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Composition, Efficacy, and Safety of Spinach Extracts

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Abstract: *Spinach leaves, containing several active components, including flavonoids, exhibit antioxidative, antiproliferative, and antiinflammatory properties in biological systems. Spinach extracts have been demonstrated to exert numerous beneficial effects, such as chemo- and central nervous system protection and anticancer and antiaging functions. In this review article, we present a compilation of data generated in our laboratories and those of other investigators describing the chemical composition of spinach, its beneficial effects, relative safety information, and its recommended inclusion in the human diet. A powerful, water-soluble, natural antioxidant mixture (NAO), which specifically inhibits the lipoxygenase enzyme, was isolated from spinach leaves. The antioxidative activity of NAO has been compared to that of other known antioxidants and found to be superior in vitro and in vivo to that of green tea,* N*-acetylcysteine (NAC), butylated hydroxytoluene (BHT), and vitamin E. NAO has been tested for safety and is well tolerated in several species, such as mouse, rat, and rabbit. NAO has been found to be nonmutagenic and has shown promising anticarcinogenic effects in a few experimental models, such as skin and prostate cancer; it has not shown any target-organ toxicity or side effects. The current review provides epidemiological and preclinical data supporting the efficacy of extracts of spinach and the safety of its consumption.*

Introduction

Considerable evidence exists for the role of antioxidative constituents of fruits and vegetables in the maintenance of health and disease prevention (1). Spinach (*Spinacia oleracea*) is one of the most important antioxidative vegetables, usually consumed after boiling either fresh or frozen leaves. Freshly cut spinach leaves contain approximately 1,000 mg of total flavonoids per kilogram. The possible presence of flavonoid-like compounds in spinach was first reported in 1943 (2), but nearly 20 yr elapsed before the structure of the flavonol isolated from spinach leaves was established as patuletin (3,5,7,3′,4′-penthahydroxy-6-methoxyflavone) and the presence of spinacetin was confirmed (3). In addition, the existence of several flavonol glycosides in a methanolic extract of spinach leaves was reported (4,5). The occurrence of at least 10 flavonoid glycosides has now been reported in spinach. These are glucuronides and acylated di-and triglycosides of methylated and methylene dioxide derivatives of 6-oxygenated flavonols (4–6). Glucuronides are more water-soluble than glycosides and acylated compounds that remain in the tissue after cooking in boiling water.

Flavonoids and other phenolic constituents act as antioxidants by the free-radical scavenging properties of their hydroxyl groups. Extensive conjugation across the flavonoid structure and numerous hydroxyl groups enhance their antioxidative properties, allowing them to act as reducing agents, hydrogen- or electron-donating agents, or singlet-oxygen scavengers (7–9). Results from the in vitro oxygen radical absorbance capacity (ORAC) assay have shown that, among various fruit and vegetable extracts, foods with the highest ORAC activity include spinach, strawberries (10,11), and blueberries (12). The antioxidant capacity of spinach flavonoids has been determined by the free-radical scavenging assay using DPPH (2,2-diphenyl-1-picrylhydrazyl) radical (13) and was compared with that of Trolox, a synthetic analogue of vitamin E. The most active products were those derived from patuletin with a 3′,4′-dihydroxyl group. The incorporation of a feruloyl residue increased the free-radical scavenging activity. During storage of spinach leaves, a decrease in the total antioxidant activity was observed. Boiling of fresh-cut spinach leaves extracted approximately 50% of the total flavonoids and 60% of the vitamin C in cooking water; however, flavonoid glucuronides were extracted more than other glycosides (13).

The purpose of the current review is to summarize previous reports of the beneficial effects of consumption of spinach leaves or spinach extracts on human health. The review provides epidemiological and preclinical data supporting the efficacy and safety of spinach consumption.

Isolation of Natural Antioxidant Mixture From Spinach Leaves

While screening for inhibitors of lipid peroxidation, we discovered a powerful, water-soluble, natural antioxidant mixture (NAO) in spinach leaves, which specifically inhibited the lipoxygenase enzyme (14,15). After further purifica-

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tion and characterization by our group, the chemical composition of NAO was described (16).

Spinach leaves were homogenized with water, and the 20,000 *g* supernatant containing the antioxidant activity was extracted with a water:acetone (1:9) solution and further purified on reverse phase HPLC using a C-8 semipreparative column. Elution with 0.1% TFA resulted in the revelation of five hydrophilic peaks. Further elution with acetonitrile in TFA resulted in revealing seven additional hydrophobic peaks. The overall recovery of NAO comprised 3 g from 1 kg of wet spinach leaves.

Chemical Characterization of NAO

Based on 1H and 13C NMR spectroscopy, four of the seven hydrophobic fractions were identified as glucuronic acid derivatives of flavonoids, three additional fractions as *trans* and *cis* isomers of *p*-coumaric acid, and others as mesotartarate derivatives of *p*-coumaric acid. The work of Bergman et al. (16) demonstrated for the first time the presence of both flavonoids and *p*-coumaric acid derivatives as antioxidant components of the aqueous extract of spinach leaves. The mean molecular weight of NAO, based on NMR analysis, was calculated as 500–1,000 g/mol.

The active antioxidant peaks identified can be separated into three categories of chemicals: flavonoid derivatives; coumaric acid derivatives; and hydrophilic components, one identified as the nucleoside, uridine. Each fraction was tested for its antioxidant activity using the thiobarbituric acid (TBA) and Xylenol Orange (FOX) assays (16) monitoring malondialdehyde (MDA) and hydroperoxide levels, respectively. Approximately seven individual active fractions were isolated in the aromatic polyphenol group. Although each isolated fraction exhibits antioxidant activity, we have shown that the combination of these fractions contributes to the synergistic efficacy of the NAO extract as a powerful antioxidant protecting against oxidation damage in various biological systems (16). The NAO mixture and a glucurinated flavonoid that has the chemical structure of 6-(3,4-dihydroxy-phenyl)-9-hydroxy-7-methoxy-[1,3]dioxolo[4,5-g]chromen-8-one 4′-β-glucuronid, which was recently isolated and purified from NAO, showed activity in the scavenging of reactive oxygen species, such as superoxide, OH anion radical, and singlet oxygen (17).

All of the aromatic polyphenols that we isolated exhibited glycosylation, which is expected to increase their intestinal absorption and bioavailability in a manner similar to that of the flavonoid quercetin, demonstrated to be absorbed in greater amounts than pure aglycone (18).

Pharmacological Models in Which Spinach Extracts and NAO Were Tested

The efficacy of NAO and its antioxidant capacity was studied in both in vitro and in vivo models and found to be superior to that of the well-known antioxidants vitamin E, butylated hydroxytoluene (BHT) (15), *N*-acetylcysteine (NAC), and green tea (19). NAO has shown promising beneficial effects in a few experimental models, such as skin (15,16) and prostate cancer (19), as well as antiseptic and chemoprotective properties (16,20–24).

In this review article, we present a compilation of data generated in our laboratories and those of other investigators describing the efficaciousness of spinach extracts as well as a water-soluble fraction isolated from spinach leaves (NAO) in various animal models of human diseases.

Models Showing Antiinflammatory Effect of NAO

Septic shock, a systemic response to infection, is characterized by distinctive pathological events occurring at specific target organs (25,26). The antiinflammatory effect of NAO has been tested in lipopolysaccharide (LPS)-induced septic shock in rat and rabbit. NAO significantly reduced the high mortality rate in rats caused by the synergism of LPS and glycerol (20), endotoxin-induced renal failure (21), and an increased sensitivity to endotoxemia resulting from bilateral nephrectomy (22).

In another study (23), histopathologic changes in several organs were compared among groups of male Wistar rats that had been injected with LPS after prophylactic pretreatment with NAO (10 mg/kg, ip) and control groups injected with LPS alone. Exposure to LPS was associated only with multifocal hepatocellular necrosis and acute inflammation; thymic and splenic lymphoid necrosis; ocular retinal hemorrhage and acute endophthalmitis; adrenal medullary vacuolation, necrosis, and acute inflammation; and decreased adrenal cortical cytoplasmic vacuolation. Pretreatment with NAO for 8 consecutive days significantly reduced the necrotic and inflammatory changes in the various organs associated with the LPS challenge. When NAO was further tested in rabbit (24), LPS injection into New Zealand rabbits induced a variety of necrotic and inflammatory histological changes in organs and systems; some were comparable to those seen in humans. NAO treatment in rabbit (10 mg/kg, ip) showed effective prophylactic capacities when it was administered for 8 consecutive days followed by a single LPS injection. Significant protective effects against LPS-related lesions in the liver, thymus, spleen, eyes, and adrenal were obtained in rabbit. In the liver and eye, NAO appeared to exert a beneficial effect by reducing the incidence and severity of inflammation, necrosis, and hemorrhage induced by LPS administration. In the spleen and thymus, NAO conferred significant protection upon the B- and T-lymphocytes. It was also effective in reducing levels of blood chemistry parameters that are indicative of liver damage (total bilirubin, aminotransferase, and alanine aminotransferase). These results indicate the possible therapeutic efficacy of NAO in the treatment of clinical sepsis,

which is known to be associated, in its pathological manifestations, with oxidative stress (25,26).

Application of the terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling technique to LPS-treated rabbits demonstrated no increase in apoptosis outside necrotic foci. Apoptotic hepatocytes, however, were observed within areas of focal necrosis in animals exposed to LPS alone. Hepatocyte cellular proliferation was tested by the proliferating cell nuclear antigen (PCNA) tool, which indicated a proliferative effect in the LPS group but a disappearance of the effect in the NAO-treated groups (27).

A few studies have been performed to investigate the mechanism by which NAO promotes its antiinflammatory effect. Reactive oxygen species (ROS) play a key role in LPS-induced septic shock (28). LPS injection enhances the formation of ROS, such as superoxide anion radicals (O_2^-), peroxides ($ROO^\bullet$), and reactive nitrogen compounds like nitric oxide ($NO^\bullet$) radicals and peroxynitrite ($ONOO^-$) in lung, liver, and other susceptible organs (28). The beneficial effect of NAO conferring protection from cellular damage may be mediated through its antioxidative and/or antiinflammatory properties. We demonstrated that LPS promotes hepatic oxidative stress in rat and rabbit (29). Liver, heart muscle, and plasma samples collected from animals exposed to LPS exhibited elevated levels of the lipid peroxidation products, MDA and hydroperoxides, whereas NAO caused significant reductions in these (27,30). Immunohistochemical staining has revealed a significant reduction in i-NOS and COX-2 protein expression in rat liver after NAO plus LPS treatment when compared with LPS treatment alone (31). The prophylactic effects of the tested compounds are more likely to decrease ROS levels (hydrogen peroxide and NO radicals) than trigger formation of the tested endogenous antioxidative enzymes, superoxide dismutase, catalase, and glutathione peroxidase (27,30).

Antimutagenic Effects of Spinach Extracts

Spinach extracts generally have been reported to possess antimutagenic potential, which was tested in bacteria by various mutation assays (32,33). Strong protective effects of spinach were also exerted against clastogenicity of benzo[a]pyrene and cyclophosphamide in the in vivo mouse bone marrow micronucleus assay; spinach homogenate (0.5 ml) was administered by oral gavage to mice, and a moderate protective effect was obtained (34).

Thirteen compounds isolated from spinach acted as antimutagens against a dietary carcinogen in *Salmonella typhimurium* TA98. The antimutagens were purified from a methanol/water extract (70:30, vol/vol) of dry spinach after removal of lipophilic compounds such as chlorophylls and carotenoids. All of these active compounds were flavonoids and related substances (Fig. 1) (35).

IC-50 values (indicators of antimutagenic potencies) of the flavonol glucuronides (Fig. 1, compounds 1–6) ranged between 24 and 58 μM. The total antimutagenicity of the original spinach juice can be explained by the presence of the flavonol glucuronides and, to lesser extent, by the flavonol disaccharides (Fig. 1, compounds 7 and 8) and flavanones (Fig. 1, compounds 9 and 10), which were only weakly active (35).

Antineoplastic Effects of Spinach Extracts and NAO

Spinach is considered a beneficial source for various carotenoids and lipophilic active compounds (i.e., neoxanthin, lutein, zeaxanthin, and chlorophylls). Dietary intake of

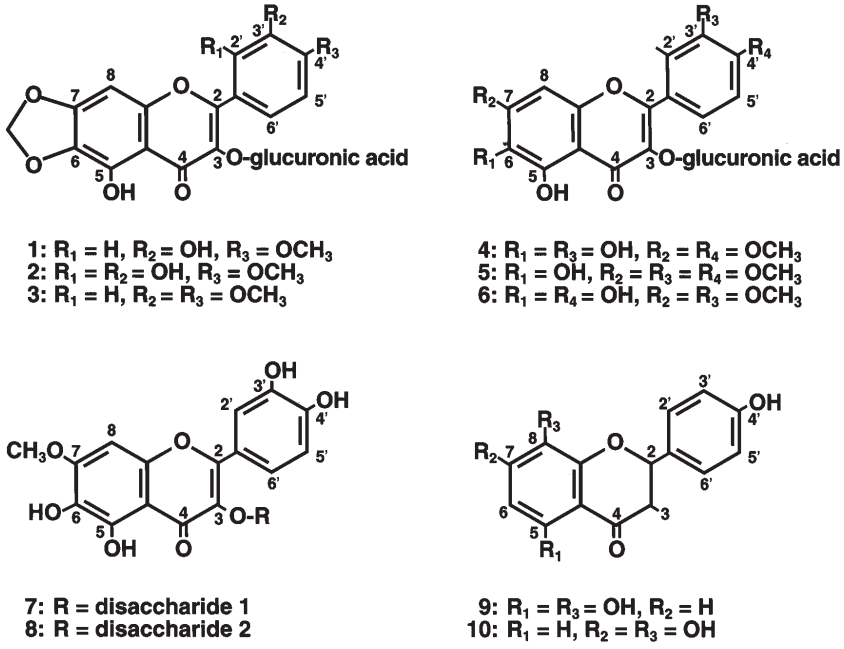

Figure 1. Chemical structures of antimutagenic flavonoids isolated from spinach. Adapted with permission from Edenharder et al. (35).

spinach extract has been reported to have beneficial effects on various types of cancer, such as ovarian (36), lung (37), prostatic (38), breast (39,40), and colon (41). The potential antineoplastic effects of NAO isolated from spinach were tested by our group in animal models described subsequently.

Effects of Spinach Consumption and NAO Administration on Prostatic Cancer

Many laboratory studies and human epidemiological data suggest that most prostate cancer (PC) deaths are attributable to lifestyle, including nutritional factors with diet playing a major role in initiation as well as subsequent progression of the disease. Epidemiological studies have shown a lower incidence of PC in Asians linked to the presence of isoflavonoids derived from soy and found at high concentrations in prostatic fluids (42,43). Moreover, soybean phytochemicals, including genistein, inhibited in vivo growth of PC tumors that resulted from the SC injection of PC cells in mice and rats (44,45). We recently investigated the use of the spinach extract, NAO, for prostatic cancer chemoprevention (19). NAO was tested both in vitro and in vivo in cell lines DU145 and PC3 derived from human prostate and in the TRAMP (Transgenic Adenocarcinoma Mouse Prostate) model. NAO exerted an antiproliferative effect on DU145 and PC3 cells. Inhibition of cellular proliferation occurred in a dose-dependent manner (IC-50 in the range of 2–4 mM), increasing numbers of G1 cells and reducing hydrogen peroxide and peroxide levels. The efficacy of NAO (200 mg/kg) was compared with that of the green tea polyphenol, epigallocatechin-3-gallate (EGCG) (200 mg/kg), and *N*-acetylcysteine, NAC (125 mg/kg). Hyperplasia was ranked by a combination of severity grade and distribution (focal, multifocal, diffuse) on Wk 5 and 9. Although significant effects were exerted on different prostate lobes by the three antioxidants, when the most severe hyperplasia in all four lobes of TRAMP mice was evaluated, only NAO reduced hyperplasia at Wk 9 and 13. The most pronounced effect of NAO was obtained in the dorsal and lateral prostatic lobes. The greater efficacy of NAO in slowing spontaneous prostatic carcinogenesis in the TRAMP, and its effects on the cultured cells may be explicable by its antioxidative and antiproliferative properties.

Effects of NAO in Mouse Skin Papilloma Model

The Tg.AC mouse carrying the v-Ha-ras structural gene is a useful model for the study of chemical carcinogens, especially those acting via nongenotoxic mechanisms. Mice carrying this oncogene readily exhibit epithelial proliferation when challenged with classic tumor promoters like 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (46). Initiation is highly correlated with activation of the v-Ha-ras oncogene.

After hemizygous Tg.AC mice were treated dermally five times over 2.5 wk with 2.5 µg TPA and NAO was administered either topically (2 mg) or orally (100 mg/kg) 5 days/wk for 5 wk, papilloma counts made macroscopically during the clinical observations revealed a significant decrease in multiplicity in the NAO topically treated group. Histological examination showed that papilloma multiplicity was lower in both topical and oral NAO-treated groups (47). On the basis of the reported antioxidative effects of NAO in skin (15,16), we suggest that the effect of NAO in the Tg.AC model may be related to its ability to detoxify peroxides and free radicals generated by TPA exposure.

Antioxidative Effect of NAO in UV-Irradiated Skin

The effect of NAO on dermal lipid peroxidation was tested in mice exposed to UV irradiation (15). NAO (1% suspension) or vitamin E (5% suspension) was applied topically to the skin of mice exposed to UV irradiation; 4 h later, the mice were killed and epidermal samples were tested biochemically. NAO was significantly more effective than vitamin E in reducing the levels of the lipid peroxidation end product, MDA.

The effect of UV irradiation on lipoxygenase activity in mouse skin in the presence and absence of topical NAO was also tested. NAO significantly inhibited lipoxygenase activity and the levels of the arachidonic acid derivatives, 12 (S)-hydroperoxyeicosatetraenoic acid (12-HpETE), and 15 (S)-hydroperoxyeicosatetraenoic acid (15-HpETE) were decreased by 70%, compared with untreated irradiated mice (15).

Protection of NAO in Doxorubicin-Induced Cardiotoxicity

Doxorubicin (DOX) produces clinically restorative responses in numerous human cancers, but its cardiotoxicity has limited its usefulness. Because ROS may affect DOX-induced antitumor activity and cardiotoxicity, we evaluated the prophylactic effect of NAO on DOX-induced cardiotoxicity (48). BalbC mice were treated with NAO (10 mg/kg, ip) 7 consecutive days before and/or 6 days after DOX administration. Light and electron microscopy of DOX-treated heart revealed myocardial degeneration. NAO treatment (cumulative dose of NAO: 130 mg/kg) conferred significant cardiac protection and did not hinder the effectiveness of DOX on survival in mice implanted with B16 cells. The effects of NAO on the lipid peroxidation product MDA and H_2O_2/hydroperoxides were examined on Day 6 after DOX administration; levels of both were elevated in heart muscle of DOX-treated mice, whereas pretreatment with NAO prevented these changes. Our results suggest the usage of NAO in combination with DOX as a prophylactic strategy to protect heart muscle from DOX-induced cellular damage (48).

Effects of Spinach Extracts on the Central Nervous System

Although few studies have documented the beneficial effect of spinach extract consumption on aging and motor learning, Joseph and colleagues demonstrated that long-term feeding of F344 rats (aged 6–15 mo) with a spinach-supplemented diet prevented the onset of age-related deficits in several indices, such as oxotremorine-enhanced striatal dopamine release and cognitive behavior measured by the Morris water maze performance test (49). Additional experiments using dietary supplementation for 8 wk with spinach, strawberry, or blueberry extracts (14.8, 9.1, 18.6 g dried aqueous extract per kilogram of diet, respectively) revealed the efficacy of these supplements in reversing age-related deficits in motor and cognitive functions in aged (19 mo) F344 rats once they have occurred (50,51).

In another model (51), diets supplemented with spinach, strawberries, or blueberries reversed age-induced declines in β-adrenergic receptor function in cerebellar Purkinje neurons measured using electrophysiological techniques. The spinach diet (9.1 g freeze-dried spinach extract/kg diet mix) was the only one that significantly ($P < 0.001$) improved learning on a runway motor task modulated by cerebellar norepinephrine. These studies indicate that age-related deficits in motor learning and memory can be reversed with nutritional interventions.

Poor performance on delay eye-blink conditioning, known to measure cerebellar-dependent learning, has been suggested as a predictor of Alzheimer's disease (52). Cartford et al. (53) examined the mechanism involved in the beneficial effect of a spinach-enriched diet in rats showing improvement in conditioning for classic eye-blink delay. They reported that cerebelli from old, 18-mo F344 rats fed a spinach-enriched diet (0.02% wt/wt dry spinach, freeze-dried powder) for 6 wk displayed significantly less mRNA expression of the proinflammatory cytokines TNF-α and TNF-β, compared with cerebelli from animals fed a control diet. These results suggest that one mechanism by which an enriched spinach diet works is modulation of an age-related increase in inflammatory responses. Dietary intake of spinach antioxidants therefore may confer similar protective effects on CNS function to those involving sepsis and cancer.

Effects of Spinach Consumption on Ophthalmic System

Dietary intervention with spinach consumption in humans has revealed beneficial effects on the ophthalmic system. Ingestion of spinach and collard greens was associated with a lower risk of age-related ocular macular degeneration and cataracts (54–57). Although the pathophysiology is complex and encompasses both environmental and genetic components, research suggests that dietary factors, including antioxidants, may contribute to a reduction in the risk of these degenerative eye diseases.

Safety Information for Consumption of Spinach Extracts

Spinach extracts generally are considered nontoxic for human consumption (58). In herbal medicine, preparations of fresh or dried spinach leaves have been used for many years for ailments and complaints of the gastrointestinal tract, as a blood-generating remedy, to stimulate growth in children, as an appetite stimulator, for fatigue, and for accelerating convalescence (58). No health hazards or side effects are known to be associated with the proper administration of designated therapeutic dosages; however, overconsumption may pose a potential risk. Generally, the presence of nitrate in vegetables, as in water and other food products, is a serious threat to human health, not because of its direct toxicity, which is low, but because of the dangerous compounds it generates in the organism, such as methemoglobin and nitrosamines (59). Recently, a case–control study of dietary factors and gastric cancer in Korean patients was reported (60). The conclusion summarized that high consumption of food rich in nitrate, including cooked spinach, increases the risk of gastric cancer. The relatively high nitrate content of spinach (0.3–0.6%, fresh leaves; 80–360 mg/kg, cooked) makes the consumption of spinach as a foodstuff too frequently inadvisable. Circumstances that lead to reduction (e.g., storage condition, high temperature) should also be avoided to prevent nitrite formation.

Because the oxalate content of spinach (in young leaves 6–8%, older leaves up to 16%) could reduce calcium resorption, the recommendation for pediatric use is that infants should not receive spinach as a foodstuff until after their fourth month because there may be a danger of methemoglobin formation through nitrites (58).

The effect of consumption of spinach products on antioxidant activity in human blood was tested in healthy volunteers. The spinach groups received 20 g/day/subject of whole-leaf, minced, liquid, or liquified spinach for 3 wk and were compared with the control group that received a basic diet. No adverse effects were reported. The consumption of spinach resulted in greater erythrocytic glutathione reductase activity and lower erythrocytic catalase and serum α-tocopherol responses (61).

Allergy Syndromes Induced by Spinach Consumption

Allergy to fresh fruits and vegetables is the most frequent cause of the oral allergy syndrome (62). Normally, it affects patients who are also allergic to pollen, and only occasionally is it not associated with that sensitivity (63). Spinach contains histamine (approximately 37 mg/100 g fresh weight), which can cause pesudoallergic reactions (63). The IgE-mediated allergy to spinach has been also described, although only a few cases have been reported (64). Only four cases have been described thus far for allergic reactions after consumption of spinach; in three of these, the allergy was related to latex sensitization (65). Contact dermatitis of hands (65) and hypersensi-

tivity pneumonitis to spinach powder (66) were also reported in a few patients; however, these allergic responses toward spinach by dermal contact or inhalation are considered very rare.

Safety Information From Preclinical Studies

NAO was found to produce no toxicity in animals following intraperitoneal, oral, or dermal administrations (19,23,24,27,30,31,47,48).

Acute Toxicity

Acute toxicity studies performed in mice revealed that the LD-50 in mouse by oral gavage was above 2,000 mg/kg and by ip 681 mg/kg (67).

Mutagenicity

By the Ames test, NAO was found nonmutagenic in bacteria at 3,500 µg with or without metabolic activation (unpublished data).

Eye and Dermal Irritation

Toxicological studies were conducted to support dermal application of NAO for cosmetic usage. These studies performed in our laboratories (unpublished data) revealed that 10% NAO caused no ocular irritation; at 100%, a slight irritation was indicated. At 10% strength NAO initiated no irritating effect upon rabbit skin; no dermal irritation was caused by 14 cumulative applications in the guinea pig (unpublished data).

A series of repeated dose studies were performed in mice, rats, and rabbits in which mortality, clinical signs, hematology, and blood chemistry markers were recorded and microscopic examination was performed. Generally, no toxicity, adverse effects, or abnormalities related to the natural antioxidant, NAO, were indicated.

Preclinical Studies

Safety information derived from preclinical studies with repeated administration of NAO is detailed subsequently and summarized in Table 1. Female Balb/C mice treated with NAO (10 mg/kg/day in saline, ip, $n = 18$ mice/group) for 13 days manifested no mortality or clinical signs. The body weight gain was comparable to that of the control group. Microscopic examinations performed for heart, liver, and spleen revealed a normal histological appearance with no target organ (48).

Female hemizygous TgAC mice were treated topically with TPA plus NAO. Mice treated with NAO administered topically (2 mg, 22 animals/group) or orally (100 mg/kg, 24 animals/group) 5 days/wk for 5 wk were compared with controls (TPA only). No mortality or clinical signs related to NAO treatment were observed (47). TRAMP mice and wild-type mice (32 mice/group) were treated with NAO by oral gavage administration of 200 mg/kg/day for 13 wk and compared with control animals. Interim death was conducted on Wk 9 and terminal death on Wk 13. Animals were observed for mortality and moribundity; body weights and clinical signs of all animals were recorded before treatment and weekly thereafter. Food consumption was measured for 3 days before the first day of treatment and twice a week thereafter until the study termination. A full necropsy was performed that included examination and removal of all organs. Mean group body weights and body weight gains in TRAMP or wild-type mice treated with NAO were similar to those in TRAMP or wild-type controls. NAO significantly reduced hyperplasia severity/focalness at Wk 9 ($P = 0.03$) and 13 ($P = 0.02$) and exerted no deleterious effect on survival, body weight gain, organ weight, or animal behavior (19).

Male Wistar rats ip-injected with NAO (10 mg/kg in saline, 10 animals/group) for 7 days were compared with controls during an investigative search for pathological alterations. Body weight and clinical signs were recorded on Days 1 and 8. A complete necropsy was performed in all rats. Brain, thymus, heart, lungs, liver, stomach, duodenum, ileum, cecum, colon, spleen, kidney, adrenal glands, and skeletal muscle were collected, and histopathological evaluation was done in tissue sections stained with hematoxylin and eosin. No effect of

Table 1. Safety Information Derived From Preclinical Studies With Repeated Administration of NAO

Species/Strain	Study/Model	Duration	Route	Dose Level	Ref.
Mouse					
Mouse/BalbC	Doxorubicin-induced cardiotoxicity	13 days	ip	10 mg/kg/day	48
Mouse/Tg.AC	Tg.AC mice	5 wk with 13 wk follow-up	Topical and oral	Topical (2 mg/day) and oral (100 mg/kg/day)	47[a]
Mouse/TRAMP		4 mo	Oral gavage	200 mg/kg/day	19[a]
Rat					
Rat/Wistar	LPS model	7 days	ip	10 mg/kg/day	23,30,31
Rabbit					
Rabbit/New Zealand	LPS model	8 days	ip	10 mg/kg/day	24,27[a]

a: These studies were conducted in compliance with Good Laboratory Practice regulations. NAO, natural antioxidant mixture; TRAMP, Transgenic Adenocarcinoma Mouse Prostate; LPS, lipopolysaccharide.

NAO on body weight gain, behavior, or gross pathology was indicated. No pathological effect was noted in organs examined from animals exposed to NAO alone (23,30,31).

Male New Zealand rabbits were injected ip with NAO (10 mg/kg, 5 animals/group) for 8 days and compared with the control group. Animals were inspected twice daily for mortality. Physical appearance, behavior, and any abnormal clinical signs were recorded daily. Body weights were recorded before the start of dosing and on Days 4 and 8 before death. Parameters of clinical chemistry, organ weight, and histopathology were examined. Blood samples were taken before and at the end of dosing from each animal fasted overnight and analyzed for glucose, total bilirubin, serum glutamic oxalacetic transaminase (GOT), serum glutamic pyruvic transaminase (GPT), alkaline phosphatase (AP), total cholesterol, triglycerides, albumin, and globulin (Table 2). The mean and standard deviations of clinical chemistry parameters noted in male rabbits on Day 9 and before the start of the experiment are presented in Table 2. Significant differences in total cholesterol values in the NAO group compared with control were not considered induced by treatment, because all individual values were within the laboratory limits of normality for the rabbit, and similar values were already present in these animals at the pretest measurements. A significant effect of NAO on triglyceride levels was obtained; the biological significance of that result is not clear. A complete necropsy was performed in all rabbits. Tissues were collected from brain, eyes, liver, kidneys, thymus, adrenals, spleen, heart, lungs, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, thyroids, femur, and skeletal muscle from the foreleg. Organ weight values (absolute and relative to final body weight) compared with control are presented in Table 3. A slight effect of NAO was noted in the increased relative weights of kidney and spleen. Whether these increases hold any biological significance is not clear, and these changes are not supported by any histological alterations. All absolute values (total organ weight) were within the range of controls. The pathological examination was conducted independently by four qualified toxicological pathologists in a blind evaluation of hematolyxin and eosin-stained sections. No lesions or target organs were indicated in NAO-treated animals (24). Overall, the no-observed-effect-level (NOEL) for oral gavage administration in mouse and rat was at least 200 mg/kg/day. The NOEL for ip in mouse, rat, and rabbit was at least 10 mg/kg/day.

Table 2. The Effect of NAO on Selected Blood Chemistry Tests Conducted in Rabbits Pretrial and at End of Study (Day 9)[a]

Parameter	Control (Saline)	NAO (10 mg/kg, ip)
Glucose (mg/100 ml)	107.1 ± 8	100.0 ± 9
	(110.7 ± 10)	(118.4 ± 10)
Total bilirubin (mg/100 ml)	0.29 ± 0.24	0.44 ± 0.50
	(0.49 ± 0.29)	(0.46 ± 0.24)
GOT (IU/L)	28.0 ± 12.8	25.9 ± 19.6
	(34.0 ± 13.5)	(25.2 ± 4.7)
GPT (IU/L)	32.9 ± 14.5	34.8 ± 16.8
	(28.8 ± 6.3)	(47.1 ± 12.7)
AP (IU/L)	220.3 ± 53	107.0 ± 27[b]
	(229.0 ± 85)	(183.5 ± 40)
Total cholesterol (mg/100 ml)	54.8 ± 11.9	81.9 ± 12.9[b]
	(76.8 ± 18.7)	(81.8 ± 10.1)
Triglycerides (mg/100 ml)	73 ± 32	244 ± 217
	(141 ± 46)	(125 ± 36)
Albumin (%)	69.7 ± 2.7	61.3 ± 1.7[c]
	(66.1 ± 6.2)	(66.3 ± 0.5)
Globulin (%)	30.3 ± 2.7	38.7 ± 1.7[c]
	(33.9 ± 6.2)	(33.7 ± 0.5)

a: Basal values obtained before the start of the experiment are shown in parentheses; GOT, glutamic oxalacetic transaminase; GPT, glutamic pyruvic transaminase; AP, alkaline phosphatase.
b: Significantly different ($P < 0.01$) from control group (Student *t*-test).
c: Significantly different ($P < 0.001$) from control group (Student *t*-test).

Female Reproductive System—Estrogenic and Antiestrogenic Potential

The developing fetus is uniquely sensitive to perturbation by chemicals with estrogenic- and/or endocrine-disrupting activity (68). During the last few years, the use of natural antioxidants and flavonoids in nutritional and pharmaceutical applications for infants and children, as well as adults, has been on the increase. Because some of these compounds, such as genistein, exhibit estrogenic activity (69,70), we investigated the estrogenic potential of NAO utilizing an in vivo rodent uterotropic bioassay and an in vitro transcriptional activation assay. Overall, our results suggest that NAO does not have estrogenic or antiestrogenic activity (71).

Table 3. The Effect of NAO on Mean Absolute and Relative Weight of Selected Organs in Male Rabbits[a]

Organ	Control (Saline)	NAO (10 mg/kg, ip)
Final fasting body wt (kg)	2.58	2.49
Liver (g)		
A	77.05	77.33
B	2.97	3.11
Kidneys (g)		
A	17.76	18.53
B	0.69	0.75[b]
Brain (g)		
A	8.83	8.47
B	0.35	0.34
Spleen (g)		
A	0.92	1.20
B	0.04	0.05[b]
Thymus (g)		
A	5.80	4.69
B	0.23	0.19
Adrenals (mg)		
A	111.40	145.00
B	4.43	5.82

a: Abbreviations are as follows: A, absolute value; B, relative organ weight in percentage of body weight.
b: Significantly different ($P < 0.05$) from control group (Student *t*-test).

In summary of the safety issues, natural antioxidant from spinach, NAO, has been tested in various species such as mouse, rat, and rabbit and was well tolerated with no toxic effects and no target organs. The NOEL was at least 200 mg/kg/day for oral gavage administration up to 4 mo in mice and 10 mg/kg/day for ip injection for 7–13 days in mouse, rat, and rabbit. The compound was found nonmutagenic in the Ames test and anticarcinogenic in experimental models such as skin and prostate cancer. The compound did not show any target organ toxicity and therefore seems to be completely safe to be used as a food ingredient.

In conclusion, the current review provides epidemiological and preclinical data supporting the beneficial effects of spinach consumption on human health. Spinach is composed of various active compounds, such as flavonoids and other polyphenolic active ingredients acting synergistically as antiinflammatory, antioxidative, and anticancer agents. The active compounds in spinach are expected to have a significant oral bioavailability based on the beneficial effects obtained in several systems, such as CNS, prostate and skin, in which oral administration of spinach extracts has been investigated.

NAO is composed of the main active compounds contained in spinach, mainly flavonoids, and cumaric acid derivatives. NAO can easily be used for chemoprevention or dietary intervention in humans because it is stable at high temperature and lacks toxicity. Based on the significant efficacy obtained in various animal models, beneficial effects in humans can be expected, and further investigations in clinical trials are highly recommended.

Future research in nutrition and disease prevention undoubtedly will give priority to investigation of the basic molecular mechanisms by which spinach antioxidants influence various steps in pathogenesis, such as regulation of the cell-division cycle in carcinogenesis. In addition, biomarkers that indicate nutrient status and systemic exposure of specific components isolated from spinach will soon be identified and validated in blood and various tissues. Such elucidation will allow detection of distribution of specific active compounds in known and new target tissues and provide us with important information concerning potential interactions of nutrients. This knowledge will be essential for developing strategies for disease prevention through dietary modifications.

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