

# Enhancement of Cardio-Protective Effects and Attenuation of Adverse Effects of Female Sex Hormones on Cultured Human Vascular Smooth Muscle Cells by a Combination of Ascorbic Acid, Lysine, Proline, Arginine, Cysteine, and Epigallocatechin Gallate

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## ABSTRACT

In this *in vitro* study, the effects of adjunctive use of a formulation containing ascorbic acid, lysine, proline, arginine, N-acetyl cysteine, and epigallocatechin gallate (NS) with female sex hormones were tested on human aortic smooth muscle cells (SMC). Estradiol and progesterone stimulated DNA synthesis in SMC 30% and 24% respectively at 25–150 nmol/L concentrations. NS (20 µg/ml) inhibited SMC growth by 30% over the control, and reversed the stimulatory effect of the sex hormones to a maximum of 25% inhibition. Dehydroepiandrosterone sulfate (DHEAS) inhibited SMC growth by 50% at 0.1 mmol/L. Addition of NS enhanced the DHEAS inhibitory effect to 70% as compared to the control. DHEAS and progesterone significantly increased SMC capacity to invade Matrigel by 20% and 60%, respectively. Addition of NS reversed the stimulatory effects, producing up to 60% inhibition of SMC invasion. Addition of NS reversed the effects of DHEAS on total collagen synthesis in SMC from 28% stimulation to 56% inhibition. Estradiol, progesterone, and DHEAS demonstrated some inhibition of tumor-necrosis-

factor-alpha-stimulated SMC secretion of interleukin (IL) 1-beta, IL-6, and monocyte chemo attractant protein 1 in cultured media; NS enhanced inhibition of these cytokines under most conditions. The results of this study imply that the specific formula of nutrients tested enhances the cardio-protective effects of female sex hormones and counteracts their adverse effects on atherogenic properties.

**KEYWORDS:** cardiovascular, estradiol, progesterone, DHEAS

## INTRODUCTION

Large numbers of menopausal women are using hormone replacement therapy (HRT) with different forms of estradiol either taken alone or supplemented with progesterone for counteracting such adverse effects as hot flashes, sudden mood changes, loss of bone mass, and others. While estradiol has been shown to exert some protective effects on the cardiovascular system, such as suppression of vascular monocyte chemotactic protein-1 expression during early atherogenesis,<sup>1</sup> hormone replacement therapy has produced major adverse effects that far outweigh these limited benefits. After a mean of 5.2 years of follow-up, the estrogen and progesterone versus placebo arm of the Women's Health Initiative study of the risk of cardiovascular disease was stopped because of adverse cardiovascular effects—higher rates of coronary heart disease (CHD), stroke, and venous

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thrombosis among women taking estrogen and progesterone compared with placebo.<sup>2</sup> In addition, recent studies evaluating the effect of estrogen alone and with progesterone on both coronary artery stenosis and carotid intima medial wall thickness have also shown little benefit from hormone therapy.<sup>3</sup> Due to these and additional adverse effects, such as increased potential of developing breast cancer, there is an urgent need for effective alternative therapy.<sup>4</sup>

The formation of an atherosclerotic lesion is associated with drastic behavioral modifications by arterial wall smooth muscle cells (SMC), including: massive migration of SMC from the vascular medial to the intimal layer, dedifferentiation of SMC to proliferating phenotype, and increased secretion of inflammatory mediating cytokines, as an autocrine response to inflammatory stimuli. These events trigger vessel wall thickening and monocyte recruitment from blood, and lead to progression of the atherogenic cascade. Pathogenic changes of the blood vessel wall in atherosclerosis are accompanied by neointimal thickening resulting from the increased deposition of extracellular matrix proteins by smooth muscle cells that migrate and proliferate in the affected blood vessel areas.<sup>5</sup> Various patho-physiologic events can promote this process, such as inflammation involving local secretion of inflammatory mediators (cytokines), oxidative processes accompanying low-density lipoprotein and lipoprotein(a) deposition, and intracellular-membrane-mediated events, such as changes in protein kinase C activity.<sup>6-7</sup> Various matrix components also affect cellular proliferation, differentiation, and expression of specific genes.<sup>8</sup>

The extracellular matrix (ECM) serves as a reservoir to which various growth factors may be bound, affecting vascular cell growth, differentiation status, and ECM production. Thus, factors affecting matrix components may influence various metabolic processes that accompany initiation and progression of atherosclerosis.

Rath et al proposed that chronic sub-clinical vitamin C deficiency is a primary cause for atherosclerotic plaque formation, as it leads to deposition of lipoprotein(a) and fibrinogen/fibrin in the vascular wall.<sup>9</sup> Ascorbic acid is essential to the synthesis and maintenance of collagen, which maintains blood vessel wall stability. Prolonged deficiency of ascorbic acid, a nutrient not produced in the body, hinders the enzymatic hydroxylation of proline and lysine residues in collagen molecules, thereby weakening the stability of the vascular wall. Thus, combined supplementation with ascorbic acid and lysine has been proposed as a preventive measure to atherosclerosis development.<sup>9-10</sup> A previous study of the direct and matrix-mediated effects of ascorbate on the proliferation rate of vascular smooth muscle cells (VSMC) isolated from guinea-pig aorta, revealed diminished cell proliferation in the presence of 0.5 – 2 mM ascorbate, in a dose-dependent manner without cytotoxic effect.<sup>11</sup> Additionally, a number of studies show cardio-protective effects, through potent antioxidant activity, from chronic tea consumption.<sup>12</sup>

Naturally occurring compounds demonstrate wider spectra of biological activity and fewer side effects than synthetic drugs. It is known that a mixture of natural compounds often produces a synergistically-enhanced therapeutic effect. The objective of this study was to investigate whether a specific formulation of ascorbic acid, lysine, proline, arginine, N-acetyl cysteine, and epigallocatechin gallate (from green tea extract) would modulate the cardiovascular effects of 17- $\beta$  estradiol and progesterone, using the cultured vascular smooth muscle cell model.

## METHODS AND MATERIALS

**Materials:** Tissue culture plastics were obtained from Becton Dickinson, (San Jose, CA). Tissue culture supplies (growth media, antibiotics, and trypsin-EDTA) were obtained from Life Technologies, (Grand Island, NY). Fetal bovine serum (FBS) was from BioWhittaker (Walkersville, MD). Scintillation fluid BetaBlend and (methyl-3H) Thymidine (25 Ci/mole) were from ICN Biomedicals (Costa Mesa, CA, USA). L-ascorbic acid, bovine serum albumin (fraction V) (BSA), 17- $\beta$  estradiol, progesterone, and other chemicals were from Sigma-Aldrich, (St. Louis, MO).

**Cell Culture:** Cell cultures of human aortic smooth muscle cells (SMC) were obtained from BioWhittaker. SMC were cultured in Dulbecco's modified Eagle medium (hereafter DMEM), supplemented with 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 10% FBS (v/v) at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>, and were split 1:3 to 1:5 upon reaching confluence. SMC at passages 5–8 were used in experiments.

**Cell proliferation assay:** SMC proliferation was assayed by (3H)-thymidine incorporation into cellular genetic material. Cells were plated in 24-well plates at a density of 10,000 cells per cm<sup>2</sup> in 0.5 ml of DMEM supplemented with 2% FBS. The attached cells were supplied every 24 hours with fresh growth medium plus additions, as specified in the protocols. A stock solution of NS (composed of vitamin C 700 mg, L-lysine 1000 mg, L-proline 750 mg, L-arginine 500 mg, N-acetyl-cysteine 200 mg, and standardized green tea extract (80% polyphenol, 20% EGCG) 1000 mg) was prepared daily immediately before addition to cell cultures by dissolving in DMEM to a concentration of 10 mg/ml, vigorously vortexed for 1 minute, and filtered through a 0.2  $\mu$ m sterile filter. Cell proliferation was measured 3 days later by the addition of 1  $\mu$ Ci/ml (3H)-thymidine to the cell culture for the last 24 hours of the experiment. Cells were washed three times with cold phosphate-buffered saline (PBS), pH 7.2, incubated with 10% trichloroacetic acid for 15 minutes at 4°C, washed with cold ethanol, air-dried, solubilized in 0.5 N sodium hydroxide, and then neutralized with hydrochloric acid. Samples were mixed with scintillation fluid and counted using a liquid scin-



tillation counter (model 6500 LS, Beckman Instruments, (Porterville, CA). Cellular DNA-incorporated radioactivity was expressed as d/min per well.

**Cell invasion assay:** SMC cultures grown in 75 cm<sup>2</sup> flasks were metabolically labeled by incubating in growth medium containing 1  $\mu$ Ci/ml (3H) thymidine for 24 hours. Cells were washed with PBS, suspended by trypsinization in DMEM supplemented with 10% FBS, and placed on upper surface of porous membrane (3  $\mu$ m pores) covered with Matrigel (Becton Dickinson). After cell attachment (1.5-2 hours) the cell culture medium was replaced by serum-free DMEM supplemented with tested compounds, as indicated. 10 ng/ml basic fibroblast growth factor (Clonetics Walkersville, MD) was added to the lower chamber. After 24 hours, incubation inserts were removed from the plate and extensively washed with PBS. SMC invasion was estimated by counting radioactivity present on the lower surface of the porous membrane after removal of cells remaining on the upper surface of the insert with cotton tissue.

**Collagen synthesis:** SMC were placed on 24-well plates and grown as a monolayer to confluence for 5-7 days, at which point growth media were replaced with DMEM containing 2% FBS and indicated compounds. Cells were incubated for 3 days with fresh media added daily. For the last 24 hours of incubation, 1  $\mu$ Ci/ml (3H) proline (Sigma-Aldrich) was added to the media. Cell layers were washed three times with PBS and solubilized with 0.5N sodium hydroxide for 18 hours at 60°C. Collagen synthesis was estimated as radioactivity retained within the cell/extracellular matrix layer.

**Cytokine secretion:** SMC were placed on 24-well plates and grown to a confluent monolayer. 5-7 days later, the growth media was replaced with serum-free DMEM and treated with NS. After 24 hours, the media were replaced with fresh media containing the same compounds and tumor necrosis factor alpha (TNF $\alpha$ , Sigma-Aldrich). After 24 hours incubation, the conditioned media were harvested for analysis and assayed for indicated cytokine presence using Quantikine ELISA kits (R&D Systems (Minneapolis, MN) according to manufacturer's protocols.

**Test Reagents:** In the design of the experiment, a stock solution of a nutrient mixture (NS), weighing 4.4 gm, was prepared daily to treat the cells, composed of the following nutrients: Vitamin C (as ascorbic acid and as Mg, Ca, and palmitate ascorbate) 700 mg; L-lysine 1000 mg; L-proline 750 mg; L-arginine 500 mg; N-acetyl cysteine 200 mg; standardized green tea extract (80% polyphenol) 1000 mg; selenium 30 mg; copper 2 mg; manganese 1 mg.

**Statistical Analysis:** All experiments were performed at least twice with cells from different passages and originating from different donors. The data represent the average ( $\pm$  S.D.) from representative experiments performed in three or more replications. Student's t-test was used for data comparison.

## RESULTS

### Effect of female sex hormones and NS on SMC growth

Estradiol and progesterone-stimulated DNA synthesis was increased by 24% and 30% respectively at progesterone and estradiol concentrations of 150 nmol/L (Figures 1A-B). Cell growth stimulatory effects were attenuated with hormone concentrations increased to 450 nmol/L. NS inhibited SMC growth by 30% at 20  $\mu$ g/ml (corresponding ascorbic acid content was 20 mmol/L) when used individually, and reversed the hormone-associated stimulatory effect to inhibitory (25% maximum inhibition) when used with estradiol or progesterone. Dehydroepiandrosterone sulfate (DHEAS), a potential metabolic precursor of estrogen, inhibited SMC growth by 48% at 0.1 mmol (Figure 1C). Addition of NS further enhanced the DHEAS inhibitory effect to 68% inhibition as compared to the control.

### Effects of female sex hormones and NS on SMC Matrigel invasion

DHEAS and progesterone significantly increased SMC Matrigel invasion by 37% and 54% respectively (Figure 2). SMC Matrigel invasion was significantly inhibited in the presence of NS when tested with all three of these female sex hormones: estradiol by 67% ( $p < 0.01$ ), progesterone by 77% ( $p < 0.01$ ), and DHEAS by 78% ( $p = 0.03$ ).

### Effects of female sex hormones and NS on total collagen synthesis by SMC

DHEAS increased SMC collagen synthesis by 28%. Addition of NS reduced collagen synthesis in the presence of all sex hormones studied, reversing the effects of DHEAS on SMC total collagen synthesis from 28% stimulation to 56% inhibition as compared to the control (Figure 3). FSH, LH, estradiol, and progesterone did not affect collagen synthesis when used individually.

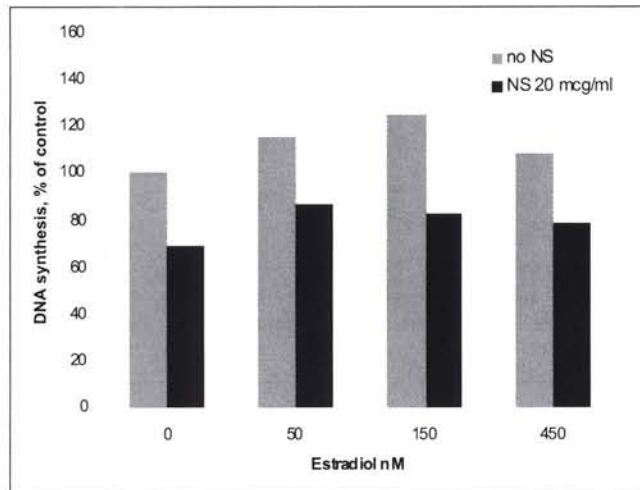
### Effects of female sex hormones and NS on the secretion of inflammatory mediators by SMC

Estradiol, progesterone, and DHEAS slightly inhibited (up to 20%) tumor-necrosis-factor-alpha stimulated SMC secretion of the inflammation mediators: interleukin 1-beta and interleukin-6 in culture media. The inhibitory effect was further enhanced by addition of NS. DHEAS inhibited TNF $\alpha$ -stimulated secretion of monocyte chemoattractant protein; however, NS did not enhance this effect.

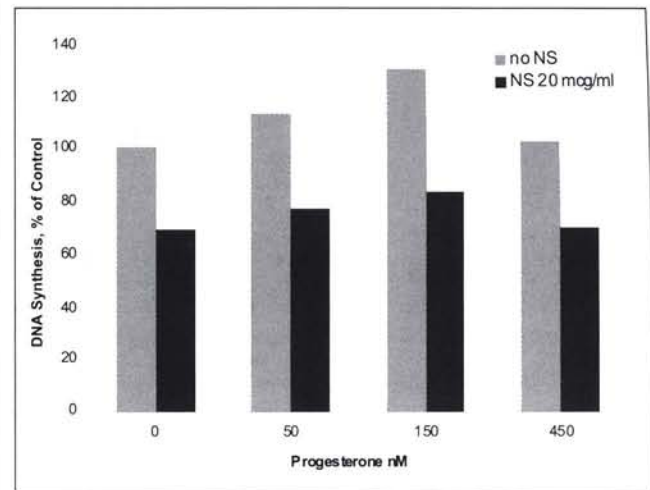
## DISCUSSION

The results from this study demonstrated that NS attenuated the pro-atherogenic modification of SMC physiological properties, such as increased growth rate and invasiveness, excessive production of extracellular matrix components, and autocrine production of inflammatory cytokines, stimulated by female sex hormones (estradiol, progesterone, and dehydroepiandrosterone sulfate). Estradiol and progesterone were found to stimulate SMC growth at 50

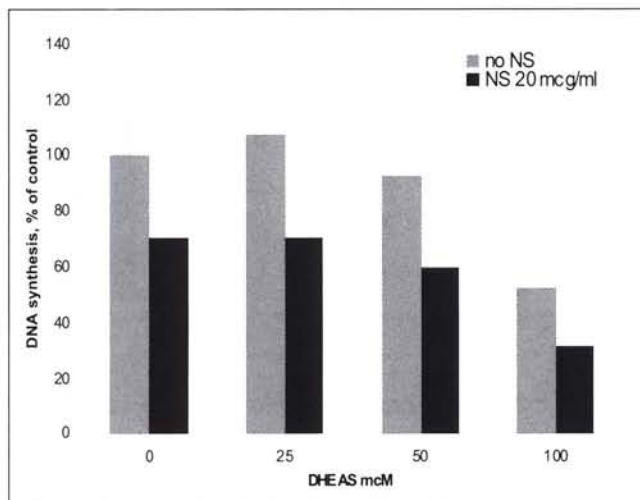
**Figure 1A:** Effect of estradiol independently and in the presence of the nutrient mixture (NS 20mcg/ml) on aortic SMC DNA synthesis (as % of control) tested at 0, 50, 150, and 450 nM concentrations of estradiol.



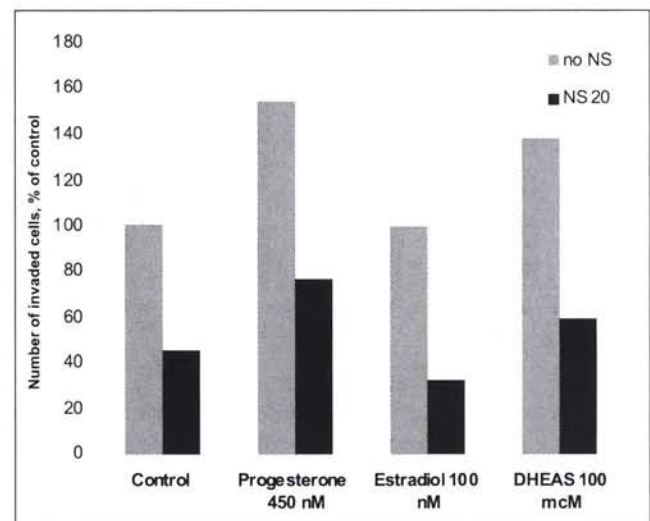
**Figure 1B:** Effect of progesterone independently and in the presence of the nutrient mixture (NS 20 mcg/ml) on aortic SMC DNA synthesis (as % of control) tested at 0, 50, 150, and 450 nM concentrations of progesterone.



**Figure 1C:** Effect of NS (mcg/ml) and DHEAS on aortic SMC DNA synthesis (as % of control) tested at 0, 25, 50, and 100 mcM concentrations of DHEAS.



**Figure 2:** Effect of estradiol, progesterone, and DHEAS on SMC invasion through extracellular matrix (as % of control) tested independently and in the presence of NS 20 mcg/ml.



and 150 nM, which was attenuated at all concentrations in the presence of NS 20  $\mu$ g/ml. These results contradict previously reported anti-mitotic and anti-migrating effects of estradiol on cultured porcine SMC.<sup>13</sup> Though DHEAS did not stimulate SMC proliferation, NS showed inhibition of SMC proliferation at all concentrations of DHEAS. Both progesterone and DHEAS significantly increased SMC invasiveness. Though estradiol did not stimulate SMC invasion at 100 nM, SMC Matrigel invasion was significantly inhibited in the presence of NS when tested with all three of these female sex hormones: estradiol by 67% ( $p < 0.01$ ), progesterone by 77% ( $p < 0.01$ ), and DHEAS by 78% ( $p = 0.03$ ).

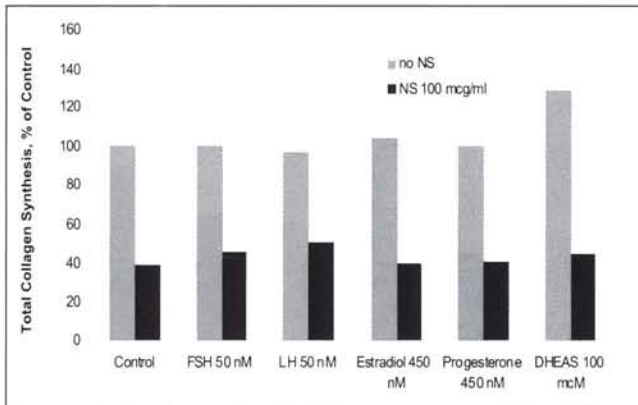
Furthermore, NS inhibited the stimulatory effect of DHEAS on collagen synthesis. Thus, the synergistic activity

of the specific nutrient mixture significantly enhanced vascular wall stability as evidenced by the significantly decreased SMC Matrigel invasion. Stability of the vascular wall is dependent upon presence of sufficient quantities of ascorbic acid, lysine, and proline for synthesis of optimal collagen structure, as discussed previously. Various studies have shown that restructuring of the vascular matrix is facilitated by ascorbate, pyridoxine, L-lysine, and L-proline.<sup>14-15</sup>

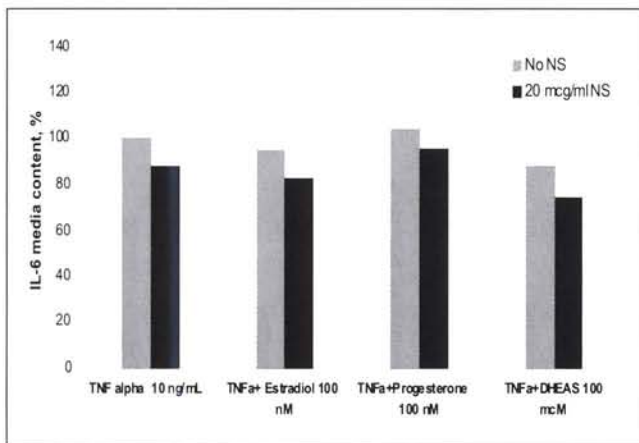
The inhibitory effects of other individual nutrients composing NS have been reported in both clinical and experimental studies. Anti-atherogenic effects of green tea extract have been demonstrated in animal and *in vitro* studies. For example, apoprotein E-deficient mice fed green tea for 14 weeks had attenuation of aortic atheromatous areas by 23%



**Figure 3:** Effect of various female sex hormones independently and in the presence of 100 mcg/ml of the nutrient mixture (NS) on aortic SMC collagen synthesis (as % of control).



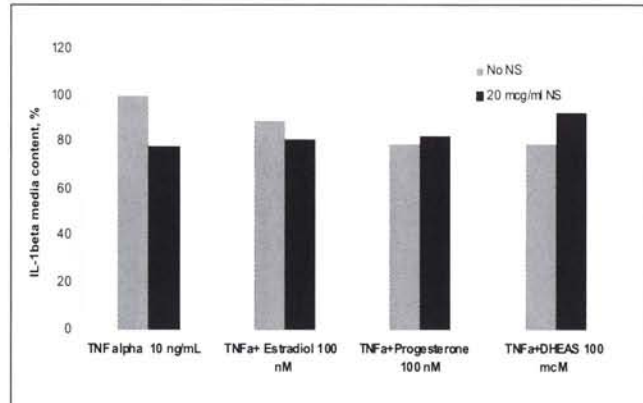
**Figure 4B:** Effect of TNF-alpha and female sex hormones independently and in the presence of the nutrient mixture (NS) on aortic SMC secretion of interleukin-6.



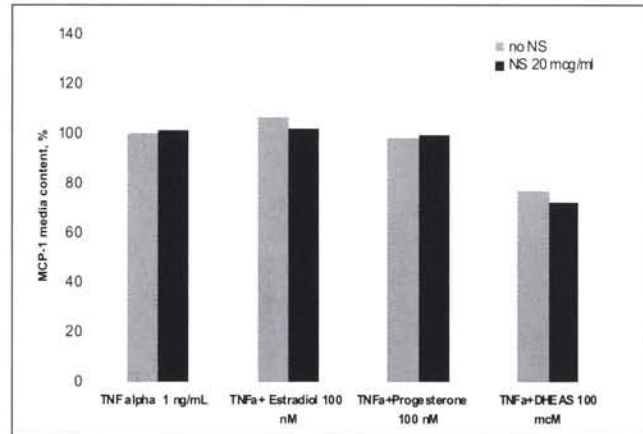
and decreased aortic cholesterol and triglyceride levels over the control group.<sup>12</sup> Furthermore, epidemiological and clinical studies have documented the benefits of individual nutrients in prevention of cardiovascular disease.<sup>16-17</sup>

However, individual nutrient effects have been shown to be enhanced when acting in synergy. Our previous studies demonstrated that the anticancer effect of ascorbic acid, proline, lysine, and EGCG on several cancer cell lines in tissue culture studies was greater than that of the individual nutrients.<sup>18</sup> Furthermore, cardio-protective effects of NS were confirmed in our study of the effect of nutrient supplementation in progression of early coronary atherosclerosis. In this pilot study, the extent of coronary calcification in 55 patients diagnosed with early coronary atherosclerosis was measured prior to nutrient supplementation and after one year of intervention, using an Imatron C-100 Ultrafast CT scanner.<sup>19</sup> Progression of coronary calcification, as determined by the CAS score, decreased significantly (from 0.49

**Figure 4A:** Effect of TNF $\alpha$  and female sex hormones independently and in the presence of the nutrient mixture (NS) on aortic SMC secretion of interleukin-1beta.



**Figure 4C:** Effect of TNF-alpha and female sex hormones independently and in the presence of the nutrient mixture (NS) on aortic SMC secretion of monocyte chemoattractant protein-1(MCP-1).



mm<sup>2</sup> to 0.28 mm<sup>2</sup> monthly growth) after one year of nutritional intervention.

## CONCLUSION

While clinical trials are necessary to examine the full cardio-protective benefits of the combination of nutrients tested, the results of this study imply that the specific combination of ascorbic acid, lysine, proline, arginine, N-acetyl cysteine, and epigallocatechin gallate tested enhances the protective effects of estradiol, progesterone, and DHEAS on the cardiovascular system and inhibits the adverse effects these hormones have on atherogenic properties. These results, coupled with prior research studies, demonstrating the cardio-protective properties of the synergistic nutrient combination of ascorbic acid, lysine, proline, arginine, and EGCG, support its potential as a strong candidate in the prevention of cardiovascular disease.

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