Lunasin-Enriched Soy Extract (LunaRich X™), in Combination with the Dietary Supplement Reliv Now, Reduces Free Fatty Acid by Increasing Plasma Leptin and Adiponectin Levels in LDL-Receptor Mutant Pigs

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ABSTRACT

Elevated levels of free fatty acids (FFA) are associated with obesity and insulin resistance and can lead to diabetes, metabolic syndrome and cardiovascular disease. Daily supplementation using 500 mg of lunasin-enriched soy extract (LES) and one serving (18.3 gm) of the dietary supplement Reliv Now® has been found to significantly reduce FFA levels by 74% and 65% after 4 and 6 weeks, respectively, on obese LDL-receptor mutant (Rapacz) pigs. The decrease in FFA levels can be explained by the coordinate increase in plasma levels of leptin by 52% and 64% and adiponectin by 20% and 60% after 4 and 6 weeks, respectively. The decrease in FFA and the increase in leptin and adiponectin correlates with slower weight gain in the obese pigs. Removal of LES and Reliv Now supplements from the basic diet of the pigs increases FFA and decreases leptin and adiponectin to the baseline, unsupplemented levels. These results suggest that supplementation with LES and Reliv Now has the potential to reduce FFA and increase leptin and adiponectin levels, which are associated with preventing obesity and insulin resistance and the related disease modalities arising from these health conditions. However, human clinical trials are needed to validate these preliminary results.

INTRODUCTION

According to the US Centers for Disease Control (CDC), there is a rising trend in obesity levels today with a parallel increase in type 2 diabetes. Elevated levels of free fatty acids (FFA) have been associated with insulin resistance in obese patients [1] and can increase inflammation [2]. Insulin resistance and pro-inflammatory response are clinically important because they can lead to several diseases like type 2 diabetes, hypertension and cardiovascular disease [3]. Although there is a close association of obesity, insulin resistance and type 2 diabetes to elevated FFA levels, there is a lack of effective treatments to lower free fatty acids [1].

Lunasin is a bioactive component in soy with a novel chromatin-binding property and epigenetic effects on gene expression [4, 5]**.** The soy peptide is heat stable, water soluble and found in significant amounts in select soy protein preparations [6]. It can get inside mammalian epithelial cells through its RGD cell adhesion motif, bind preferentially to deacetylated histones and inhibit histone H3 and H4 acetylation [4]. There is growing evidence that responses to dietary and environmental effects involve epigenetic changes in gene expression, which are modulated by the reversible processes of DNA methylation-demethylation and histone acetylation-deacetylation [7, 8].

Lunasin has been shown to have an anti-mitotic effect when expressed inside mammalian cells [5] and prevents cancer formation when applied exogenously in cell culture and in mice skin cancer model [4]. The lunasin peptide has also been shown to have anti-inflammatory [9] and anti-oxidant effects [10], to improve innate immunity (unpublished results), and to prevent metastasis of colon cancer cells to the liver [11]. The multiple health effects of the lunasin soy peptide have been attributed to its ability to bind to chromatin and affect gene expression associated with cellular health [12]. The elucidation of its mechanism of action makes lunasin an important molecule for research studies to understand the emerging role of diet on epigenetic mechanisms that can impact the development of chronic diseases, such as obesity, diabetes and metabolic syndrome.

We have previously shown the effect of lunasin-enriched soy extract in lowering LDL cholesterol [13] and we now report a novel effect of the lunasin-enriched soy extract in combination with a soy-based dietary supplement, Reliv Now, on a supplement feeding study using a LDL-receptor mutant pig model [14]. Reliv Now contains optimal blend of vitamins, minerals and herbal agents and is primarily used to lower risk factors for cardiovascular disease. Our study shows that supplementation with lunasin in combination with Reliv Now leads to a significant decrease in free fatty acid levels and the coordinated increase of leptin and adiponectin plasma levels in LDL-receptor mutant pigs.

MATERIALS AND METHODS

We chose the Rapacz pig model [15] to test the biological effect of the lunasin-enriched soy extract (LES) extract on obesity, cardiovascular disease risk and metabolic syndrome because their weight and liver function are closer to humans than any other animal models. Also, the pigs have mutations to their LDL receptor gene that predispose them to high cholesterol, obesity and increased risk for heart disease [15]. The 5 Rapacz pigs used for this study were approximately 1.5 years old and considered obese, weighing more than 20% from normal. The experiment was done with animal use permit at the Veterinary hospital at the University of Missouri, Columbia, MO.

In order to administer Reliv Now, the 18.3 gms powder was measured from a 29.6 cc scooper, mixed with 10 cc of water and formed into a dough ball that the pigs ate happily. The 500 mg dose of LES was selected based on previous study showing that 250 mg dose of LES was enough to reduce LDL cholesterol after 4 weeks in 1 -year old Rapacz pigs. We increased the dose to 500 mg to account for the increased weight of the pigs at the start of the study when they were 1.5 years of age. The 500 mg LES was put into two capsules of 250 mg each and was fed to the pigs by inserting them into a snack bar that the pigs like to eat.

To determine the bioavailability of lunasin after ingestion of 500 mg LES, we supplemented the diet of one pig with 500 mg LES and then blood draws were taken at 10 min, 30 min, 60 min and 24 hr after ingestion. The blood plasma was separated and the amount of lunasin was detected and quantitated by ELISA using a polyclonal lunasin antibody (Pacific BioLabs) and standard ELISA protocol.

To test the effects of the lunasin-enriched soy extract (LES) in combination with Reliv Now, we fed five Rapacz pigs daily with at least one kg of standard casein diet (BV233) and supplemented with 18.3 gm of Reliv Now and 500 mg of LES. The pigs were maintained on a soy-free diet (BV233 pig chow) throughout the treatment. Pigs were fed their regular diet (BV233) once per day in the morning at approximately 9:00 AM. Later in the afternoon, at approximately 4:00 PM, the Reliv Now and LES treatments were administered. Weight and blood draws were taken at pre-treatment (0), at 4 weeks, 6 weeks and 8 weeks after treatment had begun. At the end of 8 weeks, there was an additional 4 weeks washout period without treatment and weight and blood draws were also taken at the end of washout period (12 weeks).

Blood draws were taken after an overnight fast (approximately 15h). Blood samples were tested for lipid panel including plasma levels of free fatty acids (FFA) by the Analytical Laboratory at the Veterinary School of the University of Missouri. Blood samples were collected into monoject tubes with 15% EDTA, centrifuged for 20 min at 3300 rpm to separate the blood plasma, which were transferred to 1 ml cryogenic vials and stored at -70^{o}C to test for leptin and adiponectin levels. Plasma levels of leptin and adiponectin were determined using porcine leptin and adiponectin ELISA kits obtained from USCN Life Sciences, Inc. (Houston, TX USA). Standard protocols from the manufacturer were followed, including the use of 3 replicate measurements for each data point, to detect and quantify the amounts of leptin and adiponectin in the plasma.

RESULTS

Before conducting the supplementation study, we first determined whether lunasin can be detected in the blood plasma of a Rapacz pig at different time points after ingesting a 500 mg dose of lunasin-enriched soy extract (LES). Figure 1 shows that lunasin can be detected from the blood plasma as early as 10 min after ingestion and increased significantly after 30 and 60 min before peaking at 24h after ingestion. The total amount of lunasin detected in blood plasma after 24h was approximately 0.11 mg (Fig.1), which indicates that lunasin becomes bioavailable after ingestion of 500 mg LES.

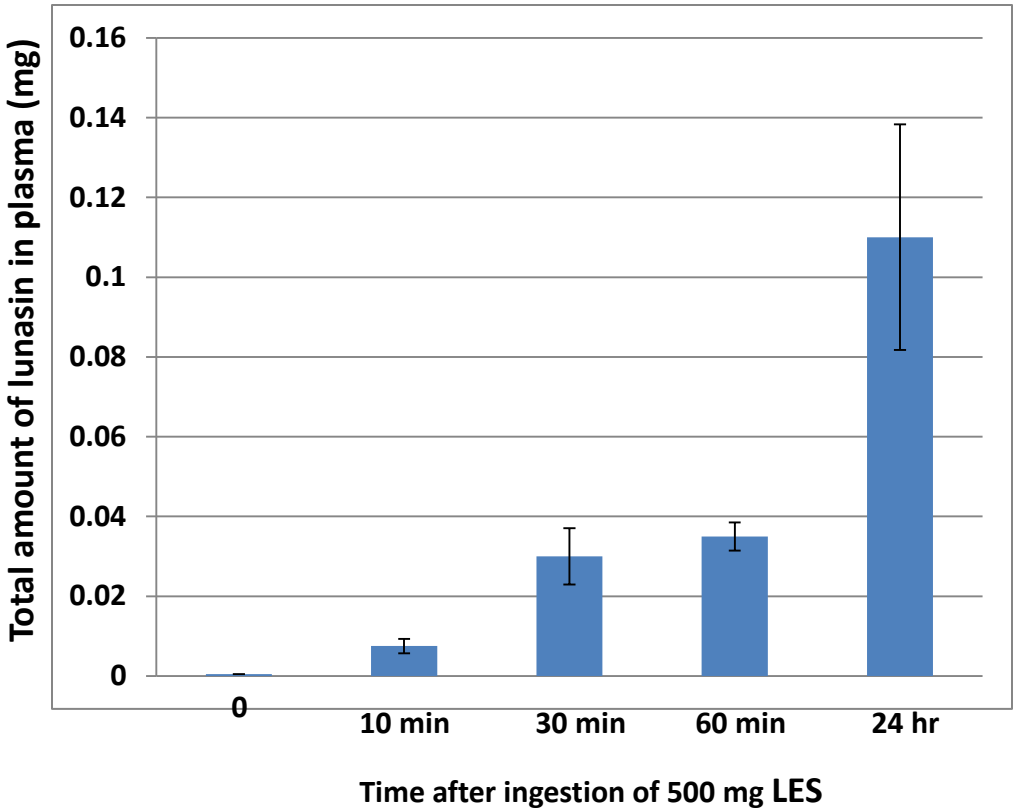

Figure 1. Bioavailability of lunasin after ingestion of 500 mg LES. Blood draws were taken at 10 min, 30 min, 60 min and 24 hr after ingestion of 500 mg LES. The total amount of lunasin in blood plasma was computed assuming a total blood volume of 3000 mL [16].

In a previous study using five 1yr-old Rapacz pigs treated only with 250 mg LES [13], the free fatty acid levels at different time points after treatment did not deviate significantly from the free fatty acid levels found in the untreated pigs at Time 0 (data not shown). The standard diet of the Rapacz pigs used in the experiment was a soy-free, casein-based pig chow (BV233). When we supplemented this standard diet daily with 500 mg of LES and added the 18 gm Reliv Now dietary supplement, we observed a dramatic decrease in free fatty acid (FFA) levels after 4 weeks of supplementation compared to the untreated pigs at Time 0 (Table 1). The reduced FFA levels persisted at 6 and 8 weeks of supplementation and then increased to Time 0 levels after 4 weeks of washout (Time: 12 weeks), when supplementation was stopped after 8 weeks (Table 1). Using Time 0 as baseline FFA level (set at 1.0), and then standardizing FFA levels at each time point after treatment by dividing from the baseline value, we can determine the percentage decrease in FFA levels at different time points with daily treatment with 500 mg of LES and 18 gm Reliv Now (Table 1 and Fig.2). The FFA levels at 4, 6 and 8 weeks of treatment were all significantly lower, at 74%, 65% and 39% respectively, than the pre-treatment levels of FFA (Table 1). When treatment ended after 8 weeks and the pigs were fed only the standard casein diet, the FFA levels went back up close to the baseline level after 4 weeks of washout (Time: 12 weeks)

(Table 1 and Fig. 2). These results show that the supplementation of the standard casein diet with 500 mg LES and 18 gm of Reliv Now can cause the significant reduction in plasma free fatty acid levels, and when the supplementation is stopped, the FFA level goes back up to pre-treatment baseline levels.

Table 1. Mean plasma levels of free fatty acid, leptin and adiponectin in 5 Rapacz pigs at different time (in weeks) after daily supplementation with 500 mg LES and 18.3 gm of Reliv Now. The amounts at Time 0 were used as baseline levels and set at 1.00. Values at different time points were standardized by dividing from baseline level and shown in parentheses. The 12-week treatment corresponds to a 4-week washout period (W/O) after the last supplementation treatment at 8 weeks.

Time	Free fatty acid (ng/mL)	Leptin (ng/mL)	Adiponectin (mg/mL)
0	0.36 (1.00)	16.19 (1.00)	29.15 (1.00)
4wk	0.09 (0.26)	24.6 (1.52)	34.98 (1.20)
6wk	0.12 (0.35)	26.55 (1.64)	46.64 (1.60)
8wk	0.22 (0.61)	20.24 (1.25)	40.81(1.40)
12wk W/O	0.33 (0.92)	16.68 (1.03)	30.32 (1.04)

To determine the mechanism of action involved in the reduction of FFA, the levels of adiponectin, an adipocyte hormone involved in FFA catabolism and oxidation [17], was measured in the blood plasma of Rapacz pigs before and after supplementation with 500 mg LES and 18 gm of Reliv Now. The adiponectin levels increased significantly after 4 weeks (20% increase), 6 weeks (60%) and 8 weeks (40%) of treatment from baseline level (Table 1 and Fig.2). After the 4 weeks washout (T=12weeks), plasma adiponectin went back down to baseline level (Table 1 and Fig. 2). This result is inverse that of free fatty acid levels, indicating that the effect of the treatment with LES and Reliv Now on reduced levels of free fatty acid can be explained by the increased amounts of adiponectin in the plasma which is important in fatty acid oxidation [18].

Leptin is another adipocyte hormone involved in lipolysis that can also reduce free fatty acid levels [19]. Leptin levels in the blood plasma of pigs after 4 and 6 weeks of treatment with LES and Reliv Now were significantly increased by 52% and 64% respectively from baseline levels, although there is a wide variation (Table 1 and Fig. 2). The mean leptin level after 8 weeks of treatment was higher than baseline level but because of the wide variation in values among the 5 pigs, it was not significantly different from baseline (Fig. 2). At 12 weeks (after 4 weeks washout), the leptin levels have gone down to baseline level, similar to the results obtained with adiponectin and FFA (Table 2 and Fig. 2). The increase in leptin level after 4 and 6 weeks and to a lesser extent 8 weeks of treatment can also explain the reduced levels of FFA. The similar trend of increased adiponectin and leptin expression levels upon

treatment with LES and Reliv Now, suggest that they may be acting synergistically to reduce FFA levels.

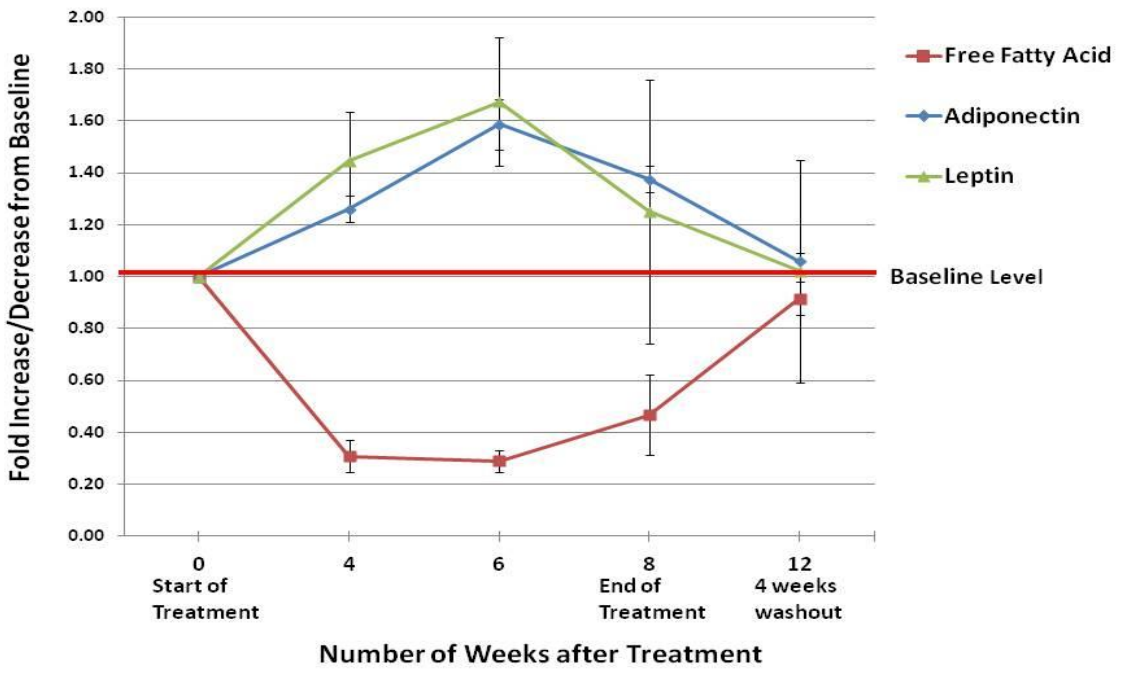

Figure 2. Effect of Lunasin-enriched Soy extract (LES) and Reliv Now daily supplementation on plasma free fatty acid (FFA), adiponectin (Adp) and leptin (Lep) levels in 5 Rapacz pigs with disposition to heart disease and obesity. Mean values from 5 pigs and their standard error were standardized using the pre-treatment (Time 0) levels of FFA, Adp and Lep as baseline levels. Values above the standard baseline level of 1.0 indicate increased levels of Adp and Lep, and below baseline level indicate decreased levels of FFA.

FFA levels are elevated in most obese subjects and are directly correlated with weight [1, 20]. To determine whether the reduced FFA levels in the pigs, upon treatment with LES and Reliv Now, had any effect on weight, we determined the average weight of the pigs at each time point and measured the weight gain in between each time point. Results shown in Fig. 6 show that the pigs did not gain any weight at the time point between 6 weeks and 8 weeks when the FFA levels were at its lowest. The highest gain in weight (4.8 kg.) occurred at 0 to 4 weeks of treatment, when FFA levels were still high at baseline levels. Between 4 weeks and 8 weeks, gain weight was at 1.8 kg which corresponds to low levels of FFA. At 4 weeks after washout (T=12 weeks), pigs gained 2.8 kgs which corresponds to the increase of FFA to baseline levels. These results indicate that the reduction of FFA due to treatment with LES and Reliv Now, led to a corresponding reduction in weight gain of the pigs.

Increase in leptin and adiponectin levels are both implicated in weight reduction and management. Leptin signals the brain when the body had enough food, producing the feeling of satiety [20]. Like leptin, adiponectin exerts some of its weight reduction also through the brain [17]. The two hormones perform complementary actions and can have additive effects on weight management. Results from our experiment show that both leptin and adiponectin levels were increased when LSE and Reliv NOW were added to the basic diet and these corresponded to a significantly lower weight gain in the Rapacz pigs.

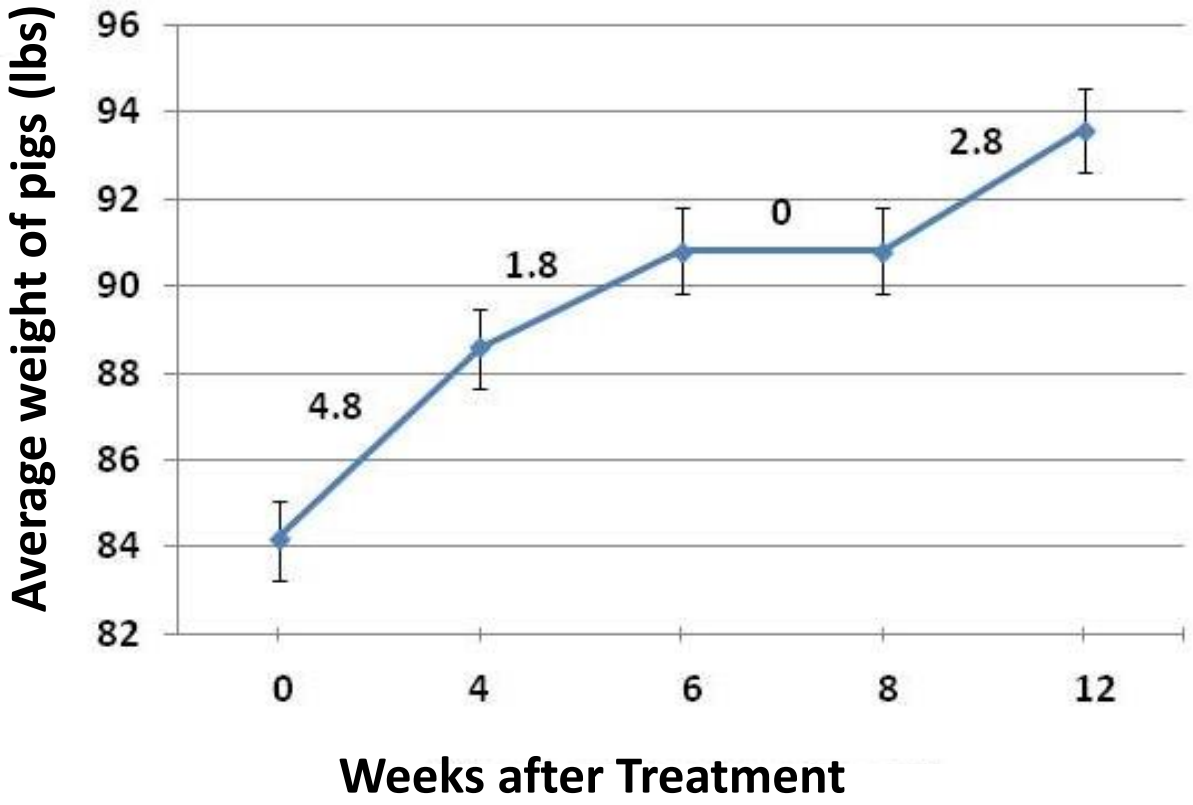

Figure 3. Effect of Lunasin-enriched Soy extract (LES) and Reliv NOW daily supplementation on the average weight of 5 Rapacz pigs. Numbers in between timepoints indicate average weight gain of the 5 pigs. Error bars are the standard error of the mean.

DISCUSSION

Lunasin is the first natural substance to be identified as a histone acetylase inhibitor, although it does not directly affect the histone acetylase enzyme [4]. It inhibits H3 and H4 acetylation by binding specifically to deacetylated lysine residues in the N-terminal tail of histones H3 and H4, making them unavailable as substrates for histone acetylation [4]. Lunasin binds to H3-Lysine 14 and inhibits its acetylation by the PCAF (p300/CBP-associated factor) histone acetylase enzyme [13]. Lunasin also binds and inhibits acetylation of H4-Lysine 8, resulting in a conformational change in the histone H4 N-terminus that promotes H4-Lysine 16 acetylation [12]. The acetylation of H4-Lysine 16 results in greater chromatin accessibility and an increase in transcriptional activity and gene expression [21, 22]. By increasing H4-Lysine 16 acetylation, lunasin has been shown to increase expression of chemopreventive genes in prostate cells [12, 23].

The Reliv Now dietary supplement has been marketed by Reliv International to provide optimal and bioavailable levels of vitamins, minerals, soy protein and other herbal agents, with emphasis on maintenance of cardiovascular health. Although containing soy protein, the amount of bioactive lunasin in one serving of Reliv Now is approximately 4-fold lower than what is found in 500 mg of LES (data not shown). It also contains Pycnogenol, a natural antioxidant proven to help fight free radical damage, improve blood circulation and lower blood glucose levels [24]. However, there are no studies that show Pycnogenol and the Reliv Now dietary supplement affecting plasma levels of free fatty acids (FFA), leptin and adiponectin.

Previous experiments using diet supplementation of 250 mg LES on Rapacz pigs have shown that lunasin can reduce LDL cholesterol by inhibiting expression of HMG-CoA reductase and cholesterol biosynthesis in the liver [13]. However, the FFA levels remained relatively unchanged with LES supplementation alone. It is only when we added one serving of the Reliv Now in combination with 500 mg LES to the pig's casein-based diet did we see a significant reduction in FFA levels. When supplementation was stopped during the washout period, FFA went back up to baseline levels after 4 weeks. These results indicate that the reduction of FFA can only be achieved by combining LES with the Reliv Now dietary supplement. Further studies need to done to identify the specific ingredient in Reliv Now that synergistically interacts with lunasin to reduce FFA levels.

The FFA lowering activity of LSE and Reliv NOW can be attributed to the increased plasma levels of leptin and adiponectin that act synergistically to reduce fatty acid by lipolysis (leptin) and by fatty acid oxidation (adiponectin). Lunasin has been shown to upregulate gene expression by promoting H4-Lysine16 acetylation but has not been shown to be a transcriptional activator [12, 23]. A working hypothesis to explain the increase in plasma levels of leptin and adiponectin is that 1) the transcriptional activation of the leptin and adiponectin genes is modulated by bioactive ingredients found in Reliv Now, and 2) lunasin then upregulates the expression of the activated leptin and adiponectin genes.

Elevated levels of free fatty acids (FFA) have been associated with obesity and insulin resistance and can lead to diseases like type 2 diabetes, metabolic syndrome and cardiovascular disease [1, 3]. Despite the need, there is a lack of effective treatments to lower plasma free fatty acids [1]. The

discovery that the combination of LSE and Reliv Now leads to the lowering of free fatty acids provides a novel way of preventing insulin resistance and the associated disease modalities arising from this health condition.

Besides lowering FFA by oxidation [17], adiponectin has also been shown to increase glucose uptake [18], to lower triglyceride [17], to increase insulin sensitivity [18], to weight loss [18] and to control energy metabolism [25]. Low level of adiponectin is an independent risk factor for developing metabolic syndrome [26] and type 2 diabetes [27]. Leptin, on the other hand, lowers free fatty acid by lipolysis [19]. Leptin has also been shown to inhibit appetite and increase energy expenditure that lead to weight loss [28] and the improvement of T cell immune response which prevents atherosclerosis [29].

There are currently no effective treatments to increase adiponectin and leptin levels, so the elevation of endogenous levels of adiponectin and leptin by LSE and Reliv Now dietary supplements provides an alternative, low-cost treatment. However, clinical studies on human subjects first need to be conducted before daily supplementation with LSE and Reliv Now can be recommended to potentially reduce obesity, type 2 diabetes, metabolic syndrome, atherosclerosis and risk for cardiovascular disease.

CONCLUSION

Supplementation with lunasin-enriched soy extract (LES) in combination with the dietary supplement Reliv Now significantly reduces free fatty acid (FFA) levels, which can be explained by the coordinate increase in endogenous leptin and adiponectin levels in the blood plasma of LDL-receptor mutant pigs. This novel effect of LES and Reliv Now supplementation provides a potentially effective, low-cost and natural alternative to therapeutic drugs to help control the current epidemic of obesity, metabolic syndrome and heart disease.

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CONFLICT OF INTEREST DISCLOSURE

Dr. Galvez is a consultant and a scientific advisor for Soy Labs, LLC and Reliv International, Inc. Drs. Matel and Bowles and Ms. Ivey have no financial conflict of interest to disclose.

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