

## Assessing Plasma Lipid Levels, Body Weight, and Hepatic and Renal Toxicity Following Chronic Oral Administration of a Water Soluble Phytostanol Compound, FM-VP4, to Gerbils.

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**Abstract:** The purpose of this project was to determine the effect of a FM-VP4 when incorporated into the diet or drinking water on plasma lipids, body weight, and hepatic and renal function following chronic oral administration to gerbils. Gerbils were administered water and food daily containing either no FM-VP4 (controls; n=6), 2% or 4% FM-VP4 incorporated into the gerbil diet (n=6 each treatment group) or 2% or 4% FM-VP4 dissolved in the drinking water (n=6 each treatment group). Body weight and food and water intake were monitored weekly. Following 8 weeks of this regimen blood was obtained *via* a cardiac puncture and all animals were sacrificed humanely. Plasma obtained from this blood was analyzed for total cholesterol, total triglyceride and high-density lipoprotein (HDL)-cholesterol levels by standard enzymatic and precipitation techniques. Low-density lipoprotein (LDL)-cholesterol levels were determined by the Friedewald equation. The plasma was also analyzed for changes in hepatic enzyme (aspartate aminotransferase [AST] and alanine aminotransferase [ALT]) and plasma creatinine (renal function) concentrations. 2% and 4% FM-VP4 administration incorporated both into the diet and in the drinking water resulted in a significant decrease in total plasma cholesterol and LDL cholesterol concentration compared to controls. Animals administered 4% FM-VP4 in either their diet or drinking water had significantly lower body weight following the 8 weeks of treatment compared to the other groups. Significant differences in daily water intake was observed in all

treatment groups with the exception of the 2% FM-VP4 in diet group compared to controls. Significant differences in daily food intake were observed in gerbils administered 2% FM-VP4 in the drinking water and 4% FM-VP4 in the diet and drinking water groups compared to controls. A significant decrease in total plasma triglyceride concentration was observed in gerbils administered 4% FM-VP4 in their drinking water compared to controls. A significant increase in HDL cholesterol concentrations was observed in gerbils administered 2% FM-VP4 in their diet and 4% FM-VP4 in their drinking water compared to controls. No significant elevations in AST, ALT and creatinine concentrations were reported for all treatment groups compared to controls. These findings suggest that FM-VP4 significantly decrease plasma lipids and body weight with no apparent hepatic or renal toxicity.

Heart disease, caused by atherosclerosis, is one of the leading causes of death in North America. It is well established that elevated plasma cholesterol levels in humans are associated with increased risk of atherosclerosis and coronary artery disease (1). Furthermore, it is proven that decreasing blood LDL-cholesterol levels prevents myocardial events in both primary and secondary prevention studies (2,3).

Increased consumption of plant sterols and stanols, known as phytosterols and phytostanols, respectively, is a recognized nutritional strategy to reduce blood cholesterol levels. Phytosterols and phytostanols are naturally occurring compounds found in edible oils, seeds, nuts, wood pulp, tall oil and soy beans (4,5) and are structurally related to cholesterol. These compounds have been shown to effectively reduce plasma

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cholesterol levels both in animal models and in humans (6-10).

Numerous studies have described phytosterol- and phytostanol-induced decreases in total plasma and lipoprotein cholesterol levels (11-14) but the mode of action is not fully understood. It is hypothesized that these compounds compete with cholesterol for absorption in the intestine, thereby reducing the amount of cholesterol absorbed into the body (11,12,15). Specifically, phytosterols and phytostanols have been shown to decrease low-density lipoprotein (LDL)-cholesterol levels in animal models and in humans (14,16-20). In addition, these compounds prevent or delay the development of atherosclerotic lesions in animal models (13,16).

Commercially available phytostanols and phytosterols are hydrophobic, hydrogenated fatty acid esters and, as food additives, are limited to oil-based foods such as margarine. Hydrophilic analogs of these compounds may prove to be effective cholesterol-lowering alternatives to these fatty acid esters. FM-VP4 is a water soluble phytostanol mixture that can be formulated into a wide range of delivery vehicles. Recently, our lab has shown that oral administration of FM-VP4 at 1% and 2% and FM-3P4 at 2% to gerbils for 4 weeks resulted in a significant decrease in total plasma cholesterol and LDL cholesterol concentration compared to controls (31). The extent of this decrease was more pronounced in animals administered 2% FM-VP4 compared to animals administered 2% FM-3P4. Animals administered 1% or 2% FM-VP4 had significantly lower body weight following the 4 weeks of treatment compared to the other groups. However, no visible signs of distress were observed in these animals. No significant differences in daily water or food intake, total plasma triglyceride and HDL cholesterol concentrations were observed in all treatment groups compared to controls.

However, to date no studies have been completed that investigate the ability of FM-VP4 to modify total plasma and lipoprotein cholesterol and triglyceride levels and hepatic and renal toxicity following 8 weeks of continuous FM-VP4 administration to gerbils. In addition, studies need to be completed to determine if the differences in weight loss attributed to 4 weeks of FM-VP4 therapy (31) continue following 8 weeks of therapy and if this weight loss is linked to animal toxicity

(i.e. liver and kidney toxicity). The specific aims of this research proposal were to determine the effect of FM-VP4 incorporated into the gerbil diet or dissolved into the drinking water on total plasma and lipoprotein cholesterol and triglyceride levels, liver function and kidney function following 8 weeks of continuous administration to gerbils. We hypothesize that FM-VP4 incorporated into the diet or dissolved in the drinking water would be equally effective in reducing total and LDL cholesterol plasma concentrations with weight loss following 8 weeks of treatment.

## METHODS AND MATERIALS

*Formulation development and animals.* Standard milling procedures (21) were used to incorporate different concentrations of FM-VP4 2% & 4% w/w into standard gerbil chow. FM-VP4 (2% & 4% w/v) was also dissolved into drinking water. Fresh drinking water and gerbil chow was replaced on a daily basis throughout the duration of the study. Mongolian gerbils were used in these studies because, similar to humans, these animals have a LDL-dominant lipid profile and exhibit sensitivity to cholesterol in the diet (22). Unlike rats, which do not have a LDL-like lipoprotein pool, Mongolian gerbils have increased LDL-cholesterol concentrations in response to high fat, high cholesterol diets (23). All gerbils used in this study were cared for in accordance with the Canadian Council on Animal Care and the University of British Columbia guidelines.

*Experimental protocol.* Thirty adult male Mongolian gerbils (70-80 g) were obtained from Charles River Breeders (Montreal, Canada). The gerbils were maintained under a 12 hr light (0700-1900)/dark cycle and supplied with a standard laboratory gerbil diet (Jameison's Pet Food Distributor, Delta, B.C.) and water *ad libitum*. After a 2-week adaptation period, the gerbils were divided into six treatment groups matched for body weight and the following treatment protocol was used throughout the duration of the study: Gerbils had access to water and food daily *ad libitum* for 8 continuous weeks (daily water and food intake were monitored and replaced), which contains either no FM-VP4 (controls; n=6), 2.0% FM-VP4 incorporated into the gerbil diet or dissolved into the drinking water (n=6) and 4.0% FM-VP4 incorporated into the gerbil diet (n=6) or dissolved into the drinking water (n=6).

**Biochemical analysis.** Following 8 weeks of this regimen blood was obtained *via* a cardiac puncture and all animals were sacrificed humanely. The blood was centrifuged to obtain plasma, which was analyzed for total cholesterol, total triglyceride and HDL-cholesterol levels by precipitation techniques and modified enzymatic procedures (24,25) from Sigma Diagnostics. LDL-cholesterol levels were calculated using the Friedewald equation (15). Lipid levels were determined using procedures standardized by the Canadian Reference Laboratory (31). Plasma was also analyzed for aspartate aminotransferase (AST) and alanine aminotransferase (ALT) by standard enzymatic assay analysis (34). The liver releases ALT and an elevation in plasma concentration are an indicator of liver damage. The liver and heart release AST and an elevation in plasma concentration are an indicator of liver and heart damage (34). Plasma creatinine levels were determined by standard enzymatic assay analysis to ascertain changes in renal function (35).

**Statistical Analysis.** Differences in daily food and water intake, total plasma cholesterol, total plasma triglyceride, LDL-cholesterol and HDL-cholesterol concentrations, AST, ALT and creatinine levels and body weight for all treated and control gerbils were determined using an analysis of variance (Instat2; GraphPad). Significant differences were determined using a Newman Keuls post-hoc test where differences were considered significant if  $p < 0.05$ . All data are expressed as mean  $\pm$  standard error of the mean.

## RESULTS AND DISCUSSION

Table 1 shows the effects of FM-VP4 incorporated into either the diet or drinking water on plasma lipid levels in gerbils after 8 weeks of FM-VP4 administration. 2%

and 4% FM-VP4 administration incorporated both into the diet and in the drinking water resulted in a significant decrease in total plasma cholesterol and LDL cholesterol concentration compared to controls. This may be due to inhibition or displacement of cholesterol by FM-VP4 from cholesterol-containing micelles, formed with bile acids in the small intestine, which are required for cholesterol absorption (12). This decrease may also be due to a decrease in facilitated cholesterol uptake by enterocyte cholesterol transporters (27,28). Another explanation may be the inhibition of pancreatic lipase that cleaves the ester bonds through which fatty acids are esterified into triglycerides, phospholipids and cholesteryl esters (29). Decreases in total plasma cholesterol levels were due exclusively to decreases in LDL-cholesterol concentrations, as HDL-cholesterol levels did not change with FM-VP4 dose. The decrease in LDL-cholesterol is consistent with previously reported results (13,14) and suggests that FM-VP4 may decrease total plasma cholesterol by inhibiting the formation of LDL-cholesterol. This inhibition may be a result of decreased activity of cholesteryl ester transfer protein (CETP), a plasma enzyme responsible for the facilitated transfer of esterified cholesterol from HDL to LDL (30). However, further investigation is required to elucidate the mechanism involved.

Animals administered 4% FM-VP4 in either their diet or drinking water had significantly lower body weight following the 8 weeks of treatment compared to the other groups (Table 2). Significant differences in daily water intake was observed in all treatment groups with the exception of the 2% FM-VP4 in diet group compared to controls (Table 3). Significant differences in daily food intake were observed in gerbils administered

**Table 1: Effect of 8-week treatment with FM-VP4 in diet and/or water on total plasma cholesterol, triglyceride, HDL**

Treatment Group	Total Chol (mmol/L)	Total TG (mmol/L)	HDL Chol (mmol/L)	LDL Chol (mmol/L)
Controls (n=6)	6.05 $\pm$ 0.57	2.54 $\pm$ 1.07	2.01 $\pm$ 0.11	3.00 $\pm$ 0.59
FM-VP4 2% in diet (n=6)	2.98 $\pm$ 0.21 <sup>a</sup>	2.47 $\pm$ 0.51	2.30 $\pm$ 0.14 <sup>a</sup>	ND
FM-VP4 4% in diet (n=6)	2.82 $\pm$ 0.11 <sup>a</sup>	1.88 $\pm$ 0.41	2.12 $\pm$ 0.09	0.07 $\pm$ 0.05 <sup>a</sup>
FM-VP4 2% in water (n=6)	2.98 $\pm$ 0.25 <sup>a</sup>	2.66 $\pm$ 0.58	2.18 $\pm$ 0.25	0.03 $\pm$ 0.02 <sup>a</sup>
FM-VP4 4% in water (n=6)	2.90 $\pm$ 0.25 <sup>a</sup>	1.01 $\pm$ 0.08 <sup>a,b,c,d</sup>	2.69 $\pm$ 0.26 <sup>a,c,d</sup>	0.05 $\pm$ 0.05 <sup>a</sup>

Data presented as mean  $\pm$  standard error of the mean (SEM); <sup>a</sup> $P < 0.05$  vs. controls; <sup>b</sup> $P < 0.05$  vs. FM-VP4 2% in diet; <sup>c</sup> $P < 0.05$  vs. FM-VP4 4% in diet; <sup>d</sup> $P < 0.05$  vs. FM-VP4 2% in water. Abbreviations: Chol, cholesterol; TG, triglycerides; HDL, high-density lipoproteins; LDL, low-density lipoproteins; ND, below limit of assay detection (undetectable).

2% FM-VP4 in the drinking water and 4% FM-VP4 in the diet and drinking water groups compared to control (Table 4). A significant decrease in total plasma triglyceride concentration was observed in gerbils administered 4% FM-VP4 in their drinking water compared to controls (Table 1). A significant increase in HDL cholesterol concentrations was observed in gerbils administered 2% FM-VP4 in their diet and 4% FM-VP4 in their drinking water compared to controls (Table 1). A significant decrease in plasma AST, ALT and creatinine concentrations were not observed in all treatment groups compared to controls (Table 5). Taken together these findings suggest that 2% FM-VP4 incorporated into the gerbil food or drinking water significantly decrease total and LDL cholesterol concentrations and body weight with no visible signs of animal distress or hepatic and renal toxicity compared to controls.

fixed-food diets over two 10-day periods with or without oil phytosterols resulted in a significant reduction in total and LDL cholesterol plasma concentrations compared to controls (33).

In this study it was observed that FM-VP4 at both 2% or 4% in either the diet or drinking water appeared to decrease total plasma cholesterol and LDL cholesterol concentrations (Table 1). A possible explanation for this decrease may be the inhibition or displacement of cholesterol from cholesterol-containing micelles formed with bile acids in the gut (which is required for cholesterol gastrointestinal absorption) (11,12) by this vegetable stanol mixture. However, this decrease may be due to the decrease in facilitated cholesterol uptake *via* enterocyte cholesterol transporters (27,28). These possible explanations warrant further investigation. Furthermore, it appears that the decrease in total

**Table 2. Effect of 8-week treatment with FM-VP4 in diet and/or water on gerbil body weight during each week of the study.**

Treatment Group	Week 0 (Body weight; g/gerbil)	Week 1 (Body weight; g/gerbil)	Week 2 (Body weight; g/gerbil)	Week 3 (Body weight; g/gerbil)	Week 4 (Body weight; g/gerbil)	Week 5 (Body weight; g/gerbil)	Week 6 (Body weight; g/gerbil)	Week 7 (Body weight; g/gerbil)	Week 8 (Body weight; g/gerbil)
Controls	81.5±2.5	83.0±2.7	86.2±3.0	86.8±3.8	88.7±4.2	89.0±4.6	88.5±4.9	88.8±5.4	90.0±5.8
FM-VP4 2% within diet	83.3±2.2	80.6±1.8	78.6±1.8 <sup>a</sup>	77.0±2.2 <sup>a</sup>	78.0±2.4 <sup>a</sup>	79.2±2.1 <sup>a</sup>	79.7±2.4 <sup>a</sup>	81.0±2.7	82.6±2.5
FM-VP4 4% within diet	81.6±1.4	74.6±1.4 <sup>ab</sup>	68.3±2.1 <sup>ab</sup>	64.7±1.8 <sup>ab</sup>	64.0±1.8 <sup>ab</sup>	63.3±2.0 <sup>ab</sup>	64.2±2.5 <sup>ab</sup>	66.7±2.4 <sup>ab</sup>	67.3±2.5 <sup>ab</sup>
FM-VP4 2% in water	77.6±1.1 <sup>bc</sup>	75.0±1.2 <sup>ab</sup>	77.3±1.2 <sup>ac</sup>	77.0±1.1 <sup>ac</sup>	78.5±1.6 <sup>ac</sup>	79.3±2.0 <sup>ac</sup>	84.0±2.1 <sup>c</sup>	84.6±2.4 <sup>c</sup>	85.0±2.8 <sup>c</sup>
FM-VP4 4% in water	81.0±2.4	69.6±1.8 <sup>ab,cd</sup>	68.0±3.0 <sup>ab,d</sup>	67.0±3.6 <sup>ab,d</sup>	65.8±4.4 <sup>ab,d</sup>	65.5±5.1 <sup>ab,d</sup>	67.7±5.4 <sup>ab,d</sup>	66.8±5.3 <sup>ab,d</sup>	68.0±5.1 <sup>ab,d</sup>

Data presented as mean ± standard error of the mean (SEM); n=6 for all treatment groups. <sup>a</sup>P<0.05 vs. controls; <sup>b</sup>P<0.05 vs. FM-VP4 2% in diet; <sup>c</sup>P<0.05 vs. FM-VP4 4% in diet; <sup>d</sup>P<0.05 vs. FM-VP4 2% in water.

Recent studies by Ostlund *et al.* have shown that sitostanol administered in lecithin micelles significantly reduced cholesterol absorption at doses lower than reported previously with sitostanol alone (32). Furthermore, in a small scale study, subjects consuming

plasma cholesterol levels was exclusively due to the decrease in LDL cholesterol concentrations (Table 1). This observation suggests that possibly FM-VP4 may further decrease total plasma cholesterol by inhibiting

**Table 3. Effect of 8-week treatment with FM-VP4 in diet and/or water on average gerbil water intake (ml/day/gerbil) during each week of the study.**

Treatment Group	Week 1 (ml/day/gerbil)	Week 2 (ml/day/gerbil)	Week 3 (ml/day/gerbil)	Week 4 (ml/day/gerbil)	Week 5 (ml/day/gerbil)	Week 6 (ml/day/gerbil)	Week 7 (ml/day/gerbil)	Week 8 (ml/day/gerbil)
Controls	7.6±0.3	8.6±1.2	9.1±1.2	9.1±0.7	8.9±1.7	10.1±1.7	9.5±1.2	9.0±0.7
FM-VP4 2% within diet	5.4±0.6 <sup>a</sup>	6.2±0.7 <sup>a</sup>	8.7±0.6	8.7±0.9	8.4±1.1	9.6±0.8	8.7±0.8	8.8±0.6
FM-VP4 4% within diet	4.3±0.3 <sup>ab</sup>	5.6±0.9 <sup>a</sup>	8.3±0.8	9.2±0.6	9.4±1.0	9.6±0.7	8.8±0.7	8.2±0.4
FM-VP4 2% in water	5.8±1.0 <sup>ac</sup>	5.3±1.0 <sup>a</sup>	5.2±0.5 <sup>ab,c</sup>	5.7±0.8 <sup>ab,c</sup>	6.0±1.3 <sup>c</sup>	6.0±0.6 <sup>ab,c</sup>	7.4±0.8 <sup>a</sup>	6.3±0.5 <sup>ab,c</sup>
FM-VP4 4% in water	5.8±0.8 <sup>ac</sup>	6.2±1.9	4.1±0.6 <sup>ab,c</sup>	5.6±0.6 <sup>ab,c</sup>	5.6±1.5 <sup>ab,c</sup>	6.9±0.8 <sup>ab,c</sup>	7.5±1.4	7.2±0.8 <sup>a</sup>

Data presented as mean ± standard error of the mean (SEM); n=6 for all treatment groups. <sup>a</sup>P<0.05 vs. controls; <sup>b</sup>P<0.05 vs. FM-VP4 2% in diet; <sup>c</sup>P<0.05 vs. FM-VP4 4% in diet; <sup>d</sup>P<0.05 vs. FM-VP4 2% in water.

the formation of LDL cholesterol. An explanation for these results requires further study.

However, to date, no information about the effectiveness of FM-VP4 to decrease lipid levels has been reported. This information would be valuable in establishing therapeutically effective dosing guidelines and may provide further insight into the mechanisms of FM-VP4.

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**Table 4. Effect of 8-week treatment with FM-VP4 in diet and/or water on average gerbil food intake (grams/day/gerbil) during each week of the study.**

Treatment Group	Week 1 (g/day/gerbil)	Week 2 (g/day/gerbil)	Week 3 (g/day/gerbil)	Week 4 (g/day/gerbil)	Week 5 (g/day/gerbil)	Week 6 (g/day/gerbil)	Week 7 (g/day/gerbil)	Week 8 (g/day/gerbil)
Controls	8.2±0.9	6.2±0.4	6.2±0.4	7.6±1.0	6.3±0.6	6.6±0.9	5.2±0.5	5.0±0.3
FM-VP4 2% within diet	5.8±0.8 <sup>a</sup>	5.6±0.5	5.7±0.8	6.7±0.7	6.8±0.9	5.7±0.7	6.4±0.4 <sup>a</sup>	6.1±0.6 <sup>a</sup>
FM-VP4 4% within diet	8.8±1.4 <sup>b</sup>	5.6±0.9	7.0±0.9	9.3±1.3 <sup>b</sup>	8.5±1.0 <sup>a</sup>	8.3±0.7 <sup>ab</sup>	8.0±0.9 <sup>ab</sup>	8.5±1.0 <sup>ab</sup>
FM-VP4 2% in water	6.7±1.2	7.8±0.9 <sup>ab,c</sup>	5.2±0.4 <sup>ac</sup>	6.2±0.5 <sup>c</sup>	5.2±0.6 <sup>bc</sup>	6.0±0.6 <sup>c</sup>	5.5±0.5 <sup>c</sup>	6.3±0.9 <sup>ac</sup>
FM-VP4 4% in water	6.9±1.5	7.7±0.9 <sup>bc</sup>	7.9±0.6 <sup>ab,d</sup>	7.1±0.3 <sup>cd</sup>	5.8±0.5 <sup>c</sup>	6.9±0.5 <sup>c</sup>	7.2±0.5 <sup>ad</sup>	5.8±0.7 <sup>c</sup>

*Data presented as mean ± standard error of the mean (SEM); n=6 for all treatment groups. <sup>a</sup>P<0.05 vs. controls; <sup>b</sup>P<0.05 vs. FM-VP4 2% in diet; <sup>c</sup>P<0.05 vs. FM-VP4 4% in diet; <sup>d</sup>P<0.05 vs. FM-VP4 2% in water*

**Table 5. Effect of 8-week treatment with FM-VP4 in diet and/or water on plasma creatinine, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) concentrations in adult male gerbils.**

Treatment Group	Creatinine* (mg/dL)	AST** (SF units/mL)	ALT** (SF units/mL)
Controls	0.49 ± 0.13 (n=6)	106.1 ± 27.2 (n=6)	38.0 ± 5.2 (n=6)
FM-VP4 2% in diet	0.36 ± 0.02 (n=4)	106.9 ± 15.0 (n=4)	23.3 ± 1.5 <sup>a</sup> (n=5)
FM-VP4 4% in diet	0.41 ± 0.02 <sup>b</sup> (n=5)	157.5 ± 15.5 <sup>ab</sup> (n=4)	35.9 ± 2.1 <sup>b</sup> (n=4)
FM-VP4 2% in water	0.43 ± 0.06 (n=5)	74.4 ± 10.8 <sup>ab,c</sup> (n=4)	25.8 ± 5.2 <sup>ac</sup> (n=5)
FM-VP4 4% in water	0.51 ± 0.17 (n=5)	65.9 ± 8.3 <sup>ab,c,d</sup> (n=3)	20.4 ± 0.7 <sup>ac</sup> (n=3)

*<sup>a</sup>P<0.05 vs. controls; <sup>b</sup>P<0.05 vs. FM-VP4 2% in diet; <sup>c</sup>P<0.05 vs. FM-VP4 4% in diet; <sup>d</sup>P<0.05 vs. FM-VP4 2% in water. Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; ND, below the detectable limit of the assay. \*Elevation in plasma creatinine concentration compared to non-treated controls indirectly suggests kidney damage, specifically the renal filtration mechanism. \*\*Elevations in plasma AST and/or ALT concentration compared to non-treated controls indirectly suggests liver damage*

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