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Prostate cancer risk and DNA damage: translational significance of selenium supplementation in a canine model

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Daily supplementation with the essential trace mineral selenium significantly reduced prostate cancer risk in men in the Nutritional Prevention of Cancer Trial. However, the optimal intake of selenium for prostate cancer prevention is unknown. We hypothesized that selenium significantly regulates the extent of genotoxic damage within the aging prostate and that the relationship between dietary selenium intake and DNA damage is non-linear, i.e. more selenium is not necessarily better. To test this hypothesis, we conducted a randomized feeding trial in which 49 elderly beagle dogs (physiologically equivalent to 62–69-year-old men) received nutritionally adequate or supranutritional levels of selenium for 7 months, in order to mimic the range of dietary selenium intake of men in the United States. Our results demonstrate an intriguing U-shaped dose–response relationship between selenium status (toenail selenium concentration) and the extent of DNA damage (alkaline Comet assay) within the prostate. Further, we demonstrate that the concentration of selenium that minimizes DNA damage in the aging dog prostate remarkably parallels the selenium concentration in men that minimizes prostate cancer risk. By studying elderly dogs, the only non-human animal model of spontaneous prostate cancer, we have established a new approach to bridge the gap between laboratory and human studies that can be used to select the appropriate dose of anticancer agents for large-scale human cancer prevention trials. From the U-shaped dose–response, it follows that not all men will necessarily benefit from increasing their selenium intake and that measurement of baseline nutrient status should be required for all individuals in prevention trials to avoid oversupplementation.

Introduction

Epidemiologic data suggest that many people could substantially reduce their cancer risk through relatively simple dietary changes, including supplementation with non-toxic doses of cancer-fighting nutrients (1,2). Prostate cancer is the second leading cause of male cancer-related mortality in the United States (3) and the identification of safe, non-toxic compounds for the prevention of prostate cancer is considered a high research priority. Selenium, an essential nutrient required for a number of metabolically important enzymes, inhibits cancer development in a variety of experimental animal models (4–6). In 1996, Clark *et al*. (7) reported the results of the Nutritional Prevention of Cancer Trial, a 13-year, randomized, placebo-controlled study of older Americans. In this study, daily supplementation of 200 μg of selenium in the form of selenium-enriched yeast was associated with a significant reduction in the risk of several cancers, most notably cancer of the prostate (63% risk reduction) (7). These results suggested that a significant reduction in cancer risk could be achieved using dietary supplementation with low, non-toxic doses of selenium and/or selenium fortification of foods.

The use of selenium supplements in the USA has grown steadily over the last 20 years, both in the number of adults who use supplements and in the amount consumed daily. But, health professionals seldom recommend that supplement users test their nutrient status prior to or after taking supplements. Growing interest in selenium as a prostate cancer preventive agent has led to a large intervention trial, selenium and vitamin E cancer prevention trial (SELECT), that is currently enrolling >32 000 men and will require 12 years to complete (8). However, it is not known what form or dose of selenium offers the most potent prostate cancer protective effects, or whether too much selenium supplementation might be harmful. Observational data from men in the Health Professionals Follow-up Study showed a strong inverse association between selenium status, as measured by toenail selenium concentration, and the risk for developing advanced prostate cancer (9). However, multivariate analysis demonstrated an apparent threshold effect, with no additional prostate cancer protective effect at toenail concentrations exceeding 0.82 p.p.m. In another study, Brooks *et al*. (10) found a similar threshold effect. Taken together, these data suggest that not all men will necessarily benefit from increasing their dietary intake of selenium.

It has been previously hypothesized that the cancer-protective effects of selenium are related to its ability to limit the accumulation of genotoxic damage within the aging prostate (11,12). However, the optimal intake of selenium or other nutrients necessary to protect the prostate from cancer is unknown because previous human and animal studies have not adequately defined the relationship between nutrient dose and genotoxic damage within the prostate. In this study, we tested the hypothesis that the relationship between selenium intake and DNA damage within the prostate and brain is non-linear, i.e. that more selenium is not necessarily better. We studied elderly beagle dogs, that were physiologically equivalent to 62–69-year-old men and free of prostate cancer, to simulate the aging human prostate and to define the dose of

Abbreviations: DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; BSS, Hanks' balanced salt solution; SELECT, selenium and vitamin E cancer prevention trial.

selenium that minimizes genotoxic damage within the prostate. This animal model was used because the influence of aging on prostatic carcinogenesis appears similar in dogs and men, the only two species in which prostate cancer occurs spontaneously with appreciable frequency (13,14). Finally, to determine whether the dose–response data from this animal model were relevant to human health, we compared our results with published data on selenium status and human prostate cancer risk from the Health Professionals Follow-up Study and the Nutritional Prevention of Cancer Trial.

Materials and methods

Experimental animals and study design

In a randomized controlled feeding trial, elderly beagle dogs, physiologically equivalent to 62–69-year-old men (15), received diets containing nutritionally adequate or supranutritional levels of selenium for 7 months to produce a range of dietary selenium exposures similar to that of healthy adult men in USA. Forty-nine elderly (8.5–10.5-year-old) sexually intact male, retired breeder dogs weighing 9–18 kg were purchased from a local supplier. After 4 weeks acclimation, dogs were randomly assigned to a control group ($n = 10$ dogs) or four daily treatment groups: 3 μg/kg/day selenomethionine (L-selenomethionine, Solgar Vitamin and Herb, Leonia, NJ) ($n = 10$ dogs), 6 μg/kg/day selenomethionine ($n = 10$ dogs), 3 μg/kg/day high selenium yeast (SelenoExcell®, Cypress Systems, Fresno, CA) ($n = 10$ dogs) and 6 μg/kg/day high selenium yeast ($n = 9$ dogs). The selenium in the high-selenium yeast product is mostly (~85%) selenomethionine. However, unlike the free L-selenomethionine product, the yeast form is protein-bound. All dogs had nutritionally adequate selenium status confirmed by plasma selenium concentration prior to the start of the experiment. Throughout the experiment, all dogs were fed a selenium-adequate maintenance diet (0.3 p.p.m as fed basis; Science Diet® Canine Maintenance, Hills Pet Nutrition, Topeka, KS). In the control group, daily selenium intake was ~6 μg/kg body weight. After 7 months of treatment, all dogs were euthanized in accordance with guidelines set forth by the American Veterinary Medical Association Panel on Euthanasia (16). All aspects of this experimental protocol were approved by the Purdue University Animal Care and Use Committee.

Measurement of genotoxic damage within the prostate

Within 15 min of euthanasia, the prostate was collected at necropsy and 50–80 mg of prostate tissue was placed in 1 ml of cold Hanks' balanced salt solution (HBSS) containing 20 mM EDTA and 10% dimethyl sulfoxide (DMSO) (17). The tissue was then minced with fine scissors and 50 μl of cell suspension was mixed with 1 ml of RPMI 1640 media containing 10% fetal bovine serum (FBS) for subsequent electrophoresis. Cytospin preparations indicated that >90% of cells had epithelial cell morphology; mean percentage cell viability estimated by trypan blue exclusion was 80%. Histopathologic evaluation of formalin-fixed, step-sectioned prostate tissue sections revealed no foci of carcinoma.

The extent of DNA damage in prostate cells, which is an index of oxidative stress and other genotoxic influences within the prostate, was measured by single cell gel electrophoresis (alkaline Comet assay) using a method previously described (17). Under the conditions of this experiment, the comet tail reflects the electrophoretic migration of DNA fragments resulting from strand breaks, alkali labile sites, crosslinks or base excision repair sites (18). The extent of DNA damage was scored in 100 randomly selected cells from each sample (50 cells from several different fields from each of two replicate slides) by an examiner who was blinded to treatment group. Analysis was performed by one slide reader (SS), thus minimizing variability attributable to intersubject scoring. SYBR green l-stained nucleoids were examined at 200× magnification with an Olympus epifluorescent microscope. Each cell was visually scored on a 0–4 scale according to its appearance using a method described by Collins (19,20) as follows: no damage (type 0), mild to moderate damage (types 1 and 2) and extensive DNA damage (types 3 and 4) (Figure 1). Using this scoring method, the extent of DNA damage within the prostate was expressed in terms of a Comet score (range 0–400) (19) and as the percentage of cells with extensive damage (sum of types 3 and 4 cells).

Measurement of genotoxic damage in brain

Immediately after euthanasia, brain tissue from the cerebral cortex was collected via craniotomy. In all cases, interval from euthanasia to brain tissue harvest was <30 min. For each dog, 50–80 mg of brain tissue was placed in 1 ml of cold HBSS containing 20 mM EDTA and 10% DMSO. The tissue was then minced with fine scissors and 50 μl of cell suspension was mixed with 1 ml of RPMI 1640 media containing 10% FBS for the alkaline Comet assay. Pyramidal neurons were the most prevalent cell type in the tissue harvested from the cerebral cortex.

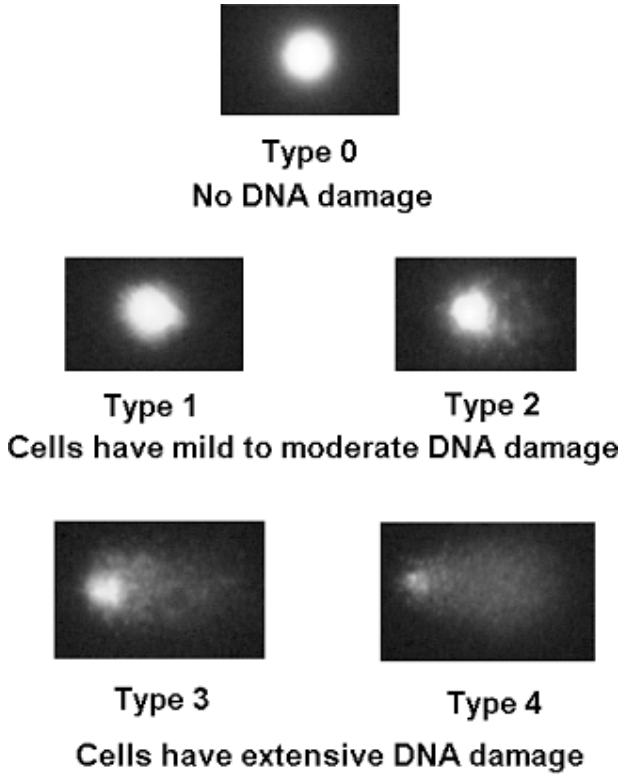

Fig. 1. Alkaline Comet assay: visual scoring method to measure the extent of DNA damage in cells.

Measurement of selenium concentration in prostate, brain and toenails

After 7 months of treatment, toenail clippings and snap frozen prostate and brain tissues were collected from dogs immediately after euthanasia. Specimens from control and selenium supplemented dogs were analyzed together, but in random order, with the supplementation status unknown to laboratory personnel. Nails (49 dogs) and tissues (25 dogs) were analyzed for selenium by instrumental neutron activation analysis at the University of Missouri-Columbia Research Reactor Center (MURR), Columbia, MO using a modification of methods previously described (21–23). Total selenium content in toenail clippings provides a reliable non-invasive measure of selenium status (24–27).

Statistical analysis

The data from selenium-treated and control dogs were combined and Pearson correlation coefficients were calculated to determine if selenium concentration within prostate or brain tissue was significantly ($P < 0.05$) correlated with toenail selenium concentration. Since the relationship between the percentage of prostate cells with extensive DNA damage and toenail selenium concentration (p.p.m) was non-linear, a polynomial regression including a quadratic term was used. To control for multicolinearity in the polynomial regression, the mean toenail selenium concentration was first subtracted from each toenail selenium concentration and then squared (28). All data analyses were done using standard statistical software [SPSS (Version 10.0, Chicago, IL) and SAS System (Version 8.1, SAS Institute, Cary, NC, 1999)].

Results

U-shaped dose–response relationship between selenium status and DNA damage in prostate and brain

There was a non-linear, U-shaped dose–response relationship between toenail selenium concentration and DNA damage within the prostate ($r^2 = 0.52$, $P < 0.0001$), with a relatively narrow range of selenium that minimized the extent of DNA damage in prostate cells (Figure 2A). When dogs with low,

moderate and high selenium status were compared, the relationship between selenium status and prostatic DNA damage could not be explained by selenium dose-dependent differences in prostatic epithelial cell proliferation or apoptosis indices or in the extent of prostatic inflammation (data not shown).

To determine whether the U-shaped relationship between selenium status and DNA damage in dogs was unique to the aging prostate, we conducted a similar analysis of DNA damage in the brain. Similar to our findings in the prostate, there was a U-shaped dose–response relationship between toenail selenium concentration and DNA damage within the aging brain. Importantly, we found the toenail selenium concentration that optimized DNA damage reduction in the prostate also minimized the extent of DNA damage within the aging brain (Figure 3A).

Toenail selenium concentration reflects selenium concentration in prostate and brain tissue

Previous studies in humans and animals did not evaluate whether differences in the tissue concentration of selenium within the prostate or brain were strongly predicted by the non-invasive measurement of selenium in toenails. In elderly dogs, we found a strong positive association between selenium concentration in toenails versus prostate ($r^2 = 0.52$; $P < 0.0001$) and brain ($r^2 = 0.53$; $P < 0.0001$); these strong associations were observed over the range of toenail selenium concentration seen in healthy adults in the USA, including the men who were likely to participate in the SELECT prostate cancer prevention trial (Figure 3B and 3C).

Dog dose–response curve parallels results from human studies

To determine whether the U-shaped dose–response in beagle dogs was relevant to human health, we compared our results with published data on selenium and human prostate cancer risk from the Health Professionals Follow-up Study (9). Toenail selenium concentrations in the lowest and highest quartiles of dogs (mean of 0.50 and 1.03 p.p.m., respectively) were similar to toenail concentrations in men (median of 0.66 p.p.m in lowest quintile; median of 1.14 p.p.m in highest quintile). Fitting the human data from the Health Professionals Follow-up Study to the dose–response curve from dogs produced an intriguing result—the same concentration of selenium that minimized prostatic DNA damage in dogs also minimized advanced prostate cancer risk in men (Figure 4). The highest risk for prostate cancer was observed in men with the lowest toenail selenium (median of 0.66 p.p.m.), which was less than the optimal concentration predicted by the dog model. The lowest risk for prostate cancer occurred in men with a median selenium level of 0.82 p.p.m., which was equivalent to the optimal concentration in the dog model. Thus, movement along the dog dose–response curve from low suboptimal to optimal selenium status (bold arrow in Figure 4) was associated with a 65% reduction in human prostate cancer risk. The canine dose–response curve also accurately predicted a cancer protection threshold, i.e. no additional reduction in prostate cancer risk with selenium >0.82 p.p.m.

We then used the canine dose–response curve to reconcile the results of the Nutritional Prevention of Cancer Trial of Clark *et al*. (7,29). In this large intervention trial, baseline selenium status prior to supplementation was strongly predictive of prostate cancer protection associated with selenium supplementation. Men with the lowest plasma selenium prior to supplementation had a significant 92% reduction in prostate cancer risk in response to daily selenium supplementation. In contrast, men with the highest plasma selenium prior to supplementation did not exhibit a significant reduction in prostate cancer risk. Instead, these men had an alarming and statistically significant 88% increase in overall cancer incidence (30). We simultaneously measured toenail and plasma selenium concentration in 12 healthy human volunteers to

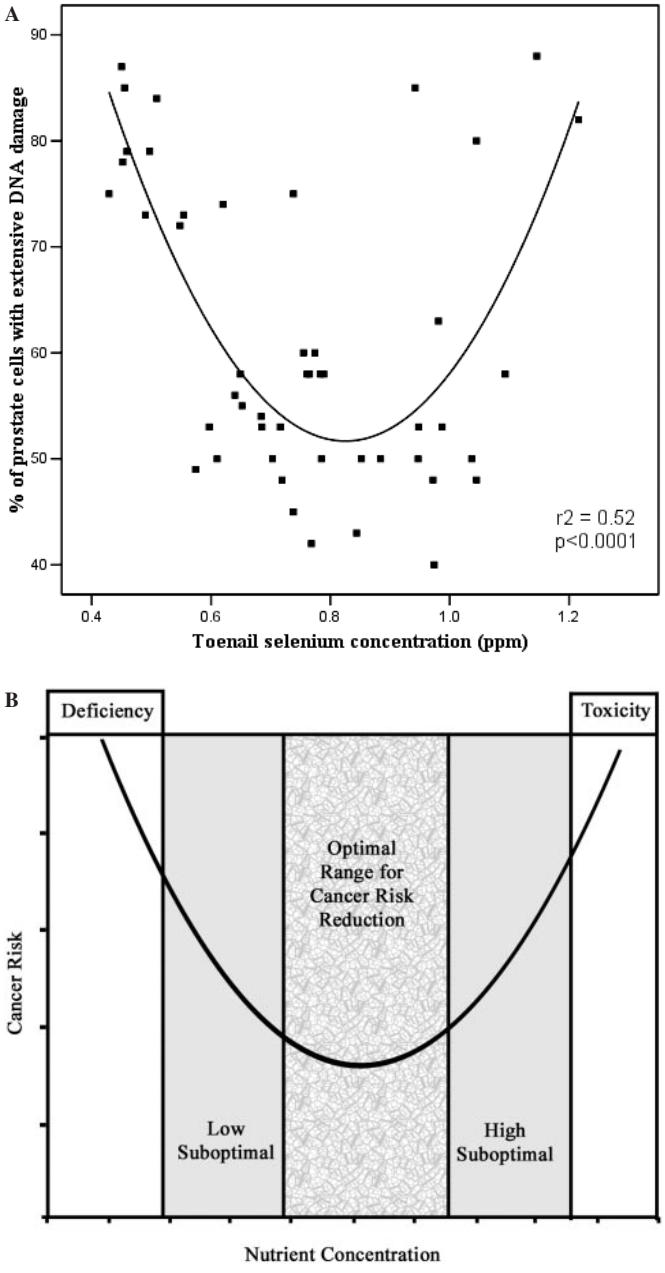

Fig. 2. A U-shaped dose–response curve defines the relationship between selenium and genotoxic stress in prostate. (**A**) U-shaped dose–response relationship between toenail selenium concentration and prostatic DNA damage in 49 elderly dogs that were physiologically equivalent to 62 to 69-year-old men. (**B**) Model adapted from Mertz (31) predicting the biological response to an essential nutrient. The data from dogs provides the first *in vivo* confirmation that the Mertz model fits for selenium and procarcinogenic events within the prostate.

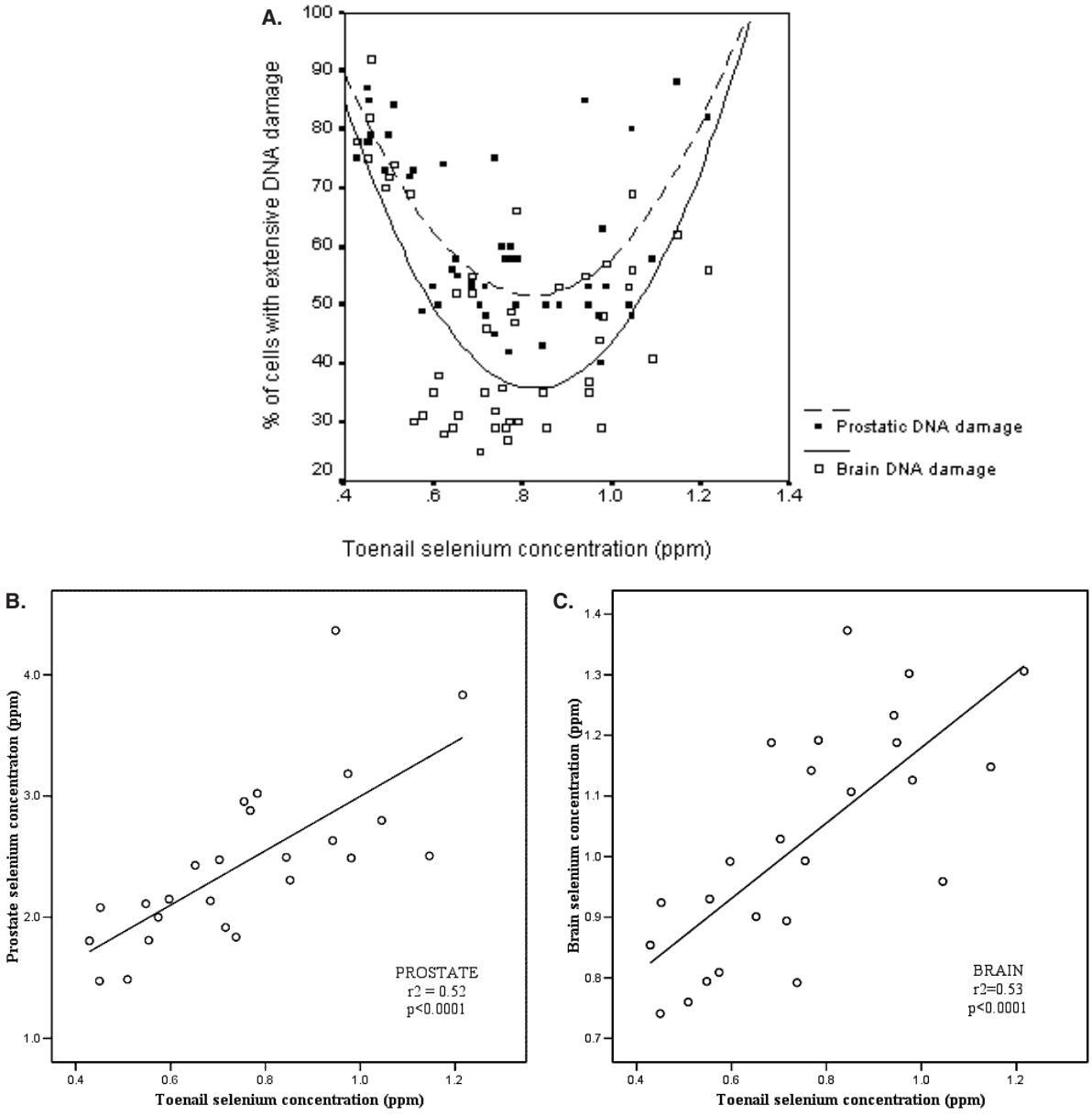

Fig. 3. (**A**) U-shaped dose–response defines the relationship between toenail selenium concentration and genotoxic stress in the brain of elderly dogs. The same concentration of selenium minimizes the extent of DNA damage within brain and prostate. (**B** and **C**) Non-invasive assessment of selenium status using toenails strongly predicts the concentration of selenium in prostate and brain tissues. Correlation between selenium concentration in prostate versus toenails (B) and brain versus toenails (C) in elderly dogs.

generate a ratio (6.7 ± 0.7) that could be used to convert plasma selenium concentration to predicted toenail values. After converting the plasma selenium levels of men in the Nutritional Prevention of Cancer Trial to an equivalent toenail selenium concentration, we found that the dog dose–response curve correctly predicted that men with the lowest baseline selenium status (<0.71 p.p.m.) would benefit from selenium supplementation (Figure 5). Men with the highest baseline selenium status (>0.81 p.p.m.) had a selenium concentration that was equivalent to or exceeded the optimal selenium concentration prior to supplementation; they did not benefit from selenium supplementation. Following supplementation, selenium concentration in these men was further elevated (median, 1.27 p.p.m.) and they experienced an increased total cancer incidence.

Discussion

The results of this study demonstrate the utility of a new approach to bridge the gap between laboratory and human studies that can be used to select the appropriate dose of anticancer nutrients for large-scale human cancer prevention trials. By studying the only non-human species that develops spontaneous prostate cancer, we documented an intriguing U-shaped dose–response relationship between the dietary intake of the essential trace mineral selenium and the extent of DNA damage within the aging prostate gland. Moreover, we found the results of two important human studies that examined selenium and prostate cancer risk—those used to justify the evaluation of selenium in the SELECT trial—were explained using this simple, cost-effective approach. More

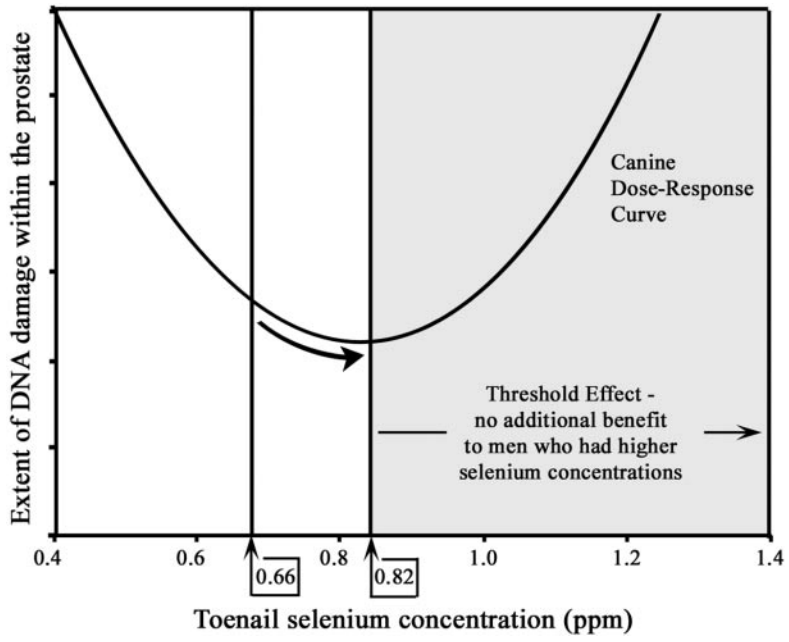

Fig. 4. Canine dose–response curve explains the effect of selenium status on human prostate cancer risk reduction in the Health Professionals Follow-up Study (9). Men with the lowest selenium status (median 0.66 p.p.m.) had lower than optimal selenium concentration predicted by the dog model; these men had the highest risk for advanced prostate cancer. Men with median selenium status of 0.82 p.p.m., a value equivalent to the optimal selenium concentration in the dog model, had the lowest prostate cancer risk. There was no additional prostate cancer risk reduction seen in men with selenium status >0.82 p.p.m., a finding predicted by the dog model. Movement along the dog curve from 0.66 to 0.82 p.p.m. (bold arrow) parallels a 65% reduction in prostate cancer risk for the men in the Health Professionals Follow-up Study.

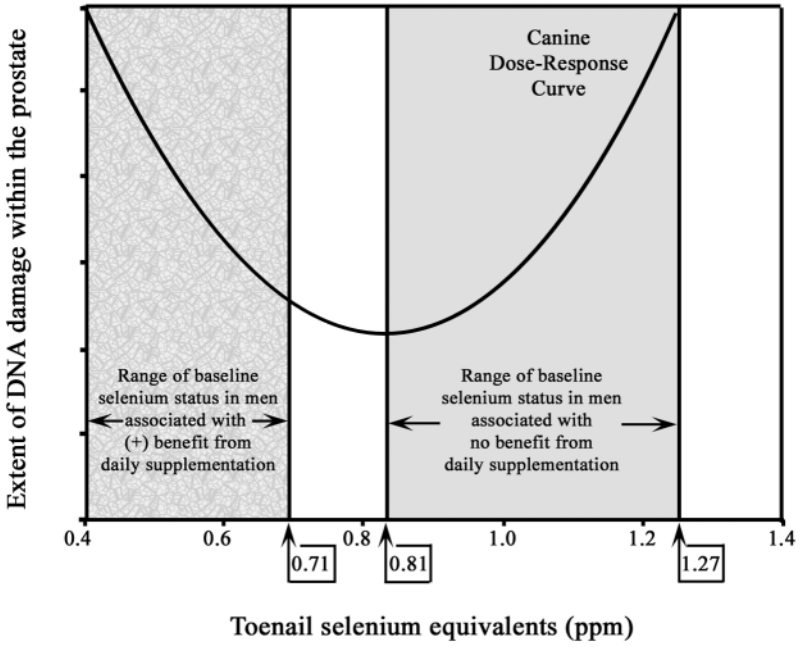

Fig. 5. Canine dose–response curve explains the effect of baseline selenium status on human prostate cancer risk reduction in the Nutritional Prevention of Cancer Trial (7,29,30). Men with baseline selenium status <0.71 p.p.m. had lower than the optimal selenium concentration predicted by the dog model; these men had a statistically significant 92% reduction in prostate cancer risk after selenium supplementation. Men with baseline selenium status >0.81 p.p.m. were already within the optimal or high suboptimal range predicted by the dog model prior to supplementation; these men had no significant reduction in prostate cancer after selenium supplementation. Following selenium supplementation, men in the highest baseline selenium tertile had a median selenium level of 1.27 p.p.m., a value clearly exceeding the selenium concentration that minimized DNA damage within the dog prostate. These men had an 88% increase in total cancer incidence compared with men with the lowest baseline selenium.

than 20 years ago, Mertz (31) proposed that the dose–response relationship between essential nutrients and biological processes was U-shaped. According to the Mertz model, a region of optimal nutrient status lies between two suboptimal (low and high) regions and the extreme values of deficiency and toxicity (Figure 2B). Our data provide further evidence that the Mertz model may indeed be correct—at least for selenium and the prostate. It follows from this new understanding that not all

men will necessarily benefit by increasing their daily selenium intake.

A similar U-shaped dose–response may also hold true for the anticancer effects of other trace minerals and carotenoids. For example, zinc is essential for prostate function (32) and it has been shown that zinc deficiency results in increased oxidative DNA damage and disruption of the p53 tumor suppressor (33). However, men with the highest intake of supplemental zinc had a significant two-fold increased risk of prostate cancer (34). Recently, Nyberg *et al*. (35) found a U-shaped dose–response between the dietary intake of β-carotene and spontaneous mutation frequency in the peripheral blood lymphocytes of humans. The aged dog model correctly predicted the human prostate's response to the anticarcinogenic effects of selenium and may also be applicable to other cancer-preventing nutrients and other anatomic cancer sites.

An important challenge facing scientists in the field of cancer prevention is to identify experimental approaches that can expeditiously define the dose-dependent effects of dietary supplements on health outcomes. Failure to recognize the U-shaped dose-dependent effects of nutritional supplements on carcinogenesis adversely impacted the design of previous cancer prevention trials. For example, in two randomized lung cancer prevention trials, subjects who received high doses of beta-carotene had an unexpected increase in lung cancer incidence compared with placebo-treated controls (36,37). Measurement of baseline nutrient status was not included as a required entry criterion in these beta-carotene trials or in any of the large selenium intervention trials. This is of particular relevance to the ongoing SELECT trial, since the average selenium status of men in the USA is roughly equivalent to a toenail concentration of 0.82 p.p.m., a value that already falls within the optimal range for prostate cancer risk reduction. Our demonstration of a U-shaped dose–response for cancer-fighting nutrients emphasizes that baseline nutrient status in the suboptimal range should be a required entry criterion for prevention trials to avoid the potential deleterious effects of oversupplementation.

Our study introduces to the field of cancer prevention research a powerful new paradigm that reflects the synthesis of three concepts: (i) the importance of using an *in vivo* model system (aging dog prostate) to mimic the aging human prostate prior to the onset of cancer; (ii) the importance of studying a broad dose range that is sufficient to define the U-shaped dose–response relationship between an essential nutrient and pro-carcinogenic processes; (iii) the use of Comet score as a measure of DNA damage that integrates prostatic exposure to genotoxic stress, the susceptibility of prostate cells to DNA damage, and prostatic DNA repair capacity (38,39). Using this approach, it is feasible to select a selenium dose that optimizes DNA damage reduction within a cancer target, such as the prostate and other organs, such as the brain. Future cancer prevention trials with humans could benefit significantly from adopting this paradigm to define the effects of nutrient dose on markers of genotoxic damage and cancer risk.

Finally, our analysis of the complex relationship between selenium, genotoxic damage and cancer risk within the prostate raises important questions regarding the currently recommended intake of this trace mineral. The current recommended daily allowance (RDA) for selenium in men is 70 μg/day, which reflects the selenium intake required to achieve maximal plasma glutathione peroxidase actvity. However, there is growing consensus that nutritionally adequate selenium intake may be suboptimal with respect to reducing disease risk (2,40). Indeed, our analysis showed that selenium status sufficient to saturate the activity of plasma glutathione peroxidase (equivalent to 0.6 p.p.m. selenium concentration in toenails) will not necessarily minimize prostatic DNA damage in the dog model or prostate cancer risk in men. Researchers are aggressively pursuing new functional markers of selenium status that can accurately reflect the biologically effective concentration of selenium that optimizes human health. Since selenium has diverse health-promoting roles, it is likely that a range of markers assessing particular biochemical functions, disease states and tissue specificity will be required. We have presented here the first evidence that prostatic DNA damage measured by Comet assay may serve as a functional marker of selenium's anticarcinogenic effect on the prostate. Importantly, our results suggest that measurement of toenail selenium concentration can provide a non-invasive method for titrating and individualizing optimal selenium intake required for prostate cancer protection.

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Conflict of Interest Statement: None declared.

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