

# DNA Repair Enhancement by a Combined Supplement of Carotenoids, Nicotinamide, and Zinc

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**ABSTRACT:** Four volunteers were involved for 5 weeks of a baseline period, followed by 7 weeks of a combined supplementation of nicotinamide, zinc, and carotenoids (Nicoplex). Blood sampling and bioassays were carried out every week during the evaluation period. The supplementation of Nicoplex resulted in statistically significant increased resistance to DNA single-strand breaks induced by  $H_2O_2$  (DNA retained on filter % from  $46.7 \pm 1.9$  to  $59.4 \pm 4.3$ ;  $p < 0.01$ ), increased DNA repair 60 min after induction of damage (DNA retained on filter % from  $74.6 \pm 4.8$  to  $88.3 \pm 4.2$ ;  $p < 0.01$ ), elevated poly (ADP-ribose) polymerase (PARP) activity ( $p < 0.05$ ), and an increased proliferative response to phytohemagglutinin (PHA) ( $p < 0.05$ ) when compared with the levels before supplementation. However, when the same subjects were supplemented with nicotinamide, zinc, and carotenoids together with another 17 nutrients or minerals, there were no changes in DNA damage, DNA repair, or proliferative response to PHA. Through the use of a rat model, DNA repair of splenocytes 3 h after 12 Gy whole-body irradiation was significantly enhanced in rats supplemented with Nicoplex for 6 weeks ( $p < 0.05$ ) and 8 weeks ( $p < 0.01$ ). Comparison of Nicoplex and its components administered separately revealed that there was an additive effect on DNA repair for both single- and double-strand breaks (both  $p < 0.05$ ). On the basis of the results, it is hypothesized that the enhanced effect of combined supplement of nicotinamide, zinc, and carotenoids on DNA repair depends on their diversified mechanisms of action while multinutrient supplementation may compromise the effects by inhibitory interactions including uptake and absorption.

**KEY WORDS:** carotenoids, DNA repair, nicotinamide, zinc.

## I. INTRODUCTION

Carotenoids, nicotinamide (NAM), and zinc are each individually well recognized to possess disease-preventative and immune-stimulatory properties. Epidemiological analysis of both case-control and cohort studies showed a remarkable consistency for the association of increased lung cancer risk with low amounts of dietary  $\beta$ -carotene or low plasma  $\beta$ -carotene.<sup>1</sup> One primary mechanism of action of carotenoids is to scavenge oxygen-derived free radicals produced either as by-products of metabolism or from exogenous environmental exposures.<sup>2-4</sup> Another mechanism of action of carotenoids could be their ability to enhance immune response.<sup>5,6</sup> Carotenoids

have been reported to possess immunomodulatory activities in humans and animals as to enhance lymphocyte blastogenesis and mitogenic responsiveness to phytohemagglutinin (PHA) induction, increase the population of specific lymphocyte subsets, increase lymphocyte cytotoxic activity, and stimulate the production of various cytokines.<sup>7-9</sup>

Nicotinamide is the precursor for the formation and maintenance of the cellular pool of NAD. NAD is essential for cellular ATP production and maintenance of the cell's redox potential, and it is also the substrate for the DNA repair enzyme, poly (ADP-ribose) polymerase (PARP). Niacin deprivation decreases the NAD pools significantly, both in animal systems<sup>10,11</sup> and in humans.<sup>12,13</sup> The NAD-depleted



cells have an increased sensitivity to DNA damage, and the levels of poly (ADP-ribose) production in cultured cells<sup>10</sup> or in a rat liver<sup>11</sup> are significantly lower after mild nicotinamide deficiency. On the other hand, when niacin is given as a supplement to dietary level the NAD pool increases and the cells are less sensitive to oxygen radicals.<sup>14</sup>

Zinc is an essential trace element for humans. More than 300 zinc-containing enzymes have been characterized for which zinc is directly involved in catalysis and interacting with the substrate molecules undergoing transformation.<sup>15,16</sup> More recently, it has been found that zinc ions are key structural components of a large number of proteins involved in gene transcription, DNA synthesis and repair, genomic plasticity, and apoptosis.<sup>17</sup> Zinc deficiency is associated with growth retardation,<sup>18</sup> impairment of immune function,<sup>19</sup> and the incidence of persistent diarrhea and dysentery,<sup>20</sup> which are both reversed when zinc is provided.<sup>21</sup> Zinc is important for DNA repair and protection of DNA damage.

There are two zinc fingers in the DNA-binding domain of PARP gene and other DNA repair proteins which contain cysteine residues, and if these cysteine residues are oxidized at their thiol constituents they would prevent DNA binding and participation in DNA repair.<sup>22-24</sup> Superoxide dismutase is an antioxidant enzyme protecting cells from the harmful superoxide anion; this enzyme also requires zinc as a cofactor.<sup>25</sup> Structural zinc ions have been found in the tumor suppressor p53,<sup>26</sup> an important protein involved in gene transcription, DNA synthesis and repair, genomic plasticity, and apoptosis. Zinc has been reported to have protective interaction with cadmium, which inhibits DNA replication and repair in mammalian cells.<sup>27</sup> Zn<sup>2+</sup> protects against DNA damage and apoptosis induced by the mycotoxin sporidesmin and the immunotoxin gliotoxin and related epipolythiodioxopiperazines in murine peritoneal macrophages and splenocytes (T blasts).<sup>28</sup> Oral zinc supplementation has been shown to be able to correct zinc deficiency and some immune defects and DNA repair rate in lymphocytes of Down syndrome children.<sup>29</sup>

It is obvious from the above review that carotenoids, nicotinamide, and zinc have different modes of action to protect against DNA damage and enhance DNA repair. The combination of natural products having well-defined, different, and thus potentially non-competitive modes of action may be useful to

obtain additive biological effects yet avoid inhibition of one another's uptake and absorption. To test this hypothesis, a supplement of carotenoids, nicotinamide, and zinc was compared with an another 20-ingredient multinutrient supplement for its abilities to enhance DNA repair in a human study. In addition, a rat model was set up to confirm the additive effect of the combined supplement of nicotinamide, zinc, and carotenoids.

## II. MATERIALS AND METHODS

### A. Subjects and Treatment Schedule

Four nonsmoking male volunteers with an age range of 30 to 40 years and no chronic medical history were included in the study. After five consecutive weeks of once-per-week measurements to establish baseline levels of the biomarkers described below, three of the subjects were given oral administration of Nicoplex (carotenoids, nicotinamide, and zinc gluconate) on a daily basis for seven consecutive weeks. One subject received no supplementation but was sampled and bioassayed at the same time to serve as a "no supplementation" control. After a "no intervention" period for 13 weeks, the baseline values for the biomarkers were reestablished over a 4-week period. Radical Fighters as described below were orally supplemented for six consecutive weeks. Compliance for both supplements was fewer than three missed daily administrations per subjects during the treatment phase. There were no major individual lifestyle and dietary custom changes before and after either supplement. The study was approved by the Ethical Committee of the Medical Faculty of the University Hospital in Lund and carried out in accordance with Helsinki Declaration of 1975.

### B. The Drugs and Dosing

Nicoplex was formulated by Oxigene Europe AB (Lund, Sweden) and provided by C. E. Jamieson, Ltd. (Ontario, Canada) as a combination of carotenoids (as Caroplex in 100-mg soft gel capsules), nicotinamide (100-mg tablets), and zinc gluconate (10-mg tablets). Caroplex is a proprietary manufactured natural source of carotenoids extracted from palm oil. It contains 60%  $\beta$ -carotene, 34%  $\alpha$ -carotene, 3%  $\gamma$ -carotene, and



3% lycopene.<sup>30</sup> These three drugs were given together during the same period by oral administration Monday through Sunday (daily) for a 7-week period. Radical Fighters is a commercially available product from Twin Laboratories, Inc. (New York, NY) which contains 20 nutrients or minerals including carotenoids, nicotinamide, and zinc (three capsules supply:  $\beta$ -carotene, 12500 IU; vitamin C, 750 mg; ascorbyl palmitate, 125 mg; vitamin E, 250 IU; L-cysteine, 250 mg; L-glutathione (reduced), 12.5 mg; selenium, 100  $\mu$ g; zinc, 10 mg; vitamin B<sub>1</sub>, 75 mg; B<sub>2</sub>, 50 mg; B<sub>5</sub>, 250 mg; B<sub>6</sub>, 62.5 mg; B<sub>12</sub>, 125  $\mu$ g; nicotinamide, 50 mg; niacin, 25 mg; folic acid, 200  $\mu$ g; biotin, 75  $\mu$ g; para-aminobenzoic acid, 150 mg; inositol, 100 mg; choline, 100 mg).

### C. Blood Sampling

Each week, about 20 ml of venous blood was collected into two heparinized vacutainers (143 U.S.P. Units/10-ml tube). Leukocyte (HML), erythrocyte, and plasma samples were separated according to the method described by Pero et al.<sup>31</sup> HML at a density of  $2 \times 10^6$ /ml were suspended in 10% fresh autologous plasma supplemented RPMI 1640 medium. This culture medium was used throughout the experiments. DNA single-strand breaks by alkaline elution, PARP activity, and PHA-induced mitogenic response in HML were prepared for analysis immediately after blood sampling. Plasma and erythrocyte samples were frozen at  $-80^\circ\text{C}$  until analysis.

### D. Alkaline Elution

HML cells were exposed on ice to zero or a standard dose of  $100 \mu\text{M}$   $\text{H}_2\text{O}_2$  for 60 min, and then the cells were incubated at  $37^\circ\text{C}$  for 0, 30, and 60 min to estimate DNA repair of single-strand breaks. DNA damage and repair at the different time points were measured by alkaline elution as described by Kohn and coworkers<sup>32</sup> with modifications to measure the unlabeled DNA by microfluorometry.<sup>33</sup>

### E. PARP Activity

PARP activities, constitutive or induced by a standard dose of  $100 \mu\text{M}$   $\text{H}_2\text{O}_2$  were measured using the procedure adapted from the permeabilized cell

technique of Berger<sup>34</sup> with modifications as previously described.<sup>35</sup> HML cells ( $1 \times 10^6$ ) were or were not exposed to  $100 \mu\text{M}$   $\text{H}_2\text{O}_2$  at  $37^\circ\text{C}$  for 30 min, and then they were harvested by centrifugation, permeabilized, and PARP activity determined by a radiometric procedure.

### F. Phytohemagglutinin-Induced Lymphocyte Mitogenic Response

$2 \times 10^5$  HML cells were incubated in microculture plates containing 200  $\mu\text{l}$  RPMI 1640 supplemented with 10% autologous plasma and 6  $\mu\text{l}$  phytohemagglutinin (PHA)/ml at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$  for 4 days. After two additional days of incubation in the presence of [ $^3\text{H}$ ]thymidine (final concentration: 2 mCi/mmol, 1  $\mu\text{Ci}/\text{ml}$ ), the cells were harvested and assayed for content of bound radiolabeled material.

### G. NAD Concentration in Erythrocytes

Frozen erythrocyte pellets (250  $\mu\text{l}$ ) were thawed on ice in the presence of 600  $\mu\text{l}$  of 1.8 M perchloric acid (PCA) and 25  $\mu\text{l}$  of 2.4 mM thymidine as an internal standard. After centrifugation at 14,000g, the supernatant was neutralized on ice with 2 M  $\text{K}_2\text{CO}_3$ . After another centrifugation, the supernatant was analyzed by HPLC, which has been described earlier.<sup>36</sup>

### H. Data Collection and Statistics

All data were collected throughout the study, but only data from the 5 weeks before the supplement were used to establish the baseline and only the data from the last 5 weeks were used to calculate the averages after supplement. Comparison of mean differences before and after supplementation was made by two-tailed *t* test with a significance level of 0.05.

### I. Animal Experiments

Female W/Fu rats weighing 175 to 200 g were used. Nicoplex (Caroplex, nicotinamide and zinc) or its components at equal doses (mg/kg body wt) for each ingredient as in the human study was dissolved in corn oil and administered daily by gavage, 7 days a week for 2 to 8 weeks. Controls were gavaged with

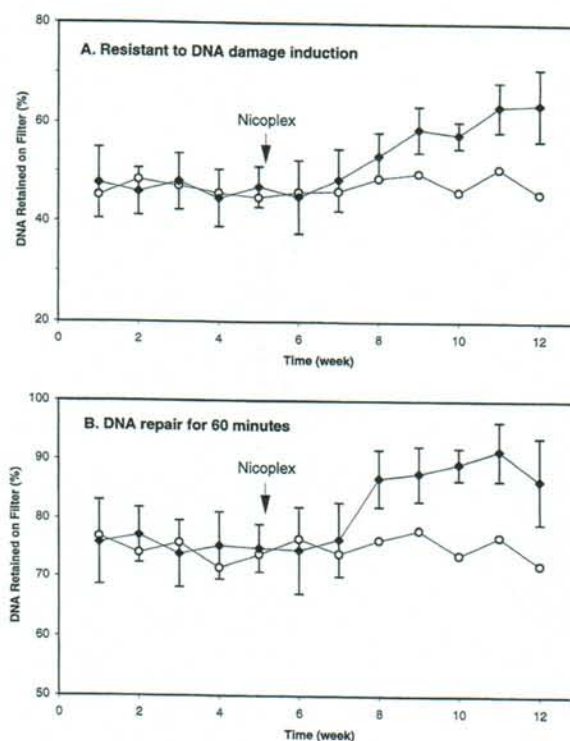


the drug carrier, corn oil. After 2, 3, 6, and 8 weeks of supplementation, the rats were treated with whole-body irradiation in a  $^{137}\text{Cs}$  source (Scanditronix, 1.56 Gy/min) and allowed to repair for 3 h. The animals were sacrificed, and blood samples were collected into heparinized tube from heart puncture and immediately analyzed by an automated hematology analyzer (Sysmex, K-1000). Spleen single-cell suspensions were prepared and frozen at  $-80^{\circ}\text{C}$  after addition of 10% dimethyl sulphoxide (DMSO). Before analysis frozen cell suspensions were rapidly thawed at  $37^{\circ}\text{C}$  and layered directly on polycarbonate filters for evaluation of DNA single-strand breaks by alkaline elution<sup>37</sup> and double-strand breaks by neutral elution.<sup>38</sup> The animals were treated according to the Swedish guidelines for humane treatment of laboratory animals and the experiments were approved by the Ethical Committee at the University Hospital in Lund, Sweden.

### III. RESULTS

#### A. The Volunteer Study

The resistance to cellular DNA damage, enhancement of DNA repair, and mitogenic response were evaluated before and after supplementation. In such a manner, each individual became his own longitudinal control. At the same time, one subject was supplemented neither with Nicoplex nor with Radical Fighters to see if there is a seasonal effect. Moreover, comparison was made between each supplementation and its correspondent baseline values to avoid such possible biases, no matter how small. The results of Nicoplex supplement on protection of DNA damage and DNA repair enhancement are shown on Figure 1. From Week 3 after Nicoplex supplement, the supplemented subjects showed an increase of resistance to DNA damage induction by a standard dose of hydrogen peroxide and enhanced DNA repair 60 min after DNA damage induction (as shown by the percentage increase of DNA retained on filter). There is no statistically significant difference of DNA damage induced by hydrogen peroxide (DNA retained on filter %:  $46.3 \pm 1.5$  compared with  $48.2 \pm 2.2$ ;  $p > 0.05$ ) and DNA repair 60 min after the induction of DNA damage (DNA retained on filter %:  $74.4 \pm 2.1$  compared

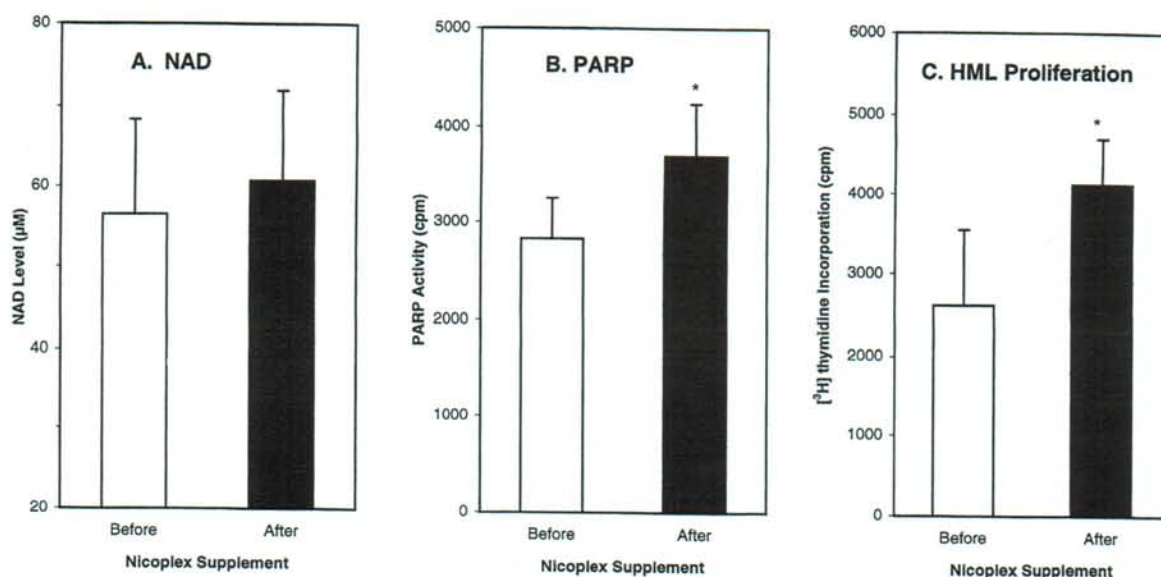


**FIGURE 1.** The *in vivo* effect of Nicoplex supplement on DNA damage and DNA repair in human mononuclear leukocytes. The DNA damage (A) was induced by 100  $\mu\text{M}$  hydrogen peroxide and assessed by alkaline elution assay. DNA repair was assessed 60 min later, after the induction of DNA damage (B). The data are expressed as mean in point and SD by error bar in supplement group ( $N = 3$ ).

with  $75.4 \pm 2.4$ ;  $p > 0.05$ ) before and after Nicoplex supplement in the control. The Nicoplex-supplemented subjects showed a significant increase in resistance to DNA damage (DNA retained on filter %:  $46.7 \pm 1.4$  before compared with  $59.4 \pm 4.3$  after the supplement;  $p < 0.001$ ) and enhanced DNA repair (DNA retained on filter %:  $74.6 \pm 2.2$  before compared with  $88.3 \pm 2.0$  after the supplement;  $p < 0.001$ ).

The enhanced DNA repair was confirmed by the statistically significant increase of PARP activity in lymphocytes ( $3696 \pm 542$  cpm after supplement compared with  $2823 \pm 428$  cpm before supplement;  $p < 0.05$ ; Figure 2B). Further more, Nicoplex supplemented subjects also showed an increased lymphocyte proliferation response to PHA induction ( $4123 \pm 580$  cpm after supplement compared with  $2616 \pm 926$  cpm before supplement,  $p < 0.05$ ; Figure 2C), which could





**FIGURE 2.** The *in vivo* effect of Nicoplex supplement on NAD pool in erythrocytes, PARP activity and proliferative response to PHA in lymphocytes. The data are expressed as mean in column, and SD by error bar (N = 3). \*A statistically significant difference ( $p < 0.05$ , two-tailed *t* test) compared with correspondent baseline level before supplement.

be the result of improved resistance to DNA damage and enhanced DNA repair.

The supplementation of Radical Fighters did not induce any statistically significant changes in HML with regard to the biomarkers of resistance to DNA damage, DNA repair, and PHA-activated lymphocyte proliferation response ( $p > 0.05$ ; Figure 3). These data support the hypothesis that megadoses of the additional nutrients in Radical Fighters block the effects of combined supplement of carotenoids, nicotinamide, and zinc.

## B. The Rat Model

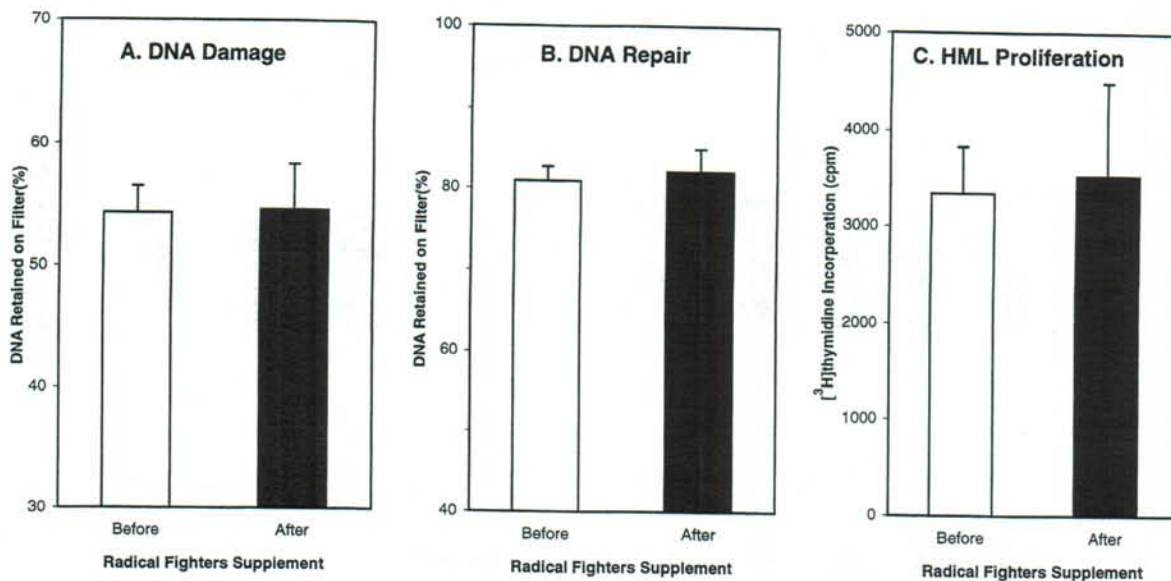
A rat model (W/Fu) was validated to test the DNA repair enhancement by Nicoplex supplementation in spleen single-cell suspensions. *In vivo* DNA repair kinetics was set up by irradiating the animals at a standard dose of 12 Gy and sacrificing them at different time points. Single-cell suspensions from spleen were used for alkaline elution estimation of DNA damage and the results are shown in Figure 4. Based on the DNA repair kinetics, 3 h after 12 Gy of radiation was chosen as the time to evaluate DNA repair capacity. Six rats in each group were evaluated.

The results in Figure 5 showed that rats supplemented for 6 or 8 weeks had a statistically significant increase in DNA repair in splenocytes compared with placebo controls, and the DNA repair enhancement was dependent on the supplement duration with the longest supplement showing the best effect.

From the above experiment, DNA repair enhancement of Nicoplex was compared with its individual components administered alone after 8-week supplementation in the rat. As shown in Figure 6, although zinc, nicotinamide, and carotenoids given separately all showed an increased tendency of DNA repair for both single-strand breaks and double-strand breaks, an additive effect was seen when the components were combined in the Nicoplex supplement. Moreover, the combination supplement group also showed a significant increased number of white blood cells ( $5.6 \pm 0.7$ ;  $p < 0.05$ ) compared with controls ( $4.3 \pm 0.9$ ), while the active component groups only showed an increased tendency but not statistically significant ( $p > 0.05$ ; Figure 7).

## IV. DISCUSSION

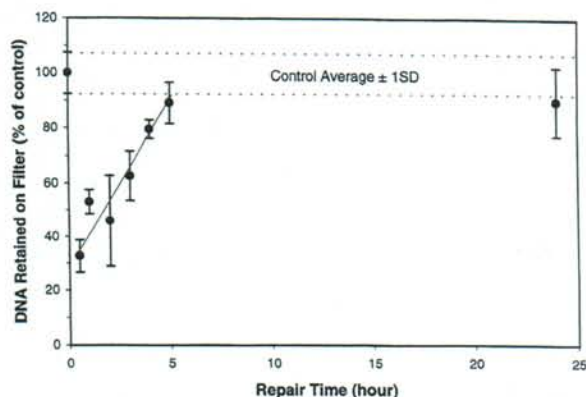
The Nicoplex supplement study involves the use of a combination consisting exclusively of carot-



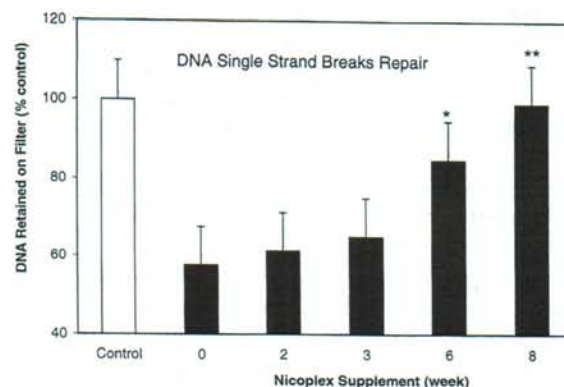
**FIGURE 3.** The *in vivo* effect of Radical Fighters supplement on resistance to DNA damage, DNA repair, and lymphocyte proliferative response to PHA. The data are expressed as mean in column, and SD by error bar.

enoids (100 mg), nicotinamide (100 mg), and zinc gluconate (10 mg), and no other active nutrient agent as a daily oral supplement over and above the normal dietary levels of carotenoids as vitamin A ( $1467 \pm 1213$  kcal), nicotinamide ( $33.1 \pm 26.7$  mg), and zinc ( $6.8 \pm 8.4$  mg)<sup>39</sup> to increase an individual's resistance to cellular DNA damage and enhance DNA repair. The hypothesis was based on combining substances with known properties to prevent cancer and stimulate immune function but with differing mechanisms of

action; for one, carotenoids are electrophilic scavengers of radicals; nicotinamide is a source of energy via increased production of NAD or ATP; and zinc is an essential cofactor to antioxidant, replicative, and

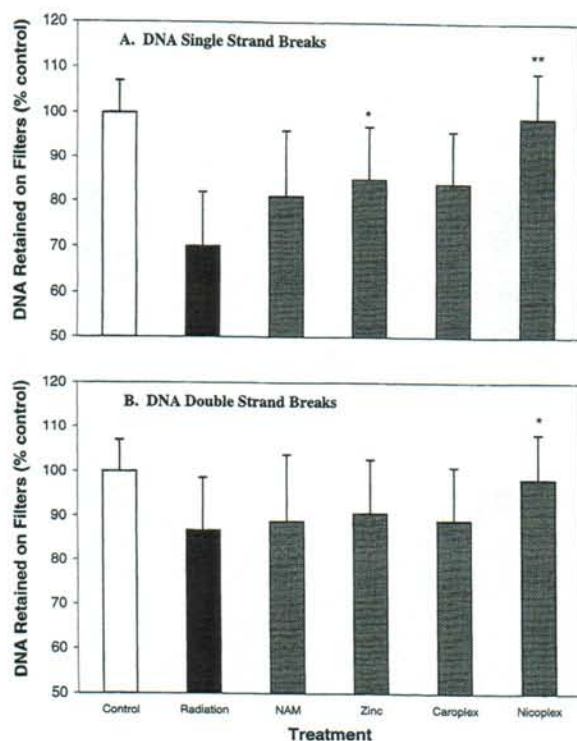


**FIGURE 4.** *In vivo* DNA repair kinetics of splenocytes of W/Fu rats irradiated whole body with a standard dose of 12 Gy and repaired at different time points. Data points are averages, and error bars standard deviation ( $N \geq 3$ ).



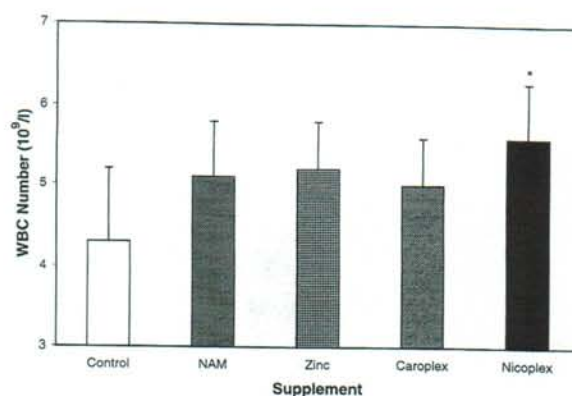
**FIGURE 5.** DNA repair enhancement by Nicoplex supplementation with different time period in rat model. DNA damage and repair were measured by alkaline elution of spleen single-cell suspension from female W/Fu rats. Nicoplex supplemented rats (0, 2, 3, 6, and 8 weeks) were irradiated whole body with 12 Gy and allowed to repair *in vivo* for 3 h. DNA retained on filter is expressed as % of controls. Column shows the averages, and error bars the standard error ( $N \geq 5$ ). \* $p < 0.05$  and \*\* $p < 0.01$  compared with no supplement (Week 0) group by two-tailed *t* test.





**FIGURE 6.** DNA repair enhancement by Nicoplex or its component supplementation in rat model. DNA damage and repair were measured by alkaline and neutral elution of spleen single-cell suspension from female W/Fu rats. After 8 weeks of supplementation with Nicoplex or its individual component, rats were irradiated whole body with 12 Gy and allowed to repair *in vivo* for 3 h. DNA retained on filter is expressed as % of controls. Columns show the averages, and error bars the standard error ( $N \geq 5$ ). \* $p < 0.05$  and \*\* $p < 0.01$  compared with control groups by two-tailed *t* test.

DNA repair enzymes in cells. Such a combination may produce an additive biological response but avoid inhibitory interaction among nutrients. This was demonstrated by the results of a Nicoplex human supplementation which resulted in significantly decreased DNA damage, increased PARP activity, and enhanced DNA repair after DNA damage induced by hydrogen peroxide (Figures 1 and 2). It is well known that DNA damage or oxidative stress inhibits immune system, and the increased lymphocyte proliferative response to PHA may be the result of the improvement of resistance to DNA damage and enhanced DNA repair of lymphocytes. Furthermore, when Nicoplex and its individual active components were administered to rats at doses equal to those given in



**FIGURE 7.** Comparison of white blood cell (WBC) of rats supplemented with Nicoplex or its active components for 8 weeks. Blood samples were collected from heart puncture into 5-ml heparinized tube 3 h after 12 Gy whole-body irradiation and then immediately analyzed by an automated hematology analyzer. Data shown are the averages in column, and SD by error bar ( $N \geq 5$ ). \* $p < 0.05$  compared with control group by two-tailed *t* test.

the human study by gavage for 8 weeks, DNA repair of both single- and double-strand breaks by  $\gamma$ -radiation was also significantly enhanced in the combination group, while the individual active component supplement groups only showed a tendency. Although the DNA damage was caused by different agents ( $H_2O_2$  and  $\gamma$ -radiation) in the different species (human and rat), Nicoplex supplement showed a consistent enhanced effect on DNA repair (Figures 1 and 5). The increase of white blood cells in rat is also consistent with the elevated lymphocyte proliferation in human.

However, when carotenoids, nicotinamide, and zinc were supplemented by the same subjects together with 17 other nutrients and minerals in the formulation of Radical Fighters, there was no statistically significant difference before and after the multinutrient supplementation for either DNA repair or proliferative response (Figure 3). These data are consistent with a recent report<sup>40</sup> in which 3000 subjects received daily supplementation with 14 vitamins and 12 minerals including  $\beta$ -carotene (15 mg), nicotinamide (40 mg), and zinc (45 mg) at two to three times the U.S. recommended dietary allowance or placebo for 6 years. No significant difference was observed between the two populations with respect to T-lymphocyte proliferative response. One possible ex-



planation for these results is that there may exist strong metabolic interactions among the compounds found in the megadoses of these natural products, so that one supplement limits the uptake and/or metabolism of the other components to protect from the toxicological consequences of overdosing. This is well illustrated by the fact that, in athletes consuming well-balanced diets, except for pyridoxine and riboflavin there were no significant changes in the blood concentrations of any other vitamins and minerals and no signs or symptoms of serious toxic side effects after 3 months of supplementation with 11 vitamins and 10 minerals.<sup>41</sup> Carotenoids and vitamin E or C are scavengers having similar mechanisms of action and are often combined into supplements. However, recent studies have shown that these natural nutrients can inhibit each other's uptake<sup>42</sup> and negate the desired induction of biological effects.<sup>43</sup> Antagonistic effects exist between vitamins E and A supplement on antibody production and phagocytosis, between vitamins D and A supplement on expression of adhesion molecules, between copper and iron on neutrophil phagocytosis, between copper and molybdenum on neutrophil function and lymphocyte proliferation, between magnesium and calcium on leukocyte adhesion (LFA-1), and between zinc and copper on lymphocyte proliferation and Jerne plate-forming cell response.<sup>44</sup>

In summary, the combination supplementation of carotenoids, nicotinamide, and zinc (Nicoplex) can protect against DNA damage and enhance DNA repair. It is suggested that these natural nutrients have additive effects, possibly due to their different modes of action. These results have also justified a larger human population study for the validation of the effects seen in volunteer study and animal model.

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