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Combined Selenium and Vitamin C Deficiency Causes Cell Death in Guinea Pig Skeletal Muscle¹

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Abstract

Combined antioxidant deficiencies of selenium and vitamin E or vitamin E and vitamin C in guinea pigs result in clinical illness. We hypothesized that combined selenium and vitamin C deficiency would have clinical consequences because in vitro interactions of these antioxidant nutrients have been reported. Since guinea pigs are dependent on dietary vitamin C, weanling male guinea pigs were fed selenium-deficient or control diet for 15 weeks prior to imposing vitamin C deficiency. Four dietary groups were formed and studied 3 weeks later: controls, vitamin C deficient, selenium deficient, and doubly deficient. Deficiencies were confirmed by determinations of glutathione peroxidase activity and vitamin C concentration in liver and skeletal muscle. Plasma creatine phosphokinase (CPK) activity and liver, kidney, heart, and quadriceps histopathology were determined. Doubly deficient animals had moderately severe skeletal muscle cell death as judged by histopathology and plasma CPK activity of 6630 ± 4400 IU/L (control 70 ± 5 ; vitamin C deficient 95 ± 110 ; selenium deficient 280 ± 250). Liver, kidney, and heart histology was normal in all groups. Muscle α -tocopherol levels were not depressed in the doubly deficient group but muscle F₂ isoprostane concentrations were elevated in them and correlated with markers of cell death. We conclude that combining selenium and vitamin C deficiencies in the guinea pig causes cell death in skeletal muscle that is more severe than the injury caused by selenium deficiency. The elevation of muscle F₂ isoprostanes is compatible with the cell death being caused by oxidative stress.

Keywords

muscle damage; guinea pig; selenium; vitamin C; combined selenium and vitamin C nutritional deficiency

1. Introduction

Selenium and vitamin C (ascorbate) are essential micronutrients. Each has a number of biochemical functions and both are classified as antioxidant nutrients because their redox properties are utilized to protect the organism against oxidative injury [1]. Selenium is a component of a number of oxidant defense enzymes, *e.g.*, the glutathione peroxidases, the

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thioredoxin reductases, methionine sulfoxide reductase B [2,3]. Ascorbate donates electrons to free radicals, including the tocopheroxyl radical (at least *in vitro*), to terminate radical chain propagation [4,5]. The resulting ascorbyl radical is recycled enzymatically to ascorbate, in part by the selenoenzyme thioredoxin reductase [6]. Thus, there is potential for interactions between selenium and ascorbate directly and involving α -tocopherol (vitamin E).

Deficiencies of these micronutrients have effects at the clinical level. Scurvy is the pathological condition caused by ascorbate deficiency. Selenium deficiency does not usually cause overt clinical injury but it predisposes animals to damage by vitamin E deficiency, certain viral infections, and some chemicals [7,8]. Based on the biochemical relationships of ascorbate and selenium, simultaneous nutritional deficiencies of them would be expected to impose a severe stress on the animal, perhaps causing clinical illness.

Guinea pigs are an excellent laboratory model for studying the interaction of vitamin C with other antioxidants, especially vitamin E and selenium, because guinea pigs are dependent on dietary intake of all three antioxidants. Vitamin C is water soluble, vitamin E is lipid soluble and selenium acts through redox functions of several selenoproteins [9]. Several selenoproteins with potential redox functions are present in muscle tissue [10,11]. Determination of dietary requirements of these antioxidants is not straightforward because of their potential interactions [12,13].

Because ascorbate is not an essential nutrient in rats or mice, guinea pigs have been used to study combined antioxidant micronutrient deficiencies [14,15]. We have developed a semi-purified guinea pig diet that can be formulated to produce single or combined deficiencies of these nutrients [15]. Since our previous studies [14,15] had shown clinical illness resulting from selenium and vitamin E combined deficiency and from vitamin E and vitamin C combined deficiency, we hypothesized that simultaneous selenium and ascorbate deficiencies would result in clinical consequences. These studies in guinea pigs provide evidence at the clinical level that the antioxidants selenium and vitamin C interact with one another and suggest that further investigation is needed at the cellular level. These results also point out the need for assessment of combined antioxidant deficiencies in human nutrition.

2. Methods

2.1 Animals

Weanling male Hartley guinea pigs (120-140 g) purchased from Elm Hill Breeding Labs (Chelmsford, MA) were housed in plastic cages with aspen shavings as bedding. They were fed pelleted *Torula* yeast-based diets formulated according to our specifications by Harlan-Teklad (Madison, WI). The control diet has been described in detail [15]. It contained 0.5 mg selenium as sodium selenate per kg, 0.8 gm ascorbic acid and 0.2 g *all-rac*- α -tocopheryl acetate per kg. The selenium-deficient versions of the diet omitted the sodium selenate and the ascorbate-deficient versions omitted the ascorbic acid. Composition of the diets is shown in Table 1 (originally reported in reference 15). Animals had free access to food and water. The Vanderbilt University Institutional Animal Care and Use Committee approved the experiments.

The primary factor determining the vitamin C requirement for guinea pigs is the prevention of scurvy [16]. Dietary ascorbate levels as low as 0.2 g/kg diet are effective while higher dietary levels have no adverse effects and will result in higher tissue concentrations [17]. Thus, the ascorbic acid supplementation level for this study and our previous studies [14,15] was chosen to ensure that an adequate amount of active vitamin C would remain after the diet was pelleted and dried. The ascorbate concentrations measured in control tissues are comparable with reported literature values [17].

Guinea pigs were anesthetized with pentobarbital (65 mg/kg intraperitoneally) and exsanguinated by removal of blood from the portal vein. The blood was treated with disodium EDTA (1 mg/ml) to prevent coagulation, and plasma was separated by centrifugation at 2,000 g for 20 min. Skeletal muscle (quadriceps) samples were excised and a piece of muscle was put into 10% buffered formalin. Pieces of liver, kidney, and heart were also put into formalin. After fixation, sections were stained with hematoxylin and eosin for microscopic analysis. Quadriceps and liver samples were quick-frozen in liquid nitrogen and stored at -70°C for biochemical analysis. A 10% liver homogenate was made in 0.1 mol/L potassium phosphate buffer, pH 7 using a Polytron tissue homogenizer (Kinematica; Lucern, Switzerland). The supernatant was separated by centrifugation of the homogenate for 30 min at 16,000 g. Muscle supernatant was obtained by centrifugation of a 20% homogenate under the same conditions.

2.2 Experimental Protocol

Scurvy develops in guinea pigs fed ascorbate-deficient diet for 3 weeks. In order to produce combined selenium and ascorbate deficiency, groups of guinea pigs were fed selenium-deficient or control diet for 15 weeks. Then each of the diet groups was subdivided into 2 groups as shown in Scheme 1. The final four groups were control (n=7), ascorbate deficient (n=8), selenium deficient (n=8), and doubly deficient (lacking both ascorbate and selenium) (n=12).

Four guinea pigs from each diet group were studied at 7 days because one of the 12 doubly deficient animals had been found dead on day 6. Animals studied at that time exhibited no clinical signs, but there was slight evidence of muscle injury by biochemical measurement (plasma CPK activities were: doubly deficient group 1100 ± 730 , selenium-deficient group 430 ± 170 , ascorbate-deficient group 190 ± 110 , and control group 134 ± 77). The remaining animals continued on the protocol and no more deaths occurred. The study was terminated at 21 days because scurvy was expected to occur in guinea pigs fed ascorbate-deficient diet for three weeks. The results presented in the tables and figures were obtained from study of these 21-day animals.

2.3 Assays

Glutathione peroxidase activity was measured as before [18] using 0.25 mmol/L H_2O_2 as substrate. Activities are reported as U/g protein with 1 unit (U) equal to 1 μ mol NADPH oxidized per min. Protein was determined with a bicinchoninic acid kit (Pierce Chemical Co., Rockford, IL) using bovine serum albumin as the standard. Plasma creatine phosphokinase (CPK) was measured using a kit (Procedure No. 45-UV, Sigma Chemical Co., St. Louis, MO). Tissue F_2 isoprostanes were measured after Folch extraction, base hydrolysis, and derivatization [19,20].

Measurement of α -tocopherol in tissue samples was carried out as described by Lang, et al. [21,22], except that heptane rather than hexane was used in the extraction step. Ascorbate was measured in tissues following homogenization of a 100 mg sample in a Teflon/glass homogenizer in 1 ml of ice-cold 80% methanol, 2 mmol/L EDTA. The homogenate was left on ice for 5 min and then was centrifuged at 6,000 g for 5 min. Aliquots of the supernatant were taken for assay of ascorbate [14].

2.4 Histopathological analysis

Muscle injury was estimated by microscopic examination of hematoxylin and eosin stained quadriceps sections by RFB. The area on the slide that was examined was approximately 1 cm^2 . Scores given were: 0, no evidence of cell injury; 1+, focal muscle cell injury present in several high-power microscopic fields; 2+, one or two areas of macrophage accumulation and/

or one or two dying muscle cells per field; 3+, more than 2 dying muscle cells per field in almost every microscopic field; 4+, confluent dying or dead cells in many fields.

2.5 Materials

HPLC solvents and perchloric acid were purchased from Fisher Scientific Co. (Atlanta, GA). Pyrogallol, tetrapentyl ammonium bromide, and glutathione reductase were purchased from Sigma Chemical Co. (St. Louis, MO). NADPH was purchased from United States Biochemical Corp. (Cleveland, OH). All other chemicals were reagent grade.

2.6 Statistics

Statistical analyses were performed using the ANOVA method in Statview software program, version 5.0.1 (SAS Institute Inc., Cary, NC) running on a Macintosh G4 computer. Scheffe's test was used as a *post hoc* test to determine statistical differences. Values are presented as means $\pm$ SD.

3. Results

3.1 Weight gain of animals and occurrence of scurvy

Weight gain appeared to have been diminished by feeding ascorbate-deficient diets but variability among animals prevented the effect from being statistically significant (Table 2). None of the animals appeared to be weak or had poor grooming.

All the guinea pigs fed the diet singly deficient in ascorbate for 21 days had hemorrhage in the connective tissues surrounding the joints. This finding is characteristic of scurvy and allows that diagnosis to be made in this group. None of the guinea pigs fed the control, selenium-deficient, or doubly deficient diets had such hemorrhage. Thus, combining selenium deficiency with ascorbate deficiency prevented or delayed the onset of this characteristic finding of scurvy.

3.2 Biochemical evidence of deficiencies

The diets had the expected effects on liver and muscle glutathione peroxidase activities and ascorbate concentrations (Table 3 and Figure 1). Liver glutathione peroxidase activity was decreased to the same degree in tissues from both groups fed selenium-deficient diet for 18 weeks (Table 3). Tissue ascorbate levels were decreased similarly by both ascorbate-deficient diets at 15 weeks and at 18 weeks (Table 3). Thus, inducing the deficiencies simultaneously did not affect the biochemical severity of either deficiency in liver (Table 3) or muscle (Figure 1).

α -Tocopherol was measured in liver and muscle at 18 weeks (Table 3 and Figure 1). Its concentration was unaffected by the deficiencies that were produced in selenium and/or ascorbate.

3.3 Muscle injury

Histological examination of muscle revealed no evidence of injury or cell death in control or ascorbate-deficient groups (Table 4 and Figure 2A-C). Minimal injury with rare evidence of cell death was noted in one selenium-deficient muscle section (Table 4). Histological examination of quadriceps muscle from all doubly deficient guinea pigs showed varying amounts of injury and cell death. The muscle damage varied from mild to severe in different animals. There was a scattered distribution of lesions within each muscle specimen (Figure 2D-F). Thus, simultaneous selenium and ascorbate deficiencies caused moderately severe skeletal muscle damage in guinea pigs. No histological abnormalities were found in liver, kidney, or heart from any of the groups.

Plasma CPK activity was measured as an index of muscle damage. It was significantly higher in plasma of guinea pigs that were doubly deficient than in plasma from the control and singly deficient animals (Table 4). CPK measured in plasma of doubly deficient guinea pigs showed a positive correlation with histological damage observed (Figure 3A). Thus, more severe injury and cell death was evident in quadriceps muscle from all doubly deficient animals.

Lipid peroxidation was assessed by measurement of F₂ isoprostanes in muscle. Neither single deficiency affected muscle F₂ isoprostane concentration (Table 4). However, doubly deficient muscle had F₂ isoprostane concentrations that averaged 2.5 times those measured in control muscle (Table 4). The F₂ isoprostane level measured in muscle of doubly deficient guinea pigs had a positive correlation with histological damage observed (Figure 3B). This finding suggests that increased lipid peroxidation occurred in the quadriceps muscle of doubly deficient guinea pigs compared with the other groups. This occurrence raises the possibility that lipid peroxidation was involved in the pathogenesis of the muscle damage.

4. Discussion

Nutritional muscular dystrophy is a term used to describe a family of muscle diseases caused by lack of dietary selenium [11,23]. The biochemical bases of these diseases are not known but are thought to be related to the loss of function of one or more selenoproteins or genetic mutations in selenoproteins. Our previous study demonstrated that combined selenium and vitamin E deficiency is fatal to guinea pigs [15]. In this study we have examined the interaction of selenium and vitamin C to determine if there is a clinical consequence resulting from simultaneous removal of both nutrients.

Inducing ascorbate deficiency in selenium-deficient guinea pigs precipitated moderately severe skeletal muscle damage (Figures 2 and 3 and Table 4), which was not evident in singly deficient animals. Thus, there is synergism of these two antioxidant micronutrients on the clinical level. The biochemical mechanism of this synergism is uncertain, however.

One candidate for the mechanism is regeneration of ascorbate from some of its oxidized forms by the selenoenzyme thioredoxin reductase-1 [6,24]. Such regeneration might maintain redox functions of ascorbate even when ascorbate levels are low.

Considerable evidence indicates that ascorbate can regenerate α -tocopherol from tocopheroxyl radical *in vitro* [4,5]. If this occurs *in vivo*, regeneration of the oxidized form of ascorbate by thioredoxin reductase might support it. Thus, a redox chain extending from NADPH through thioredoxin reductase to ascorbate and then to α -tocopherol can be envisioned. Loss of function of this redox chain would allow the oxidation and loss of α -tocopherol. However, there was no loss of α -tocopherol in skeletal muscle of doubly deficient animals when the injury occurred (Figure 1). This does not support the existence of the postulated redox chain. It is possible, however, that the pathological events occurred in a small compartment of the muscle, so that depletion of α -tocopherol was not detected when the measurement was made on whole muscle.

Another possible mechanism is that redox functions of ascorbate and selenoproteins might be distinct but overlap, creating a situation in which they can substitute for one another. Single deficiencies, then, would be tolerated without loss of the particular function, but deficiencies of both micronutrients simultaneously would lead to loss of the function with consequent tissue injury.

The elevation of F₂ isoprostanes in doubly-deficient muscle is compatible with the injury being oxidative in nature (Figure 3B). This fits with the redox functions of these micronutrients but does not distinguish between the molecular mechanisms proposed. A limitation of our study is that we have not investigated which selenoprotein(s) is directly involved with vitamin C to

maintain muscle integrity. Further experiments will be necessary to assess these and other potential mechanisms.

Simultaneous deficiency of selenium and vitamin E has devastating effects in rats [25], pigs [26], and other animals. Because it was not possible to study deficiency of the third antioxidant nutrient, vitamin C, in those animals, we initiated a project to evaluate combined deficiencies of the 3 antioxidant micronutrients in guinea pigs. The present report describes the protocol and manifestations of the last of the three combination deficiencies that we have studied.

Table 5 lists the deficiency states that we have studied in guinea pigs. Skeletal muscle appears to be highly susceptible to injury by deficiencies of antioxidant nutrients in the guinea pig. Severe selenium deficiency caused mild muscle injury and vitamin E deficiency caused moderate injury. Combining selenium and vitamin E deficiencies, however, led to massive necrosis of skeletal muscle that occurred after only a month of feeding the doubly deficient diet. The necrosis resulted in profound weakness leading to death.

The present study shows that adding ascorbate deficiency to selenium deficiency caused moderately severe muscle injury. The injury did not progress to death in the limited time of the study. The double deficiency was maintained only for 3 weeks because of the expected occurrence of scurvy. The lack of signs of scurvy in the doubly deficient group suggests that future experiments can be lengthened to determine whether muscle injury will become more severe.

The protection against scurvy manifestations by selenium deficiency is intriguing. Selenium deficiency leads to the induction of a number of oxidant defense enzymes [27,28] through the Nrf2-antioxidant response element system [29] and other mechanisms that have not yet been identified. Some of these induced enzymes might be involved in the protection against scurvy and further investigation of these relationships will likely provide insights into the antioxidant mechanisms of these micronutrients.

The other system injured by combined antioxidant micronutrient deficiencies was the central nervous system. Guinea pigs with simultaneous vitamin E and vitamin C deficiencies developed ascending paralysis that began in the lower extremities and progressed to death in 12-24 hours [14]. Neuropathology studies revealed extensive infarct-like injury in the brain and spinal cord [30]. The lesions were asymmetrical and appear to result from damage to blood vessels.

This report completes our description of combined antioxidant micronutrient deficiencies in the guinea pig. Three clinical models have been developed (Table 5). Two of them, combined deficiencies of vitamin E or of ascorbate with selenium cause skeletal muscle injury. The third, combined vitamin E and ascorbate deficiency, caused severe central nervous system injury.

These studies strengthen the concepts that antioxidant mechanisms are somewhat redundant and that they interact with one another. They provide evidence at the clinical level that those concepts need further investigation at the cellular level. They also point out the need for clinicians to repair antioxidant micronutrient deficiencies in patients, especially combined deficiencies.

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Abbreviations

CPK	creatine phosphokinase
Gpx	glutathione peroxidase
vitamin C	ascorbate

Figure 1.

Assessment of antioxidant micronutrients in quadriceps muscle of guinea pigs fed diets deficient in selenium and ascorbate for 3 weeks. The bars represent means (control, n=3; selenium deficient, n=4; ascorbate deficient, n=4; and doubly deficient, n=7) and the brackets are 1 S.D. Values within each panel that do not share the same letter (a or b) differ from one another by Scheffe's test, $p < 0.05$.

Figure 2. Photomicrographs of quadriceps muscle from guinea pigs fed the four experimental diets. Panels A-C show muscle from control, selenium-deficient, and ascorbate-deficient animals, respectively. Panels D-F show muscle from doubly deficient animals with 1+, 2+, and 4+ damage, respectively. The arrow in panel D points to an apoptotic cell, the arrows in panel E point to areas of apoptotic and dying cells. Panel F shows extensive necrosis of the tissue section. Original magnification was 400X.

Figure 3.

Muscle injury and lipid peroxidation in guinea pigs fed diets deficient in selenium and ascorbate. Muscle damage and plasma CPK (Panel A) and muscle F2 isoprostanes (Panel B) are shown for individual guinea pigs fed doubly-deficient diet for 21 days. The regression lines are $y=3561.4x - 724.1$ and $y=5.4783x + 3.8522$ for panels A and B, respectively. The r^2 values are 0.8875 and 0.7078 for panels A and B, respectively.

Scheme 1.
Diet protocol for the weanling male guinea pigs

Table 1

Control guinea pig diet (Teklad TD 98013).

Component	g/Kg diet
<i>Torula</i> yeast	400.0
DL-methionine	3.0
Sucrose	468.8
corn oil	67.0
mineral mix ^a	50.0
sodium selenate (0.1% Na ₂ SeO ₄ in sucrose) ^b	1.2
corn starch	4.9016
p-aminobenzoic acid	0.11
ascorbic acid, coated (97.5%) ^c	0.821
biotin	0.0004
vitamin B ₁₂ (0.1% trituration)	0.03
calcium pantothenate	0.066
choline dihydrogen citrate	3.5
folic acid	0.002
inositol	0.11
menadione	0.05
niacin	0.099
pyridoxine HCl	0.022
riboflavin	0.022
thiamin HCl	0.022
dry vitamin A palmitate (500000 U/g)	0.0396
dry vitamin D ₃ (500000 U/g)	0.0044
dry vitamin E acetate (500 U/g)	0.2

^aPer Kg, the mineral mix contained: 516.29 g CaCO₃, 225.2 g KH₂PO₄, 104.8 g KCl, 59.69 g NaCl, 68.0 g MgO, 20.5 g ferric ammonium citrate, 3.44 g MnSO₄·H₂O, 1.0 g NaF, 0.9 g CuSO₄, 0.1 g CrCl₃·6H₂O, and 0.08 g KI.

^bOmitted from the selenium deficient and doubly deficient diet.

^cOmitted from the vitamin C deficient and doubly deficient diet.

Table 2Body weight of guinea pigs fed diets deficient in selenium and/or ascorbate¹

diet groups	weight at 15 weeks (g)	weight change from 15 to 18 weeks (g)
control ²	786 ± 111	75 ± 17
selenium deficient ³	765 ± 57	77 ± 6
ascorbate deficient ³	798 ± 27	36 ± 18
doubly deficient ⁴	772 ± 64	-1 ± 69

¹ Values are means ± SD. No group weights were different from one another by Scheffe's test and neither were any weight changes different from one another.

² N=3

³ N=4

⁴ N=7

Table 3Liver markers of antioxidant nutrient status in guinea pigs fed diets deficient in selenium and/or ascorbate^{1,2}

diet	glutathione peroxidase (U/g protein) ³	ascorbate ($\mu\text{mol/g}$)	α -tocopherol (nmol/g)
control ⁴	14 ± 3.3^a	0.49 ± 0.02^a	9.6 ± 1.6^a
selenium deficient ⁵	2.3 ± 0.3^b	0.51 ± 0.2^a	15 ± 5.9^a
ascorbate deficient ⁵	12 ± 5.8^a	0.02 ± 0.01^b	15 ± 6.3^a
doubly deficient ⁶	1.4 ± 0.3^b	0.01 ± 0.01^b	11 ± 4.2^a

¹Values are means $\pm$ SD. Values within each column that do not share the same letter (a or b) are statistically different from one another by Scheffe's test, $p < 0.05$.

²Data presented in this table were obtained from guinea pigs euthanized at the end of the experiment (3 weeks of ascorbate deficiency, see Scheme 1). Ascorbate values measured in livers from guinea pigs fed diets for 1 week were: 0.44 ± 0.02 , $0.42 \pm .004$, 0.15 ± 0.04 , 0.11 ± 0.04 $\mu\text{mol/g}$ for control, selenium deficient, ascorbate deficient, and doubly deficient groups, respectively.

³One unit (U) of glutathione peroxidase activity is equal to 1 μmol NADPH oxidized/min.

⁴N=3

⁵N=4

⁶N=7

Table 4Indices of muscle damage in guinea pigs fed diets deficient in selenium and/or ascorbate¹

diet	necrosis score	CPK (IU/L plasma)	F ₂ isoprostanes (ng/g)
control ²	0 ± 0 ^a	70 ± 5 ^a	6.0 ± 1.8 ^a
selenium deficient ³	0.3 ± 0.3 ^a	280 ± 250 ^a	7.5 ± 1.3 ^a
ascorbate deficient ³	0 ± 0 ^a	95 ± 110 ^a	6.5 ± 0.6 ^a
doubly deficient ⁴	2.1 ± 1.2 ^b	6630 ± 4400 ^b	15 ± 7.6 ^a

¹ Values are means ± SD. Values within each row that do not share the same letter (a or b) are statistically different from one another by Scheffe's test, p<0.05. Data presented in this table was obtained from guinea pigs euthanized at the end of the experiment (3 weeks of ascorbate deficiency, see Scheme 1).

² N=3

³ N=4

⁴ N=7

Table 5

Clinical Manifestations of Antioxidant Micronutrient Deficiencies in Guinea Pigs

nutritional deficiency	manifestations		references
	clinical	biochemical and histological	
selenium	none	mild elevation of plasma CPK and mild histological muscle injury	[15], this study
vitamin E	none	moderate elevation of plasma CPK and moderate histological muscle injury	[15]
vitamin C	scurvy	hemorrhage into connective tissue	[1], this study
selenium and vitamin C	none	moderate elevation of plasma CPK and moderately severe histological muscle injury	this study
selenium and vitamin E	profound weakness and death	very high plasma CPK and severe histological muscle injury	[15]
vitamin C and vitamin E	paralysis and death	severe histological injury of brain and spinal cord	[14,30]