncture following light anesthesia, after induction of hyperlipidemia and at the end of the 8 weeks’ treatment.

2.4 Serum lipid profile

Lipid profile analyses were performed using serum from blood collected at the beginning and the end of the study by cardiac puncture after an overnight fast. Samples were analyzed using Randox analytical kits according to manufacturer’s instructions using a Selectra XL instrument (Vita Scientific, Dieren, The Netherlands).

2.5 Plasma homocysteine

Plasma homocysteine concentration was also determined by ELISA kit (Cusabio Biotech Co., Ltd. Wuhan, China) method according to the manufacturer’s instruction. Samples were prepared in triplicates. Absorbance was read using microplate reader at 450 nm. Concentrations of the samples were calculated from the standard curve equation 35.92logx + 101.48, \(R^2 = 0.9851\).

2.6 Histopathology of heart and liver

The heart and liver harvested after sacrificing the rats, were weighed, washed with normal saline, examined grossly for abnormality and then immediately placed in tissue fixative RCL2 and then transferred into 10% formalin. The tissues were processed using standard histopathological procedure and embedded into paraffin blocks, then microsectioned into about 5 \(\mu\)m thick using a microtome (Leica RM2045) and placed onto glass slides. The slides were stained by Haematoxylin and Eosin (H & E) staining method and then were viewed under optical microscope (Olympus FSX-100, Olympus Corporation, Shinjuku-ku, Tokyo, Japan).

2.7 Gene expression study

From rat livers, RNA was extracted using the Total RNA Isolation kit (RBC Bioscience Corp., Taipei, Taiwan) according to the manufacturer’s instructions. Primer sequences for PPARG and house-keeping genes were designed on the NCBI website, except for the internal control (KanR), which was supplied by Beckman Coulter. The primers (Table 1) were supplied by Biosune (Shanghai, China) and reconstituted in 1X TE buffer according to the protocol of the GenomeLab™ GeXP kit (Beckman Coulter Inc., Miami, FL, USA). Reverse transcription and PCR were performed according to the GenomeLab™ GeXP kit protocol (Beckman Coulter Inc., Miami, FL, USA) in an XP Thermal Cycler (Bioer Technology, Hangzhou, China). The PCR products were then analyzed with a GeXP genetic analysis system, and the results were normalized using eXpress Profiler software based on the manufacturer’s instructions.
2.8 Statistical analysis

The data were analyzed using SPSS version 20 statistical package (IBM Corporation, Amonk, NY, USA) by one-way analysis of variance (ANOVA) and presented as mean and standard deviation (SD). The significant difference was taken to be a value of $p < 0.05$ at 95% confidence interval.

3 Results and Discussion

3.1 Animal weight changes

The effects of the different treatments on the rat body weights are shown in Fig. 1. There was a significantly ($p < 0.05$) higher body weight gain of more than a two-fold in the HCD group compared to the ND group at the end of the study period. Simvastatin (SMV) treatment significantly ($p < 0.05$) reduced body weight gain, in agreement with a previous report on weight-reducing effect of simvastatin. Policosanol has been reported to have significant anti-obesity effect in hyperlipidemic rats, thus, the significant reduction in the weight gain demonstrated in this study in the NPOL and POL groups supports the previous reports. Moreover, NPOL showed more profound effect on weight gain reduction, which may have been as a result of enhanced bioavailability by the nanoemulsion formulation.

3.2 Heart and liver weight changes

Figure 2 depicts the organ weight changes of heart and liver in the respective treatment groups and controls. Hyperlipidemia has been implicated in the development of oxidative stress-related cardiac hypertrophy, while a diet rich in cholesterol has been reported to significantly increase liver weight due to fat accumulation in the liver. The results of the present study demonstrate higher liver and heart weights in agreement with previous findings. Policosanol and simvastatin attenuated the HCD-induced liver and heart weight gain, in keeping with their effects on oxidative stress and cholesterol. Policosanol is able to at-

Table 1 Genes used in the multiplex panel and their primers sequences.

| Genes                      | Forward primer sequence | Reverse primer sequence |
|----------------------------|-------------------------|-------------------------|
| PPARG                      | AGGTGACACTATAGAATAGTCTGG | GTACGACTCTATAGGGAGTCAACC |
|                            | GCTGTTATGG              | ATGGTATTT               |
| Beta actin                 | AGGTGACACTATAGAATAGTCTGG | GTACGACTCTATAGGGAAACATG |
|                            | CCTCCTGAGC              | CCAATCTCAGC             |
| Glyceraldehydes-3-         | AGGTGACACTATAGAATAGTCTGG | GTACGACTCTATAGGGAGTCTCCTG |
| phosphat dehydrogenase     | GTCGAGTCAAA             | CCTGGAGATG              |
| Peptidylprolyl isomerase   | AGGTGACACTATAGAATAGTCTGG | GTACGACTCTATAGGGACAGGCA |
| (cyclophilin) A           | ACATTGCAT               | GCAAAAGGCA               |
|                            |                         |                         |
| PPARG, peroxisome         |                         |                         |
| proliferator-activated     |                         |                         |
| receptor gamma; House-    |                         |                         |
| keeping genes; Normalization gene as selected by the GeXP system. RT conditions were 48°C for 1 min, 37°C for 5 min, 42°C for 60 min, and 95°C for 5 min and then hold at 4°C. PCR conditions were initial denaturation at 95°C for 10 min, followed by two-step cycles of 94°C for 30 sec and 55°C for 30 sec, ending in a single extension cycle of 68°C for 1 min.

![Fig. 1](image_url) Effect of treatments on rat body weight. Body weight as mean ± SD (n = 6 rats per group). ND, normal diet; HCD, high cholesterol diet; SMV, simvastatin; POL, rice bran wax policosanol 250 mg/kg and NPOL, rice bran wax policosanol nanoemulsion containing 250 mg/kg of POL. HCD group showed significant ($p < 0.05$) body weight gain and NPOL modest reduction in weight gain.
cosanol from other sources
strengthened the possibility of unknown variability in the
significantly reported firmed the lipid lowering effect of simvastatin as previously
pared to the HCD group, the SMV, POL and NPOL groups
The SMV group had the lowest LDL level, while the NPOL
group had the lowest TG level. The SMV and NPOL groups
had similar TC and HDL levels. This study further con-
trary to their effects on the liver and heart weight
changes.

3.3 Effect of treatment on serum lipid profile

The lipid profile results are depicted in Table 2. Com-
pared to the HCD group, the SMV, POL and NPOL groups
significantly (p<0.05) decreased TC, TG and LDL levels.
The SMV group had the lowest LDL level, while the NPOL
had the highest TG level. The SMV and NPOL groups
had similar TC and HDL levels. This study further con-
firmed the lipid lowering effect of simvastatin as previously reported[24]. Furthermore, the lipid lowering effect of rice
bran wax policosanol and its nanoemulsion in this study
further confirmed recent report of the same effect by poli-
cosanol nanoemulsion containing 250 mg/kg of POL. Data are mean ± SD. Different letters on bars denote significant difference at p<0.05.

Table 2 Comparison of lipid profile between the treatment groups.

| Groups, n = 6 | TC (mmol/L) | TG (mmol/L) | LDL (mmol/L) | HDL (mmol/L) |
|---------------|-------------|-------------|--------------|--------------|
| ND            | 0.94 ± 0.02a | 1.41 ± 0.02a | 0.41 ± 0.08a | 0.48 ± 0.13a | 0.36 ± 0.02a | 0.28 ± 0.07a | 0.26 ± 0.02a | 0.28 ± 0.03a |
| HCD           | 1.72 ± 0.24b | 2.47 ± 0.17b | 1.05 ± 0.19b | 1.46 ± 0.28b | 0.45 ± 0.05b | 0.53 ± 0.13b | 0.22 ± 0.06b | 0.22 ± 0.08b |
| SMV           | 1.67 ± 0.20c | 1.59 ± 0.33c | 1.00 ± 0.19c | 0.82 ± 0.12c | 0.49 ± 0.01c | 0.22 ± 0.10c | 0.24 ± 0.01c | 0.31 ± 0.10cd |
| POL           | 1.75 ± 0.45d | 1.72 ± 0.04d | 1.02 ± 0.21d | 1.01 ± 0.27d | 0.49 ± 0.54d | 0.42 ± 0.08d | 0.26 ± 0.11d | 0.25 ± 0.52d |
| NPOL          | 1.71 ± 0.37e | 1.57 ± 0.32e | 1.04 ± 0.33e | 0.78 ± 1.02e | 0.49 ± 0.66e | 0.33 ± 0.09e | 0.24 ± 0.21e | 0.32 ± 0.13e |

Data presented as mean ± SD. Different letters in columns represent significant difference at p<0.05 by Tukey’s multiple comparison test. Rats grouping as in Fig. 1. ND = normal diet; HCD = high cholesterol diet; SMV = simvastatin; POL = rice bran wax policosanol 250 mg/kg and NPOL = rice bran wax policosanol nanoemulsion containing 250 mg/kg of POL.
Simvastatin has been reported to markedly decrease plasma homocysteine level\(^7\) similar to what was observed in the present study. The level of homocysteine was lowest among the SMV group followed by the NPOL group. However, previous human study by Reiner et al. (2005)\(^{15}\) suggested that policosanol has no effect on plasma homocysteine level. The presence of effect in this study indicates that policosanol could lower homocysteine and that higher doses in humans may be needed, or the use of nanoemulsion could enhance bioavailability and hence its efficacy in humans. The composition of the policosanol used may also have affected the results.

3.5 Effect of treatment on histology of heart and liver

The effect of treatments on the heart and liver histology is as depicted in Figs. 4a and 4b. The histological findings closely parallel biochemical findings. In the ND group, normal liver architecture is shown with visible central vein, radiating sinusoids, hepatocytes and portal triad. On the contrary, in the HCD group, there was evidence of extent-

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**Fig. 4a** Liver and heart histology after H & E stain, 4 x objective of rat groups (n = 6 per group). The simvastatin group shows preservation of normal architecture compared to the high cholesterol diet (HCD) control group. BD = bile duct, CV = central vein, HA = hepatic artery, PT = portal triad, and PV = portal vein. ND = normal diet; and SMV = simvastatin. L = liver and H = heart sections.
sive hepatic tissue necrosis, macrovesicular steatosis parenchymal distortion, mononuclear infiltration and hepatocellular swelling. It is well-established that hyperlipidemia results in changes in liver histology. Increase of hepatic TGs is associated with steatosis. It also results in mononuclear inflammation, lobular inflammation, pericellular fibrosis and hepatocellular swelling in consequence of hyperlipidemia-induced oxidative stress. Simvastatin treatment effectively prevented the above pathological changes. This supports recent reports on simvastatin-induced liver histology preservation in high fat diet fed rats.

Similar preventive effect was observed with all treatments in this study. It is worthy to note that, NPOL treatment preserved liver architecture better than the POL.

The results of the histopathological study also showed the normal cellular architecture of the myocardium in the ND group. Histopathological sections of the HCD group showed evidence of necrosis, fibrosis, myofibril distortion, and extensive steatosis. Varying degrees of heart architecture preservation were observed in the treatment groups. There was clear preservation of myocardial architecture in simvastatin-treated group, and this finding supports previous report that showed simvastatin markedly attenuated the cardiac myofibril and capillary structure in diabetic/hyperlipidemic rats. Interestingly, treatment with NPOL showed effect that is comparable to that of SMV treatment. Thus, the histological study of the heart tissue suggests that, RBW policosanol nanoemulsion (NPOL) confers a better cardioprotection than RBW policosanol (POL).

3.6 Hepatic mRNA expression of PPARG

Figure 5 depicts the relative expression of PPARG in hepatic tissue of treated rats. Transcriptional regulation of fatty acid storage, adipogenesis, cell proliferation, and insulin sensitivity have been recognized to be under the influence of PPARG. Reports have shown that, agents which interfere with lipid and/or glucose metabolisms such as berberine, germinated brown rice, ciglitazone and rosiglitazone do that through the modulation of PPARG. In this study, only NPOL treatment group showed a profound down-regulation of hepatic mRNA of PPARG; and the expression was non-different (p > 0.05) from that of the ND group. The 3- and 2-folds increases in mRNA expression of PPARG in the SMV group compared to the ND and HCD groups could be as a result of a direct effect of simvastatin on the gene as observed by Qin et al., 2010. This supports the finding of previous study using cultured gallbladder cells. Researchers have shown that, lipid oxidation increased PPARG expression, which explains the increased expression in the HCD group compared to the ND group. The same explanation could be the reason for the increased mRNA expression in POL treatment group, and this demonstrated that POL has, to a large extent, lower effect compared to NPOL. The reduction in hepatic PPARG
mRNA in NPOL parallels with its effect on lipids especially TG reduction. It also supports the anti-obesity effect of policosanol reported in a previous study.  

4 Conclusion  
Considering the wide range of adverse effects of hyperlipidemia, our findings suggest that rice bran wax policosanol confers protection against these effects by ameliorating lipids and homocysteine levels, and reversal of liver and heart histological changes through the modulation of PPARG gene. Interestingly, these effects are more profound in the nanoemulsified form of the policosanol, likely due to increased assimilation. The findings also add to the body of works supporting hypolipidemic effect of policosanol and the potential role of carrier systems to enhance bioactivity.

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Conflict of Interest  
The authors declare no conflict of interest.

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